Biophysical characterization of the structural stability of *Helix lucorum* hemocyanin

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**ABSTRACT**

The structural stability of the hemocyanin purified from the hemolymph of garden snails *Helix lucorum* (HlH) was investigated by means of far-UV circular dichroism (CD), differential scanning calorimetry (DSC) and transmission electron microscopy (TEM). For the first time, TEM analyses showed the presence of tubular polymers in hemocyanins after three-day dialysis against a stabilizing buffer containing high concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions (100 mmol L$^{-1}$). The conformational stability study of native HlH by means of CD in a wide pH range (2.5-11.5) defined the pH stability region of HlH at pH 6.5–8.0. DSC analyses demonstrated the thermal stability of this hemocyanin. One transition, with an apparent transition temperature ($T_m$) at 82.3$^\circ$C, was detected in the heat capacity curve of HlH in 50 mmol L$^{-1}$ Tris-HCl buffer, pH 7.2, at a heating rate of 1.0$^\circ$C min$^{-1}$. The calorimetrically observed thermal transition correlates well with the unfolding transition monitored by CD measurements. The two-state kinetic model was used to analyse the process of irreversible thermal denaturation of HlH; $E_a$ of 451 ± 4 kJ mol$^{-1}$ was calculated. The obtained results on the conformational stability of HlH will facilitate the further investigation of the properties and potential biomedical applications of this respiratory protein.

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**Introduction**

Hemocyanins (Hcs) are oxygen-carrying proteins found in the hemolymph of most arthropods and mollusks [1,2]. These proteins have high molecular mass and complex quaternary structure. Thus, the molecule of native Hc from gastropods contains 20 subunits, each of which has eight functional units, the molecular mass of Hc didecamers reaching 8 MDa. The didecamer has a cylindrical structure, which is ~35 nm in length and diameter [1,2].

There are many reports in the literature about possible applications of mollusk Hcs in medicine as immunostimulants, vaccine carriers, antiviral agents, etc. [3–5]. Very recently, a study reported a significant anticancer effect of Hcs from marine snails *Rapana thomasiana* (RTH) and garden snails *Helix pomatia* (HpH) on a murine model of colon carcinoma [6].

Investigations on the Hc isolated from garden snails *Helix lucorum* (HlH) have demonstrated its immunological properties and antitumor activity [7–9].

The question of the formulation of stable preparations is of particular importance in view of the possible therapeutic applications of Hcs. This in turn requires knowledge of the structure and stability of molecules. Circular dichroism (CD) spectroscopy is one of the most sensitive biophysical techniques for studying the structure of proteins in solution, affording a direct interpretation of changes in the protein secondary structure [10]. Differential scanning calorimetry (DSC) has been regarded as the best quantitative assay for thermal stability, employed in the characterization of biopharmaceutical proteins [11,12]. Most proteins denature irreversibly due to some changes, such as autolysis, aggregation or chemical alteration of residues, after which they are unable to fold back.
to their native structure. The DSC analysis of irreversible protein denaturation provides parameters, including melting temperature ($T_m$), that are not actual thermodynamic parameters, but the rank-ordering of $T_m$ is useful for screening qualitative parameter stability. Melting temperatures in the range from 78 to 84 °C have been observed for the Hcs isolated from different mollusk organisms [13–17].

In the present study, we used a combination of biophysical techniques: high-sensitive DSC, far-UV CD spectroscopy and transmission electron microscopy, to characterize the conformational stability and dissociation-association behavior of the Hc isolated from garden snails $H. lucorum$. The new data on the stability of HIH make it possible to perform comparative analysis with the data for other Hcs and to draw conclusions about the factors determining the stability of the group of oxygen-transport proteins from invertebrates.

**Materials and methods**

**Purification of native HIH**

The hemolymph was collected from the foot of garden snails $H. lucorum$, filtrated and centrifuged at 5000 $g$ for 20 min, at 4 °C, for removal of rough particles and hemocytes. The crude Hc in the supernatant was concentrated by ultrafiltration using a 100-kDa membrane (Millipore Ultrafiltration Membrane Filters, Regenerated cellulose). The fraction above 100 kDa, which contained mostly Hc, was centrifuged at 1,80,000 $g$ (ultracentrifuge Beckman LM-80, rotor Ti 45), for 4 h, at 4 °C. The Hc pellets were suspended and subjected to gel filtration chromatography on a Sepharose 4B column (90 x 2.4 cm) equilibrated and eluted with 50 mol $L^{-1}$ Tris-HCl buffer, pH 7.2. Hc solution was filtered through 0.22-μm disposable Millipore filters prior to measurements. Protein concentration was determined using the Bradford method [18].

**Transmission electron microscopy**

Stability and dissociation-reassociation behaviour of HIH was studied by means of transmission electron microscopy (TEM) in the presence of different concentrations of $Ca^{2+}$ and $Mg^{2+}$ ions and different pH values. Dissociated HIH was produced by dialysis against 130 mmol $L^{-1}$ Gly-NaOH buffer, at pH 9.6. Electron micrographs were taken with a Philips®CM10 transmission electron microscope with a 30 mm objective aperture. HIH samples were adsorbed for 60 s to a glow-discharged pitoform/carbon-coated support film, washed three times with droplets of distilled water to remove buffer salts, and finally negatively stained with 1% uranyl acetate. Electron micrographs were routinely recorded at an instrumental magnification of 52 000, as previously described [19].

**Circular dichroism measurements**

CD measurements were performed on a Jasco J-720 spectropolarimeter (Tokyo, Japan) equipped with a Peltier temperature control system. The temperature was thermostatically-controlled using a NESLAB thermostat model RTE-110 connected to a digital programming controller and a thermocouple placed inside the optical cell. All CD spectra and temperature-dependent traces were measured in a cylindrical temperature-controlled quartz cells with a path length of 5 mm and recorded in the UV-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. Specific absorption coefficient $a_{278} \text{nm} = 1.413 \text{mL mg}^{-1} \text{cm}^{-1}$ [20] was used for determining the protein concentration. Four scans were averaged for each sample. The results were expressed as Mean Residue Ellipticity ($[\theta]_L$) in deg cm$^2$ dmol$^{-1}$. Two approaches were applied for evaluation of effective thermodynamic functions and determination of cross-overlapped CD data: (i) For temperature denaturation studies, the CD spectra of HIH solution in 50 mmol $L^{-1}$ Tris buffer, containing 10 mmol $L^{-1}$ CaCl$_2$, pH 7.2, were recorded at different temperatures from 20 to 95 °C (in 5 °C increments), after incubation of 10 min. (ii) CD spectra of standardized HIH solution in “cocktail buffer” were recorded at different pH values from 2.5 to 11.5 (in 0.5 °C increments) after incubation of 10 min at 25 °C. More precisely, the extreme values at 222 nm were digitalized and recalculated in $[\theta]_{222}$ (deg cm$^2$ dmol$^{-1}$) units. Protein solutions were prepared in 20 mmol $L^{-1}$ “cocktail buffer” (by equal stocks of 20 mmol $L^{-1}$ sodium phosphate buffer (pH 7.4–5.5), 20 mmol $L^{-1}$ sodium acetate buffer (pH 5.4–3.6), and 20 mmol $L^{-1}$ Tris–HCl buffer (6.4–11.0)) in the presence of 10 mmol $L^{-1}$ CaCl$_2$. The final solution mixture with protein concentration of 20 μmol $L^{-1}$ at given adjusted pH was incubated for 20 min at room temperature before optical measurements.

**Differential scanning calorimetry**

Calorimetric measurements were performed on a high-sensitivity differential adiabatic scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with capillary platinum cells and a volume of 0.47 mL.
Different heating rates (0.2, 0.5, 1.0 and 1.6 °C min⁻¹) were employed. In order to assay the reversibility of the calorimetric transitions, all samples were cooled down to 35 °C after the first scanning run and reheated. Since an irreversibility of the process was found in all cases, the second scan was used as a baseline. The excess heat capacity curves were obtained after concentration normalization and baseline subtraction, as described in our previous studies [13,14]. The temperature at the maximum of the excess heat capacity curve was taken as the transition temperature ($T_{m}$).

**Data analysis**

The DSC data were analyzed by means of the non-linear least-squares method using the Origin 8 software package (OriginLab Corp.). In all fitting procedures, the correlation coefficient $r$, used as a criterion for the accuracy of fitting, was not less than 0.99. In the calculation of molar quantities, the molecular mass used for the protein was 9 000 kDa.

DSC transitions were analyzed in terms of the two-state kinetic model:

$$N \xrightarrow{k} D,$$

(1)

which is a particular case of the Lumry-Eyring model [21]. This model considered only two significantly populated macroscopic states: the initial or native state (N) and the final or denatured state (D), the transition between which is determined by a strongly temperature-dependent first-order rate constant ($k$). The Arrhenius equation represents the temperature dependence of $k$:

$$k = \exp \left[ \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T^*} \right) \right],$$

(2)

where $E_a$ is the activation energy of the denaturation.
process, \( R \) is the gas constant, and \( T' \) is the temperature at which \( k \) is equal to \( 1 \text{ min}^{-1} \).

In this case, the excess heat capacity \( C_p^\text{ex} \) is given by the following equation [22]:

\[
C_p^\text{ex} = \frac{1}{V} \Delta H \exp \left\{ \frac{E_a}{R \left( \frac{1}{T} - \frac{1}{T'} \right)} \right\} \times \exp \left\{ -\frac{1}{V} \int_{T_0}^{T'} \exp \left[ \frac{E_a}{R \left( \frac{1}{T} - \frac{1}{T'} \right)} dT \right] \right\},
\]

(3)
where $\nu = \frac{dT}{dt}$ (K min$^{-1}$) is a heating rate value, $\Delta H$ the enthalpy difference between the denatured and native states.

**Results and discussion**

**Electron microscopic measurements of native HlH**

The morphological features of HlH were determined by negative staining of the molecules by means of a transmission electron microscope [23]. TEM images can be used to confirm that the native protein structure of gastropodan Hcs is mainly composed of didecamers (20 subunits) [1,2,23-25].

Transmission electron micrographs of the negatively stained specimen prepared from purified native HlH, at different conditions are shown in Figure 1. Predominantly, didecamers (20 subunits assembled) and rarely some decamers were observed (Figure 1(A)), which is the typical quaternary structure of gastropodan Hcs. This is proof that the isolated protein was in a homogeneous state, and no dissociated material or tubular structures were observed. Hcs can lose their quaternary structure under non-physiological conditions. Upon increasing the pH of the medium to $\sim$ 9 and/or removing bivalent metal ions (such as $\text{Ca}^{2+}$, $\text{Mg}^{2+}$), the Hc molecule dissociates into subunits, without losing the ability to reversibly bind molecular oxygen [1,2]. After overnight dialysis against 130 mmol L$^{-1}$ Gly-NaOH buffer, pH 9.6, the TEM analysis of dissociated HlH showed disorganized structures, as the decameric forms dissociated into subunits (Figure 1(B)). After changing the conditions, using dialysis against the stabilizing buffer (SB), pH 7.2, containing 20 mmol L$^{-1}$ CaCl$_2$ and 20 mmol L$^{-1}$ MgCl$_2$, the obtained structural subunits mostly reassociated to form didecamers (black arrows) and short multidecamers (white arrows) (Figure 1(C)). By increasing the concentrations of both divalent ions, $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$, to 100 mmol L$^{-1}$, the reassociation increased and, in addition to the didecamers, longer multidecamers were formed. The multidecamers consisted of a “nucleating” didecamer with attached decamers at one or both sides (Figure 1(D)). For the first time, tubular polymers were observed for HlH after three-day dialysis against SB, containing 10 mmol L$^{-1}$ CaCl$_2$ and MgCl$_2$, in contrast to the Hc from garden snails *Cornu aspersum* (CaH) and previous analysis of HlH subunits [19,26]. The reassociation of isolated individual subunits $\beta$-HlH in the SB, containing 10 mmol L$^{-1}$ CaCl$_2$, led to formation of short tubules, as was observed at previous TEM analysis [26]. The $\alpha$-HlH subunits reassociated into didecamers and tubules of different length, but shorter ones than in reassociated $\beta$-HlH [26].

**CD Study of conformational stability of HlH**

Information about the secondary structure of proteins can be derived from CD signals in the far-UV spectral region between 190 and 250 nm, due to the amide chromophores of the peptide bonds. Two negative Cotton effects at 222 and 208 nm were distinguished in the CD spectrum of native HlH (Figure 2(A)), as observed for other gastropodan Hcs, due to their $\alpha$-helix and $\beta$-sheet structures [17,19,27,28]. The thermal stability of the native HlH was investigated based on the changes in the ellipticity at 222 nm with increasing the temperature in the range of 20-95 °C, at pH 7.2 (Figure 2(B)). The negative ellipticity decreased at temperatures above 65°C. The thermal denaturation of HlH was irreversible, thus the thermal stability was characterized by the transition or “melting” temperature - $T_m$, which is the midpoint of the sigmoidal denaturation curve (Figure 2(B)). The $T_m$ value of 76.3°C, at pH 7.2, determined for native HlH, defines it as a thermostable protein. The data are consistent with those obtained by CD for other investigated Hcs: *Haliotis tuberculata* Hc (HtH) ($T_m$=76.0°C), Hcs from *Viviparus ater* ($T_m$=77.0°C) and *Concholepas concholepas* Hc ($T_m$=78.0°C) [15,27]. The native HlH possesses higher thermostability compared to the native CaH ($T_m$ = 67.0°C, at pH 7.5), native KLH ($T_m$ = 67.0°C) and its structural subunits KLH1 ($T_m$ = 56.0°C) and KLH2 ($T_m$ = 52.0°C) [19,29]. The higher $T_m$ values of native Hcs, compared to their subunits, are an excellent indication for the stabilization caused by oligomerization, which introduces additional factors like non-ionic forces (intra-subunit interactions, hydrophobic and hydrogen-bonded networks and carbohydrate moieties interactions) [30]. The relatively small changes of initial $\theta_{222}$ at high temperatures and neutral pH (Figure 2(A)) indicate that many structural elements are preserved even at extreme high temperatures. The thermal-induced unfolding of HlH was found to be irreversible in nature, as reported also for other studied Hcs [19,26,27].

The results from the conformational stability study of native HlH, at different pH values in the range of 2.5-11.5, at 25°C, by means of CD are presented in Figure 2(C). The $\theta_{222}$ versus pH plot represents a smooth and partially bell-shaped curve with a maximum in the pH region between 6.0-8.5 (with an extremum at pH 7.2) and non-symmetric acidic and alkaline extremes, but without any obvious sigmoid feature at
extreme pH. The results showed that pH-transitions (acid and alkaline denaturation) were poorly presented in all the extremes within the datasets of native oligomeric HlH, and can be accounted as an increased stability which is due to the quaternary structure of the protein (Figure 2(C)). In the alkaline range (pH 8–12), the relative changes were too small and non-cooperative (within a wide pH range), indicating that alkaline denaturation cannot occur as a reversible process. The relative changes were small and almost non-cooperative (in the vicinity of pH 2.5), indicating that acidic denaturation cannot occur as a reversible process either, as observed in other Hcs, too [19].

**DSC study of the thermal stability of HlH**

DSC is an established biophysical technique for characterizing temperature-induced conformational changes in biomolecules and measuring directly the thermodynamic parameters associated with these processes.

**Irreversibility of the thermal denaturation**

The experimental $C_p$ transition curve of HlH in 50 mmol L$^{-1}$ Tris-HCl buffer, pH 7.2, at a heating rate of 1.0 °C min$^{-1}$, is shown in Figure 3 (line a). The thermal denaturation of HlH was irreversible, since no thermal effect was detected in a reheating scan (Figure 3, line b).

The parameters characterizing the process of irreversible thermal denaturation of HlH are respectively: an apparent $T_m$ 82.3 °C; a calorimetric enthalpy ($\Delta H_{cal}$) 108.7 MJ mol$^{-1}$. The irreversibility of the thermal denaturation was observed in the DSC measurements of all of the studied Hcs to date [13–17,31]. Kinetic models have been applied to analyze heat capacity curves if the model-derived transition temperatures and enthalpies are dependent on the heating rate [22,32,33].

**Scan rate dependence**

Figure 4 shows the excess heat capacity function vs. temperature curves for HlH at four different scan rates (0.2, 0.5, 1.0 and 1.6 °C min$^{-1}$). The transition temperature of the irreversible thermal denaturation of HlH is scan-rate dependent: the maximum of the endotherms is shifted toward lower temperatures with a reduction in the scan rate. The kinetic theory of irreversible denaturation requires a dependence of peak position on the scan rate [22,33]. This happens due to the heating rate and denaturation rate being of a similar scale. The thermal unfolding of the investigated HlH is therefore under kinetic control.

The irreversible denaturation observed in the DSC experiments coincided with the irreversible conformational unfolding of HlH observed by CD ellipticity. The value of $T_m$ determined by CD-spectrometry was only 3 °C lower than the $T_m$ value of 79.3 °C determined by DSC, at a heating rate of 0.2 °C min$^{-1}$ (50 mmol L$^{-1}$ Tris-HCl buffer, pH 7.2).
Concentration dependence of the temperature of denaturation

The quaternary structure of the HlH molecule consists of twenty subunits forming an oligomer (didecamer). According to the equilibrium thermodynamics, any change in the oligomeric state of the protein during the denaturation process should produce a concentration dependence of $T_m$.

In order to determine whether this occurred, DSC experiments were performed at varying protein concentrations (1.5, 3.0 and 4.1 mg/mL), at a constant heating rate of 1 °C min$^{-1}$. As shown in Figure 5, the $T_m$ and $\Delta H_{cal}$ values were independent of the protein concentration. This leads to the conclusion that the rate-determining step of the process of thermal unfolding of HlH does not involve a change in its oligomeric state. Consistent with our previous studies, the heat absorption observed in the DSC curve ($T_m \approx 82.3$) is connected to the melting of the compact quaternary structure of the Hc without simultaneous dissociation into subunits [13–15,17].

Successive annealing procedure

Analysis of the heating profile of HlH revealed asymmetry of the shape (Figure 3, line 1) due to overlapping transitions, which was expected in view of the complex structure of this protein. The $C_p$ transition curve of HlH was analysed applying a successive annealing procedure, which was shown to be useful for experimental deconvolution of complicated completely or partially irreversible thermal transitions [34,35]. Figure 6 shows an example of this procedure, which provides two irreversible transitions. The first complete DSC scan (Figure 6, panel A, line a) offered insight into the number and $T_m$ of the expected transitions (close to 82 and 86 °C, respectively). After that, a new sample was heated slightly above the main transition, to 83 °C (Figure 6, panel A, line b). After cooling to 35 °C, the sample was reheated up to 96 °C (Figure 6, panel A, line d). To determine the ascending part of the first transition, the second scan was subtracted from the first one (Figure 6, panel B, solid line). The descending part of the curve was obtained by extrapolation (Figure 6, panel B, dashed lines).

Figure 7 shows the experimental deconvolution of the $C_p$ transition curves of HlH recorded at different
heating rates, as a result of the applied successive annealing procedure.

Analysis of the calorimetric data
As evident from Figure 4, the DSC traces for HiH were dependent on the scan rate, which allowed analyzing this non-equilibrium process using the two-state model of irreversible thermal denaturation of proteins (eq. 1). Previous studies have applied this simplest model in the interpretation of the DSC data for the thermal denaturation of Hcs from different species [13–15,17,31]. To analyze the excess heat capacity functions, we fitted the data to the two-state irreversible model according to Equation (3) (Figures 4 and 6). The theoretical curves are practically superimposed on the experimental ones, confirming that the thermal denaturation of HiH follows the proposed kinetic model (Figure 8).

Estimated Arrenius parameters are shown in Table 1. The $E_a$ values determined for HiH are in good agreement with the values reported for Hcs in related species [13–15,17,31]. Although the two-state model has described experimental data satisfactorily, the actual protein denaturation mechanism may be more complex [36]. Therefore, it should be emphasized that

![Figure 7. Experimental deconvolution of the $C_p$ transition curves of HiH at a scan rate of: 0.2 °C min$^{-1}$ (A); 0.5 °C min$^{-1}$ (B); 1.0 °C min$^{-1}$ (C); 1.6 °C min$^{-1}$ (D). Symbols (.) depict the experimental data; dash-dot lines show the individual components, results of the annealing process, and solid lines represent the sum of the corresponding individual components. In all cases the protein concentration was 3 mg mL$^{-1}$.](image)

![Figure 8. $C_p$ transition curve of HiH in Tris-HCl buffer (pH 7.2) recorded at a heating rate of 1.0 °C min$^{-1}$ (black line). The solid lines (red and blue) represent the theoretical fitting curves based on Equation (3); lines with symbols (.) show the curves obtained from the deconvolution procedure.](image)
the values for $E_a$ are apparent and characterize the initial rate-limiting step of the process.

Table 2 summarizes the DSC data for the process of irreversible thermal denaturation of the Hc isolated from mollusk *H. lucorum*. The data are compared with those obtained up to now for other mollusk Hcs.

Recently, the thermal denaturation of Hc isolated from garden snails *H. aspersa maxima* was studied in various buffer systems and differences in respective DSC parameters ($T_m$ and $D_{H cal}$) were observed [17]. Tris and HEPES were found to be stabilizing buffers. Salts at some concentrations also have shown to promote the stability of Hcs (e.g. 100 mmol L$^{-1}$ NaCl, divalent cations). Therefore, when comparing the thermal stability of Hcs, respective experiments should be performed in the same buffer system in addition to the same heating rate (Table 2). As can be seen from Table 2, there are differences in the thermal stability of the Hcs from mollusks *H. pomatia*, *R. thomasiana* and *C. concholepas*, studied by DSC at the same experimental conditions. The quaternary structure of the studied Hc molecules is similar (didecamer), so it is difficult to define a structural basis for the observed differences. Hcs are glycoproteins with different carbohydrate content and structure of the oligosaccharide chains. The most thermostable $\beta$-Hc of *H. pomatia* contains 7% (w/w) carbohydrates [37], whereas 2.6% (w/w) carbohydrate content has been reported for *R. thomasiana* Hc [38]. Therefore, a correlation between the thermal stability of Hcs and their carbohydrate content could be supposed.

### Conclusions

The results from the present investigation by means of several biophysical techniques on the HlH isolated from garden snails *H. lucorum*, classified this Hc as a thermostable protein ($T_m$ 82.5$^\circ$C). The pH stability range of HlH is at pH values 6.5 – 8.0. Higher concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions (100 mmol L$^{-1}$) promote the stability of the protein molecule. The data obtained will serve as a basis for creating a stable Hc formulation and will facilitate the further investigation of the properties and potential biomedical applications of this respiratory protein.

### Disclosure statement

No potential conflict of interest was reported by the author(s).
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