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

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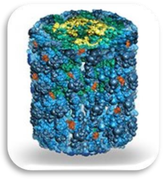
Snail *Helix lucorum*

Hemocyanin



Structure of Hc molecule

Hemocyanins (Hcs) are oligomeric copper-containing respiratory proteins, freely dissolved in the hemolymph of many arthropod and mollusc species.

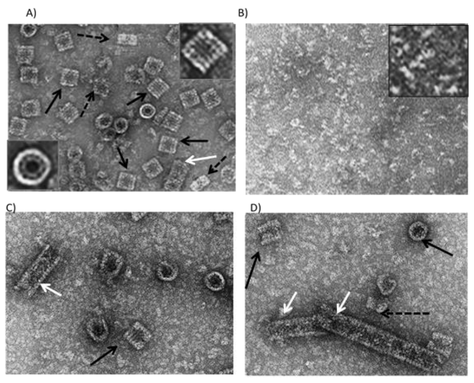
Various aspects of biomedical applications of molluscan Hcs, associated with their immunogenic properties and antitumor activity, promoted us to perform structural studies on a representative of these proteins.

The structural stability of the Hc, 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 microroscopy (TEM).

CD measurements were performed on a Jasco J-720 spectropolarimeter (Tokyo, Japan), equipped with a Peltier temperature control system. Calorimetric measurements were performed on a DASM-4 (Biopribor, Pushchino, Russia) high sensitivity differential scanning microcalorimeter. Four different scan rates (0.2, 0.5, 1.0 and 1.6 ^{o}C min $^{-1}$) were used. Electron micrographs were taken with a Philips®CM10 transmission electron microscope with a 30 mm objective aperture.

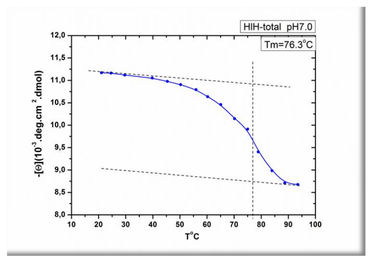
Results

Electron microscopic measurements of native HlH

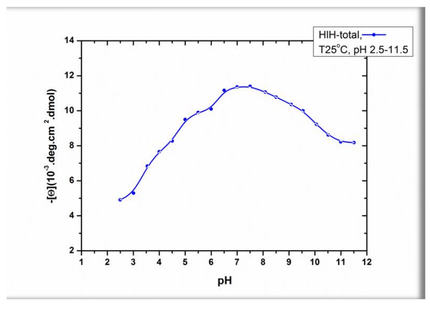


Gallery of transmission electron micrographs of the purified negatively stained HlH. A) The molecule of native HlH is didecamer (black arrow), visible in side views (rectangular) and in top views (circles). In top view orientation (insert), the outer wall and the internal collar are directly visible. Few decamers (black dash arrow) and one short multidecamer (white arrows) are observed; B) dissociated protein in 130 mM Gly-NaOH buffer, at pH 9.6; C) reassociated HlH after dialysis against SB, pH 7.2, containing 20 mM $CaCl_2$ and 20 mM $MgCl_2$; D) reassociated HlH after dialysis against the SB, pH 7.2, containing 100 mM $CaCl_2$ and 100 mM $MgCl_2$.

CD study of conformational stability of HlH



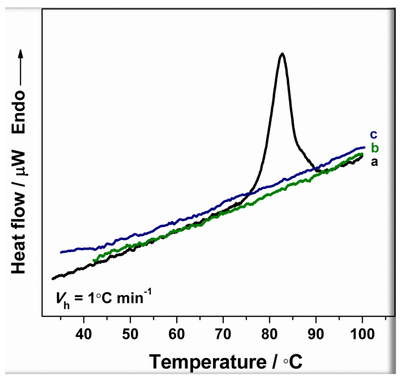
Influence of the temperature on the unfolding of native HlH at pH 7.2, and determination of a melting temperature T_m=76.3 °C.



Influence of pH values (2.5-11.5), at temperature 25 ^{o}C, on unfolding of native HlH.

The thermal denaturation of Hcs is an irreversible process

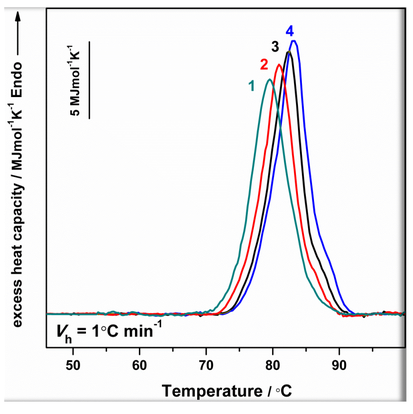
T_m ~ 82.5 ^{o}C



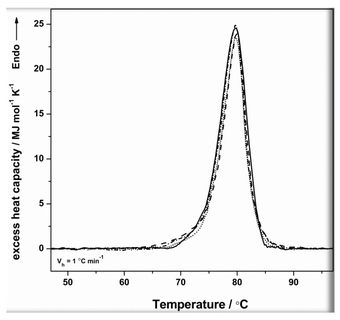
(a) Experimental Cp transition curve of HlH in 50 mM Tris buffer (pH 7.2), at a heating; rate of 1.0 ^{o}C min^{-1}; (b) Reheating run; (c) Buffer-buffer base line.

Scan-rate dependence

The scan rate dependence of the calorimetric profiles indicates that the thermal unfolding of the investigated Hcs is kinetically controlled.

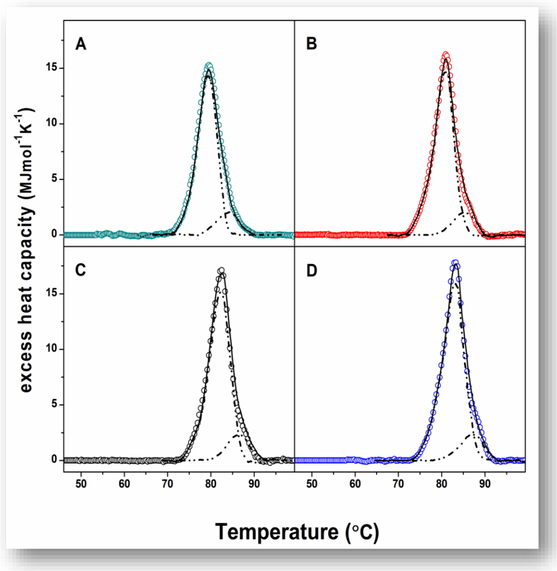


Dependence of the Cp transition curves of HlH in 50 mM Tris buffer pH 7.2) with heating rate: (1) 0.2 ^{o}C min^{-1} ; (2) 0.5 ^{o}C min^{-1}; (3) 1 ^{o}C min^{-1}; (4) 1.6 ^{o}C min^{-1}. In all cases the protein concentration was 2.8 mg ml^{-1}.



Cp transition curves of HaH in 20 mM HEPES buffer, pH 7.2, recorded at a heating rate of 1 ^{o}C min^{-1} and at different protein concentrations: 1.5, 3.0 and 4.1 mg ml^{-1}.

Experimental deconvolution of HlH as a result of successive annealing procedure



Dependence of the C_p transition curves of HlH, with a scan rate: 0.2 ^{o}C min^{-1} (A); 0.5 ^{o}C min^{-1} (B); 1.0 ^{o}C min^{-1} (C); 1.6 ^{o}C min^{-1} (D). Symbols (o) depict the experimental data; dash dot lines show the individual components, result of the annealing process, and continuous lines represent the result of the sum of the corresponded individual components. In all cases the protein concentration was 3 mg ml^{-1}.

Effect of protein concentration

The T_m and the specific enthalpy values (ΔH_{cal}) for the thermal denaturation of Hcs were found to be independent of the protein concentration, indicating that the dissociation of the Hc into subunits does not take place prior the start of the rate-determining step i.e. the process of thermal unfolding.

Determination of the activation parameters from the experimental heat capacity functions

The thermal denaturation of HlH can be described by the two-state irreversible model:

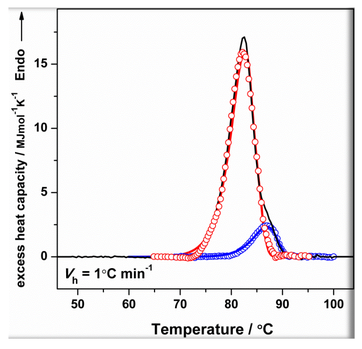
$N \xrightarrow{k} F$ (1), k - temperature-dependent first-order rate constant

$k = \exp\{E_a (1/T^* - 1/T) / R\}$ (2), E_a - activation energy;

R – gas constant, and T* - temperature at which k is equal to 1 min^{1}

$$C_p^{ex} = \frac{1}{v}\Delta H \exp\left\{\frac{E_a}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)\right\} \times \exp\left\{-\frac{1}{v}\int_{To}^{T} \exp\left[\frac{E_a}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)dT\right\}\right.$$

Theoretical fitting curves based on eq.(C_p)



C_p transition curve of HlH in tris buffer recorded at scan rate 1.0 ^{o}C min^{-1}. The solid lines (red and blue) represent the theoretical fitting curves based on eq. (C_p); lines with symbols (o) show the curves obtained from the deconvolution procedure; experimental C_p transition curve (black line).

Arrhenius equation parameters estimated for the two-state irreversible model of the thermal denaturation of Hc, isolated from HlH

	Parameters	Temperature scan rate [^{o}C min^{-1}]			
		0.2	0.5	1.0	1.6
First Transition♦	T_m [^{o}C]	79.3	80.7	82.3	83.3
	ΔH_{cal} [MJ mol^{-1}]	86.5 ± 1.3	89.6 ± 1.1	99.2± 0.6	104.5 ± 3.1
	E_a [kJ mol^{-1}]	453± 9	466 ± 8	451 ± 4	453 ± 5
	T^*[^{o}C]	83.6 ± 0.5	83.8 ± 0.3	83.8 ± 0.4	83.9 ± 0.3
Second Transition	T_m [^{o}C]	83.9	85.1	86.2	86.9
	ΔH_{cal} [MJ mol^{-1}]	11.7	12.3	14.2	16.1
	E_a [kJ mol^{-1}]	475 ± 12	471 ± 8	472 ± 10	462 ± 14
	T^*[^{o}C]	86.6 ± 0.2	86.5 ± 0.2	86.4± 0.2	86.7 ± 0.2

Conclusion

In conclusion, the results of the present investigation by means of several biophysical techniques on the HlH, isolated from garden snails *H. lucorum*, allow classifying this Hc as a thermostable protein (T_m 82.5 ^{o}C). The pH stability region of HlH is at pH values 6.5 – 8.0. Higher concentrations of Ca^{2+} and Mg^{2+} ions (100 mM) 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.

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