Original Article

Enhanced structural stability of oxidized *Helix aspersa* maxima hemocyanin

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ABSTRACT

Hemocyanins (Hcs) are large glycoproteins present in the blood of some mollusks and arthropods, whose biological function is mainly related with the oxygen transport to the tissues. In addition, mollusk Hcs are used as carriers/adjuvants and non-specific immunostimulants in biomedicine. Achieving structural stabilization in proteins having therapeutic application is an important task. In the present study, the carbohydrate moieties of the hemocyanin, isolated from the garden snail *Helix aspersa* maxima and its isoforms, were oxidized with sodium periodate. The changes in the microenvironment around the chromophores in the protein molecules and the structural stability were evaluated and discussed. The oxidized proteins demonstrated increased resistance to proteolytic cleavage, as well as enhanced thermal stability.

KEYWORDS: hemocyanin, periodate oxidation, structural stability.

1. INTRODUCTION

Hemocyanins (Hcs) are large oligomeric proteins present in the blood of some mollusks and arthropods, whose biological function is mainly related with the oxygen transport to the tissues [1]. Hcs also play a role in the innate immune system of the animals as they possess some phenoloxidase activity. It has been shown that the oxygen-binding function of Hc can be converted to phenoloxidase (PO) activity and furthermore that PO activity can be induced in Hcs by *in vivo* and *in vitro* activation [2, 3]. In addition, mollusk Hcs have shown promising properties, making them useful in the development of various medicinal products including antiviral agents, conjugate vaccines and immunotherapeutic agents for cancer [4-6]. Thus, it has been revealed that the Hcs isolated from marine snail *Rapana thomasiana* (RtH) and from terrestrial snail *Helix pomatia* (HpH) were able to elicit strong antiviral or antibacterial immune response in mouse models when combined with bacterial and viral antigens [7, 8]. Furthermore, it has been demonstrated that these Hcs expressed strong *in vivo* anti-cancer and anti-proliferative effects in a murine model of colon carcinoma [9]. Recently, the Hc of garden snails *Helix aspersa* maxima (HaH) and its isoforms, β-HaH and α_{D+N}-HaH, were isolated and characterized [10, 11]. Expression of functionally distinct Hc isoforms in some mollusks, e.g. *Helix*, is supposed to be related to the development of the species [12]. On the basis of the o-diphenoloxidase activity of HaH, a model of biosensor for quantitative determination of phenols in aqueous solutions has been developed [10]. An antiproliferative effect of the total molecule HaH and structural subunit β-HaH, against bladder cancer cell lines, has been observed [13]. Moreover, strong antimicrobial activity has been reported for the β-HaH [14].

From the point of view of the potential for therapeutic and other applications of Hcs, the issue of formulation of stable preparations is of particular

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importance. Chemical modification is one approach to improve the protein's stability. Hcs, isolated from various gastropodan organisms, have a carbohydrate content of 2-9% w/w, with mannose being the major monosaccharide found in these structures [15]. It has been reported that the periodate oxidation of the carbohydrate moieties of Hcs with sodium periodate has induced structural stabilization and enhanced their immunogenicity and/or anti-tumor activity [16-18].

The aim of the present study is to enhance the structural stability of the Hc, isolated from the garden snail *H. aspersa* maxima and its isoforms, by chemical oxidation of their sugar moieties. The native and oxidized Hcs were characterized by various spectroscopic methods, electrophoresis and differential scanning calorimetry (DSC).

2. MATERIALS AND METHODS

2.1. Reagents

Sodium periodate, ethylene glycol and phenylmethylsulfonyl fluoride (PMSF) were purchased from Merck, Darmstadt, Germany. Trypsin and proteinase K were supplied by Sigma–Aldrich Chemie GmbH. All other chemicals used were of analytical grade.

2.2. Biochemical methods

2.2.1. Isolation and purification of Hcs

Native HaH was isolated according to the procedure described in [10]. Briefly, the Hc was obtained from the hemolymph, collected from the snail *H. aspersa* maxima, by ultracentrifugation at 180 000 x g (ultracentrifuge Beckman LM-80, rotor Ti 45), for 4 hours, at 4 °C. The pellets were suspended in 50 mM phosphate buffer, pH 7.2, and HaH was purified by gel filtration chromatography on a Sepharose 4B column (90 x 2.4 cm). Further, HaH was separated into its isoforms (β-HaH and $α_{D+N}$-HaH) by ion exchange chromatography on a DEAE-Sepharose CL-6B column (32 x 1.2 cm), equilibrated and eluted with buffer 50 mM Tris-HCl, pH 8.0, using a linear gradient 0.1-0.45 M NaCl.

2.2.2. Chemical modification of Hcs

The chemical oxidation using the sodium periodate method was performed to modify HaH and its isoforms, β-HaH and $α_{D+N}$-HaH, as described in [18].

Briefly, each Hc (protein concentration 2 mg ml^{-1}) was dissolved in 0.1 M sodium acetate buffer, pH 5.5, containing 15 mM sodium periodate and incubated for 1 h in the dark at room temperature. Next, 25 μl of ethylene glycol solution was added to each 2 ml protein and incubated overnight at 4 °C. At the last step, the protein samples were concentrated by ultrafiltration using an Amicon stirred cell, dialyzed against buffer 50 mM Tris-HCl, pH 8.0, and filtered through 0.22-μm membrane filter.

2.2.3. Protease digestion of Hcs

Modified Hcs and their respective native forms were digested with trypsin and proteinase K at a concentration of 0.2% (w/w). The enzymatic reactions were performed in a buffer 50 mM Tris-HCl, pH 8.0, at 37 °C, up to 60 min, and stopped with an addition of 1% PMSF.

2.2.4. Native and SDS-PAGE

Hc samples were analyzed by SDS-PAGE on 7.5% separating gel, as described by Laemmli [19]. Native PAGE was conducted using the same conditions, but omitting the SDS from all buffers. Electrophoresis was performed using a Mini Protean electrophoresis system (Bio-Rad). Sensitive silver staining was used to detect the proteins after electrophoretic separation on polyacrylamide gels.

2.2.5. Carbohydrate analysis

The phenol-sulfuric acid method was used to determine the carbohydrate content (%) of native HaH [20].

2.3. Spectroscopic methods

2.3.1. Absorption spectroscopy

Absorption spectra of Hcs were recorded using Evolution™ 300 UV-Vis spectrophotometer (Thermo Electron Corporation). Specific absorption coefficient a_{278} nm = 1.413 ml mg^{-1} cm^{-1} for HaH [21] was used for determining the protein concentration.

2.3.2. Steady-state fluorescence measurements

The fluorescence spectra of HaH and its isoforms were recorded by means of a spectrofluorimeter Perkin Elmer model LS55, at an excitation wavelength of 295 nm. The optical density of the solutions was kept lower than 0.05 at the excitation wavelength to avoid inner filter effects.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were recorded on Bruker Vertex 70 spectrometer by direct deposition of the samples on a diamond attenuated total reflectance (ATR) accessory. The spectra were collected in the frequency region 4000-600 cm^{-1} with 128 scanning at a resolution of 1 cm^{-1}. The absorptions of the water/ aqueous solution were subtracted from the sample spectra. Second derivative spectra were obtained using the Savitzky-Golay algorithm based on 25 smoothing points. The protein secondary structure content was estimated by the curve fitting procedure implemented in Opus software version 5.5. In the fitting, the number of components and the initial values of their position were set as determined from the second derivative spectra. The initial bandwidth of all components was set to 12 cm^{-1} and the components were approximated by mixed Lorentzian/Gaussian functions. The curve-fitting was performed according to the Local Least Squares algorithm.

2.3.4. Differential scanning calorimetry (DSC)

Calorimetric measurements were performed on a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with sensitivity greater than 0.017 mJ K^{-1} and a noise level less than ±0.05 µW. A constant pressure of 2 atm was maintained during all experiments to prevent possible degassing of the solution on heating. The protein solution in the calorimetric cell was reheated after the cooling from the first run to estimate the reversibility of the thermally induced transitions. The calorimetric data were evaluated using the ORIGIN (MicroCal Software) program package. Molecular mass of 9 000 kDa for Hc was used in the calculation of molar quantities.

3. RESULTS AND DISCUSSION

Native HaH was purified from the hemolymph of snail *H. aspersa* maxima according to a well-established protocol. The HaH isoforms were further isolated by ion exchange chromatography. The isoform β-HaH (pI 5.2) was eluted as a single symmetric peak, followed by a peak containing both α-isoforms with similar electrophoretic properties (pI 4.6), which eluted together as α_{D+N}-HaH (Fig. 1). At neutral pH and in the presence of Ca^{2+} and Mg^{2+}, Hc isoforms occur as a didecamers of ~450 kDa subunits. Important difference between HaH isoforms is that the didecameric molecule of β-HaH consists of one type of subunits, while each of the two α-Hcs is a heterooligomer of two types α and α' subunits [21].

3.1. Carbohydrate content of native HaH

Molluscan Hcs are glycoproteins with a carbohydrate content between 2% and 9% (w/w) and variable monosaccharide composition being a possible source of microheterogeneity of these proteins [22]. Besides the commonly occurring sugars D-mannose, D-galactose, L-fucose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, the Hcs also contain D-xylose and 3-*O*-methyl-D-galactose, which are unusual carbohydrates for animal glycoproteins [22, 23]. The high antigenic potency of molluscan Hcs is related to their bound carbohydrate chains [24, 25].

By means of the classic phenol-sulfuric acid method, a carbohydrate content of 2.7 ± 0.5 % (w/w) was obtained for the purified native HaH. Similar carbohydrate content of 2.6% (w/w) was determined for RtH [23], while 7% (w/w) carbohydrates had been reported for the β-isoform of HpH [26].

### 3.2. Modification of HaH and its isoforms

To stabilize the structure of the investigated Hcs, the carbohydrate chains from the surface of molecules were oxidized with sodium periodate to generate Schiff bases between the free amine groups from proteins (N-terminus of polypeptides and the side chain of lysines) and the reactive aldehydes, formed by the oxidation procedure.

Electrophoretic analysis was used to characterize modified (oxidized) Hcs. Native PAGE showed differences in the electrophoretic mobility between native and oxidized (Ox) Hcs. Ox-Hcs did not enter the resolving portion of the gel (Fig. 2a, lane 2 and 4). This effect was attributed to the internal cross-linking within Hc molecules and/or change in the charge of the molecules as a result of the periodate treatment [16, 18].

Digestion of investigated Hcs with trypsin and proteinase K were used to assess whether Schiff bases were formed in Ox-Hcs. Both proteolytic enzymes possess different specificity. Trypsin cleaves polypeptide chains mainly at the carboxyl
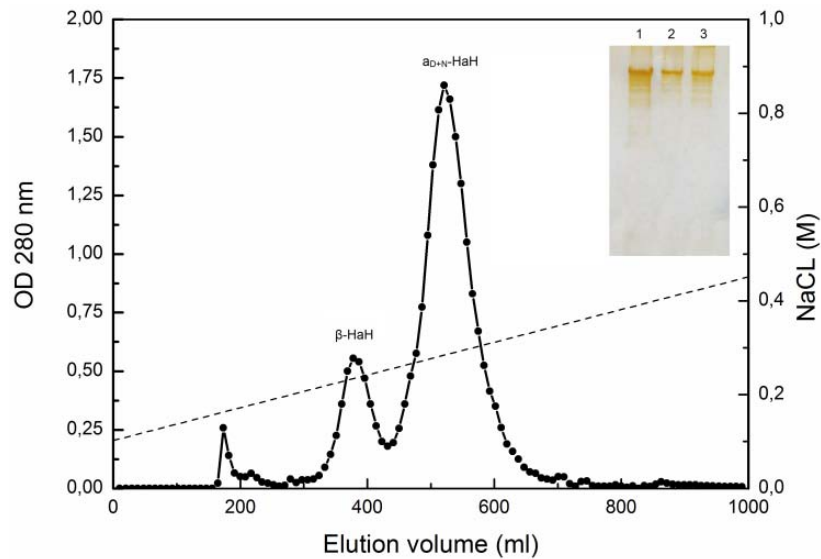
Fig. 1. Isolation of Hc isoforms (β-HaH and α_{D+N}-HaH) by ion exchange chromatography on a DEAE-Sepharose CL-6B column (32 x 1.2 cm), equilibrated and eluted with buffer 50 mM Tris-HCl, pH 8.0, using a linear gradient 0.1-0.45 M NaCl; flow rate 32 ml h^{-1}. Inset: 7.5% SDS-PAGE: lane 1, native HaH; lane 2, isoform β-HaH; lane 3, isoform α_{D+N}-HaH.
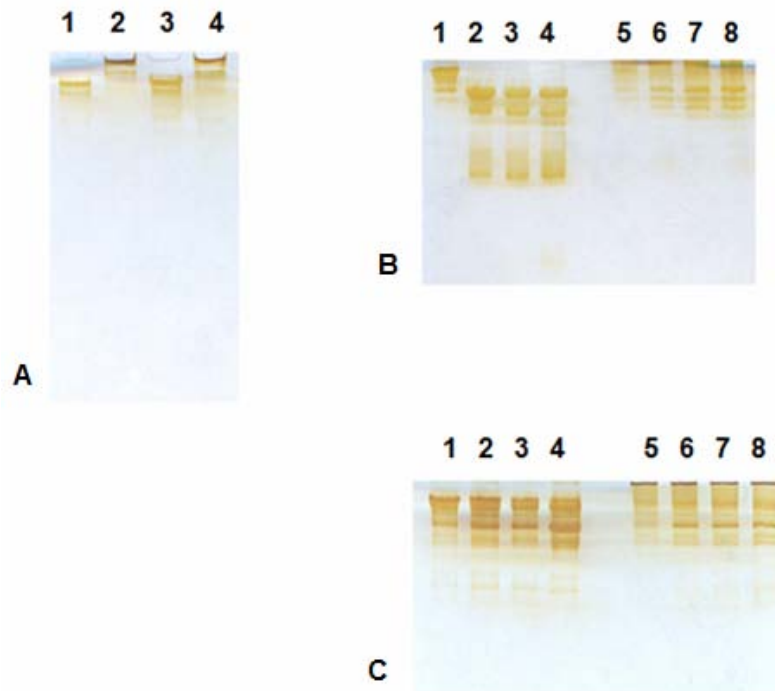
Fig. 2. A. Native PAGE in 7.5% acrylamide gel: lane 1, native HaH; lane 2, Ox-HaH; lane 3, native β-HaH; lane 4, Ox-β-HaH. **B.** 7.5% SDS-PAGE: lane 1, native β-HaH; lanes 2-4, β-HaH digested with trypsin (0.2% w/w) for 10, 30 and 60 minutes, respectively, at 37 °C; lane 5, Ox-β-HaH; lanes 6- 8, Ox-β-HaH digested with trypsin (0.2% w/w) for 10, 30 and 60 minutes, respectively, at 37 °C. **C.** 7.5% SDS-PAGE: lane 1, native β-HaH; lanes 2-4, β-HaH hydrolysed with proteinase K (0.2% w/w) for 10, 30 and 60 minutes, respectively, at 37 °C; lane 5, Ox-β-HaH; lanes 6-8, oxy-β-HaH hydrolysed with proteinase K (0.2% w/w) for 10, 30 and 60 minutes, respectively, at 37 °C.

side of the amino acids Lys or Arg. Proteinase K has broad specificity – it preferentially cleaves peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids. We assumed that trypsin would not be able to digest Ox-Hcs because the ε-amino groups of Lys were involved in the formation of Schiff bases. Indeed, the results showed that the native Hc molecules were rapidly degraded after incubation with trypsin (0.2% w/w) (Fig. 2b, lane 2-4). By contrast, Ox-Hcs were only partially degraded and retained in the stacking portion of the gel (Fig. 2b, lane 6-8). Proteinase K (0.2% w/w) almost equally cleaved native and Ox-Hcs, confirming the conclusion made above (Fig. 2c).

3.3. Spectroscopic characterization of modified Hcs

Fluorescence spectroscopy is one of the most sensitive methods for studying the protein structure and conformation in solution. After excitation at 295 nm, where the tryptophan side chains are selectively excited, the fluorescence spectra of native HaH and its isoforms showed an emission maximum position (λ_{max}) in the region 335-337 nm, which is typical for "buried" tryptophyl side chains in a hydrophobic environment within the protein [27]. Fluorescence spectra of Ox-Hcs did not show shift in the λ_{max} position (Fig. 3). An increase in the intensity of tryptophan fluorescence of Ox-Hcs was observed, indicative of conformational

changes, which made tryptophan residues more exposed to the polar solvent (water).

The changes in the absorption spectra can be assigned to the changes in the microenvironment near the chromophores (aromatic amino acid residues) or/and near the Cu (II) active site, which can be due to denaturation or rearrangement of the protein secondary structure (refolding). Absorption spectra taken for native and Ox-Hcs showed that the intensity of the characteristic copper (II)-peroxide complex band at 345 nm slightly decreased as a result of modification with sodium periodate (Fig. 4). Therefore, induced local conformational changes did not affect the integrity of copper active sites in Hc molecules.

The effect of the modification with sodium periodate on the secondary structure of HaH was followed using ATR-FTIR spectroscopy in the region 1720-1580 cm^{-1} [28]. Analysis of the amide I adsorption band was done in order to determine the secondary structure content of the native and Ox-HaH. The band area of each structural element was presented as a percentage of the total area (Table 1). Rearrangement in the Ox-HaH molecule, but still close to the native conformation, was observed. In comparison to the native protein, Ox-HaH characterizes with an increase in the random coil and β-turns mostly at expenses of the native β-sheets. An increase in the intensity of the absorption
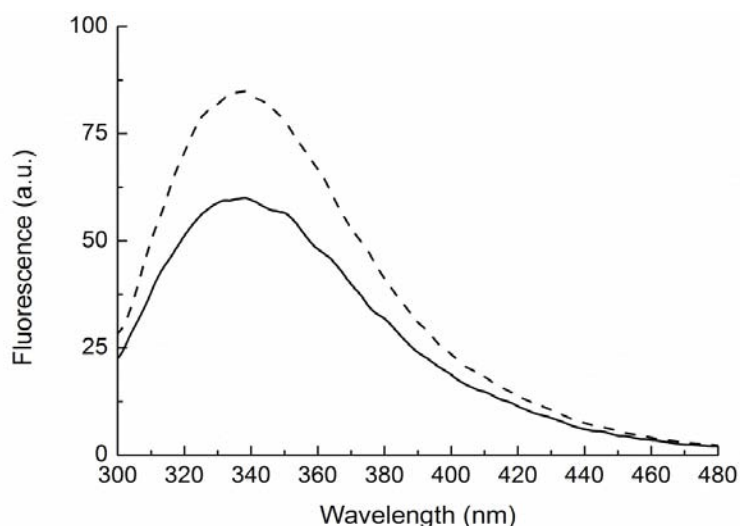
Fig. 3. Fluorescence spectra of native isoform β-HaH (straight line) and Ox-β-HaH (dash line) in buffer 50 mM Tris-HCl, pH 8.0, excited at 295 nm.
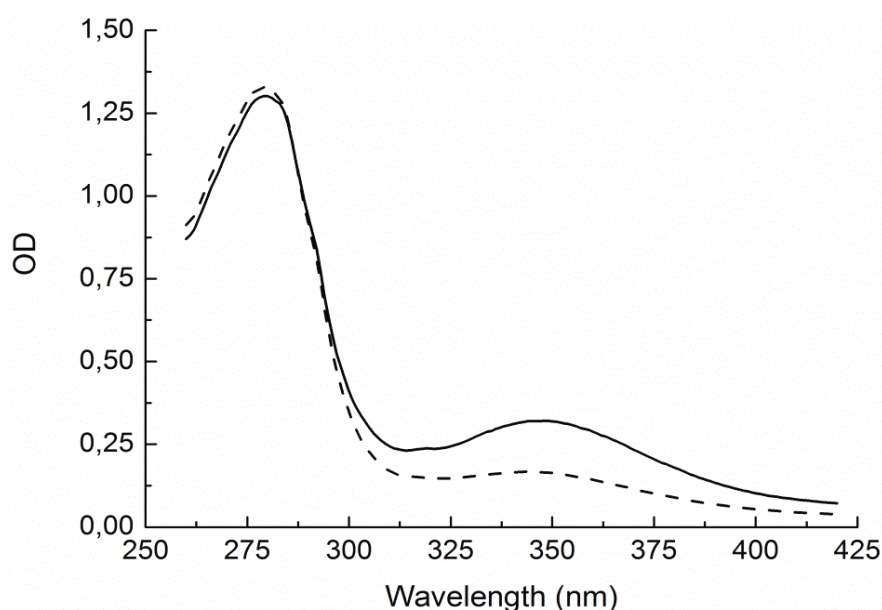
Fig. 4. Absorption spectra of native isoform α_{D+N}-HaH (straight line) and Ox-α_{D+N}-HaH (dash line) in buffer 50 mM Tris-HCl, pH 8.0.

Table 1. Secondary structural elements calculated from the curve-fitted FTIR spectra of native and oxidized HaH.
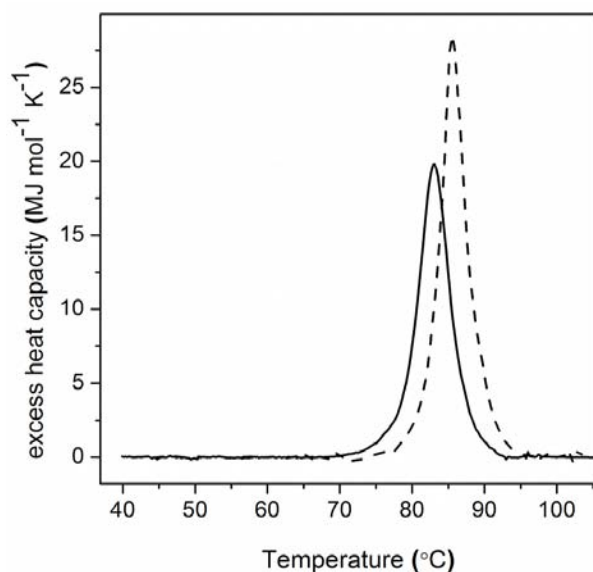
Native HaH		Ox-HaH		
ν [cm^{-1}]	Band area [%]	ν [cm^{-1}]	Band area [%]	Assignment
1606	5.81	1600	5.64	tyrosine residues
1616	7.82	1617	12.21	aggregated strands / side chains
1625	12.32	-	-	β-sheets
1634	14.73	1631	15.06	β-sheets
1643	15.52	1642	19.70	α-helix / random coil
1651	15.07	1652	23.84	α-helix
1659	9.52	-	-	α-helix
1669	9.68	1668	13.79	β-turns
1679	6.25	1682	7.53	β-turns / intermolecular β-sheets
1690	3.29	1696	2.23	intermolecular β-sheets

bands at 1617 and 1682 cm^{-1} were observed, which were typical of the formation of intramolecular anti-parallel β-sheet aggregates.

3.4. Differential scanning calorimetry (DSC)

Intramolecular cross-linking has been shown to increase thermal stability of different proteins [29, 30]. Differential scanning calorimetry (DSC) is the most useful technique for characterizing thermal stability of proteins in terms of their thermodynamic characteristics [31]. DSC measurements of the native and Ox-Hcs were performed in buffer 50 mM Tris-HCl, pH 8.0, at a heating rate of 1 °C min^{-1}. In all cases the thermal unfolding was found to be calorimetrically irreversible, as no thermal effect was observed in a second heating of the protein solutions. Consistent with our previous study [32], one main transition with apparent transition

Fig. 5. Experimental C_p transition curves of native HaH (straight line) and Ox-HaH (dash line) in buffer 50 mM Tris-HCl, pH 8.0, recorded at a heating rate of 1 °C min^{-1}. Protein concentration was 3.2 mg ml^{-1}.

Table 2. DSC parameters for the thermal denaturation of native and modified Hcs from *H. aspersa* maxima in buffer 50 mM Tris-HCl (pH 8.0), at a heating rate of 1 °C min^{-1}.

Hemocyanin	ΔH_{cal} [MJ mol^{-1}]	C_p^{ex} [MJ mol^{-1} K^{-1}]	T_m [°C]
Native HaH	118.6 ± 1.0	19.78	83.0
Ox-HaH	145.0 ± 1.0	28.55	85.6
Native β-HaH	69.9 ± 1.0	8.51	82.1
Ox-β-HaH	81.6 ± 1.0	9.98	85.4
Native α$_{D+N}$-HaH	109.0 ± 1.0	21.96	83.0
Ox-α$_{D+N}$-HaH	135.5 ± 1.0	27.52	85.8

temperatures (T_m) at 83.0 °C, were detected in the thermogram of native HaH at the above-mentioned conditions (Fig. 5).

The thermal stability of Ox-HaH was higher compared with native Hc, the T_m value increased to 85.6 °C. Integration of the heat capacity (C_p) of the protein sample *vs.* temperature yields the enthalpy (ΔH) of the unfolding process, which is due to endothermic events such as the breaking of hydrogen bonds, and exothermic processes such as the disruption of hydrophobic interactions [33]. The ΔH_{cal} value for native HaH is 118.6 MJ mol^{-1}, while higher ΔH_{cal} value of 145.0 MJ mol^{-1} was determined for Ox-HaH. This effect is most likely related to the induced cross-linking in the protein molecule as a result of modification. The values of T_m and ΔH_{cal} obtained for the modified

isoforms Ox-β-HaH and Ox-α$_{D+N}$-HaH also increased in comparison with the native ones. Table 2 summarizes DSC data for the process of thermal denaturation of native and Ox-Hcs, obtained at the same experimental conditions (buffer 50 mM Tris-HCl, pH 8.0; heating rate of 1 °C min^{-1}).

4. CONCLUSION

In conclusion, the results of the present investigation reveal that the oxidation of carbohydrate moieties in the Hc, purified from garden snail *H. aspersa* maxima, with sodium periodate leads to the enhancement of the structural stability, in particular the resistance to proteolytic cleavage, as well as the thermal stability of this oxygen-transport protein. Hcs isoforms were influenced in a similar way from the modification. Further evaluation of

the impact of the increased structural stability of modified Hcs on their immunological properties and/or antitumor activity would be of interest.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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