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Modification with sodium periodate increases the structural stability of molluscan hemocyanins

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th anniversary

Hemocyanins (Hcs) are large glycoproteins present in the blood of some mollusks and arthropods. In addition to their biological function, molluscan Hcs have shown promising properties in the development of various medicinal products. In the present study, the carbohydrate moieties of two representatives of Hcs from molluscan species, namely those isolated from the marine snails *Rapana thomasiana* and terrestrial snails *Helix pomatia*, were oxidized with sodium periodate. This chemical modification led to increased structural and thermal stability of oxygen-transport proteins.

Key words: hemocyanin; periodate oxidation; thermal stability

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 are multifunctional proteins. 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, molluscan Hcs have indicated promising properties in the development of various medicinal products including antiviral agents, conjugate vaccines and immunotherapy of cancer [4-6]. Thus, it was 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]. Moreover, it was demonstrated that these Hcs expressed strong *in vivo* anti-cancer and anti-proliferative effects in murine model of colon carcinoma [9].

Achieving structural stabilization in proteins having therapeutic application is an important task. Chemical modification is one approach to improve the protein's stability. It has been reported that the periodate oxidation of the carbohydrate moieties of proteins with sodium periodate has induced structural stabilization and enhanced their immunogenicity [10, 11].

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 [12]. The aim of the present study is to enhance the structural stability of two representatives of gastropodan Hcs, namely RtH and HpH, by chemical oxidation of their sugar moieties.

EXPERIMENTAL

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.

Isolation and purification of Hcs

RtH was purified from the hemolymph, collected from marine snails *R. thomasiana*, according to the procedure described in [13]. The β-isoform of HpH was isolated from the hemolymph of terrestrial snails *H. pomatia* as described elsewhere [14,15].

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Absorption spectroscopy

Absorption spectra of Hcs were recorded using Evolution™ 300 UV-Vis spectrophotometer (Thermo Electron Corporation). The concentration of protein solutions was determined spectrophotometrically using specific absorption coefficient $A_{278}^{0.1\%}$ = 1.36 ml.mg^{-1}.cm^{-1} for RtH [13] and $A_{278}^{0.1\%}$ = 1.416 ml.mg^{-1}.cm^{-1} for β-HpH [16], respectively.

Chemical modification of Hcs

The chemical oxidation with sodium periodate method was performed to modify RtH and β-HpH as described in [11]. Briefly, each Hc (protein concentration 2 mg/ml) 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 a solution of ethylene glycol was added to each 2 ml of protein and incubated overnight at 4 ˚C. At the last step, the protein samples were concentrated by ultrafiltration, dialyzed against buffer 50 mM Tris-HCl, pH 8.0, and filtered through 0.22-µm membrane filter.

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 at 37 ˚C for 30 min in a buffer 50 mM Tris-HCl, pH 8.6, and stopped with an addition of 1 % PMSF.

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 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 9 000 000 Da for Hc was used in the calculation of molar quantities.

SDS-PAGE

Hc samples were analyzed by SDS-PAGE on 10% separating gel, as described by Laemmli [17]. 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.

RESULTS AND DISCUSSION

Carbohydrate content of 2.6 % (w/w) has been determined for RtH [12], while β-HpH contains 7 % (w/w) carbohydrates [16]. These Hcs contain, besides the commonly occurring sugars D-mannose, D-galactose, L-fucose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine also D-xylose and 3-*O*-methyl-D-galactose, unusual carbohydrates for animal glycoproteins. To stabilize the structure of 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 and the reactive aldehydes, formed by the oxidation procedure (Fig. 1).

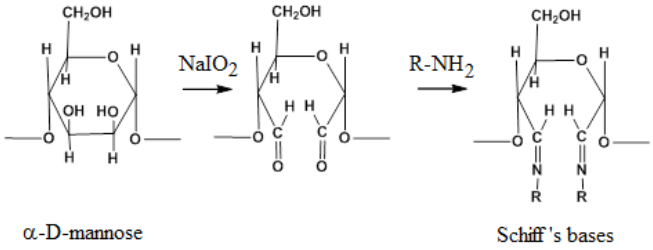

Fig. 1. Chemical strategy for oxidation of carbohydrates with sodium periodate and Shiff's base formation.

SDS-PAGE analysis showed differences in the mobility pattern between the native and modified Hcs. Oxidized Hcs (Ox-Hcs) did not enter the resolving portion of the gel (Fig. 2, lane 4). This effect was attributed to the internal cross-linking within Hc molecules as a result of periodate treatment [11].

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 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.

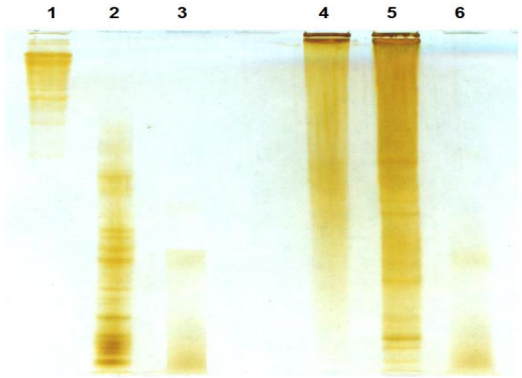

Fig. 2. 10 % SDS-PAGE: lane 1, native RtH; lane 2 and 3, native RtH digested with trypsin (0.2 % w/w) and proteinase K (0.2 % w/w) for 10 min at 37 °C, respectively; lane 4, oxy-RtH; lane 5 and 6, oxy-RtH digested with (0.2 % w/w) and proteinase K (0.2 % w/w) for 10 min at 37 °C, respectively.

We assumed that trypsin would not be able to digest Ox-Hcs because the ε-amino groups of Lys were involved in formation of Schiff bases. Indeed, the results showed that the native Hc molecules were rapidly degraded after incubation with trypsin (0.2% w/w) for 30 min (Fig. 2, lane 2). By contrast, Ox-Hcs were only partially degraded and retained in the stacking portion of the gel (Fig. 2, lane 5). Proteinase K (0.2% w/w) equally cleaved native and Ox-Hcs, confirming the conclusion made above (Fig. 2, lane 3 and 6).

Absorption spectra taken for native and Ox-Hcs showed that the intensity of the characteristic copper-dioxygen band at 345 nm slightly decreased as a result of modification with sodium periodate (Fig. 3). Therefore, induced local conformational changes did not affect the integrity of copper active sites in Hc molecules.

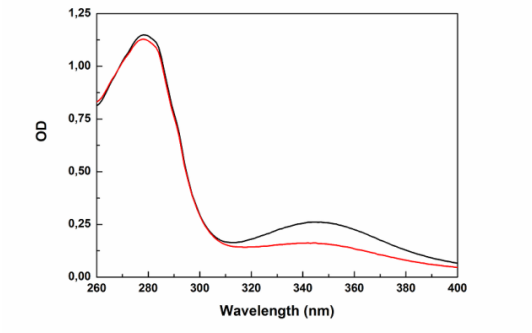

Fig. 3. Absorption spectra of native β-HpH (black line) and Ox-β-HpH (red line) in buffer 50 mM Tris-HCl, pH 8.

Intramolecular cross-linking has shown to increase thermal stability of different proteins [18, 19]. Differential scanning calorimetry (DSC) is the most useful technique for characterizing thermal stability of proteins in terms of their thermodynamic characteristics [20]. DSC measurements of the native and modified Hcs were performed in buffer 50 mM Tris-HCl, pH 8.0, at a heating rate of 1 °C/min. 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 [21], one main transition and a shoulder with apparent transition temperatures (T_m) at 77.49 °C and 88.6 °C, were detected in the thermogram of native RtH (Fig. 4).

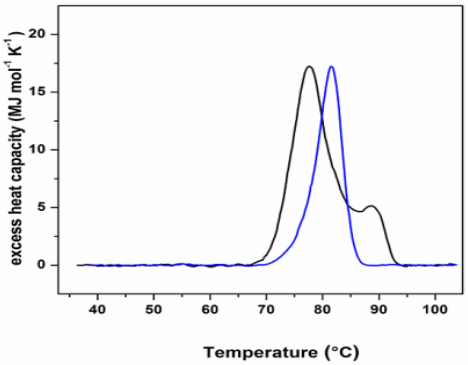

Fig. 4. Experimental C_p transition curves of native RtH (black line) and Ox-RtH (blue line) in buffer 50 mM Tris-HCl, pH 8.0, recorded at a heating rate of 1 °C/min. Protein concentration was 2.8 mg/ml.

The thermal stability of Ox-RtH is higher compared with native Hc. The T_m value for the main transition increased to 81.61°C. Moreover, the thermogram of Ox-RtH has more cooperative character with $T_{1/2}$ of 5.13 °C (Table 1).

Table 1. Parameters for the thermal denaturation of native and modified Hcs from *R. thomasiana* and *H. pomatia*, obtained by DSC, at a heating rate of 1 °C/min.

Hemocyanin	ΔH_{cal} [MJmol^{-1}]	C_P^{ex} [MJ mol^{-1}K^{-1}]	T_m, [°C]	$T_{1/2}$ [°C]
Native RtH	173.1	17.22	77.49*	7.67
Ox-RtH	102.2	17.21	81.61	5.13
Native β-HpH	110.6	20.1	82.4	4.64
Ox-β-HpH	96.3	14.7	84.2	5.46

* Main transition

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 [22]. The ΔH_{cal} value for native RtH is 70.9 MJ mol^{-1} higher than that determined for Ox-RtH. This effect is probably related to the induced cross-linking in the

protein molecule as a result of modification. One transition with T_m value of 82.4 °C was detected in the thermogram of native β-HpH [23]. The T_m value obtained for Ox-β-HpH also was shifted towards the higher temperature of 84.2 °C (Fig. 5).

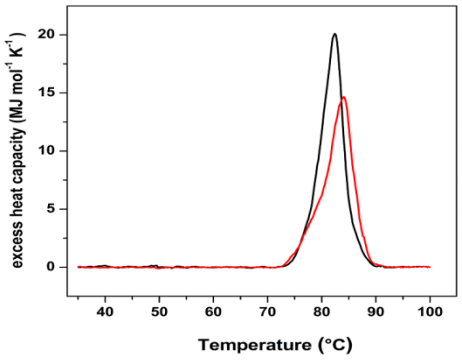

Fig. 5. Experimental C_P transition curves of native β-HpH (black line) and Ox-β-HpH (red line) in buffer 50 mM Tris-HCl, pH 8.0, recorded at a heating rate of 1 °C/min. protein concentration was 2.35 mg/ml.

The ΔH_{cal} value for modified β-HpH decreases with 14.3 MJ mol^{-1}. It seems structural differences between both Hcs in terms of reactive functional groups have determined the effect of modification. Table 1 summarizes DSC data for the process of thermal denaturation of native and modified Hcs.

CONCLUSION

In conclusion, the results of the present investigation reveal that the oxidation of carbohydrate moieties in Hcs with sodium periodate leads to enhance of the structural stability, in particular the resistance to proteolytic cleavage, as well as the thermal stability of these oxygen-transport proteins. Further evaluation the influence of the increased structural stability of modified Hcs on their immunological properties would be of interest.

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МОДИФИКАЦИЯ С НАТРИЕВ ПЕРЙОДАТ УВЕЛИЧАВА СТРУКТУРНАТА СТАБИЛНОСТ НА ХЕМОЦИАНИН ОТ МОЛЮСКОВИ ОРГАНИЗМИ

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(Резюме)

Хемоцианините са олигомерни гликопротеини присъстващи в кръвта на някои мекотели и членестоноги. В допълнение към тяхната важна биологична функция, хемоцианините от молюскови организми показват обещаващи свойства в разработването на различни лекарствени продукти. В настоящото изследване, въглехидратните вериги на два представителя на молюсковите хемоцианини, а именно тези, изолирани от морски охлюви *Rapana thomasiana* и градински охлюви *Helix pomatia*, бяха окислени с натриев перйодат. Тази химична модификация доведе до повишена структурна и термична стабилност на изследваните кислород-пренасящи протеини.