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Structural destabilization and enhanced cytotoxicity on murine fibroblasts of *Helix pomatia* ß-hemocyanin in presence of four cholinium amino acids

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

Four complexes of ß-hemocyanin from *Helix pomatia* (ß-HpH) with ionic liquids (ILs) based on cholinium cation and amino acid anion were prepared. Using FTIR spectroscopy we observed that the tested ILs were able to induce conformational changes in the protein molecule. In the presence of cholinium methionate we detected the most significant changes in ß-HpH secondary structure, which is expressed in a 2-fold increase of the intensity of the absorption band that is assigned to the side-chain amino acid residues and a complete loss of the α-helical structures at expense of the ß-structures. Interestingly, the aggregation in this case seemed to be suppressed. In an experiment in vivo using 3T3 cells (fibroblasts), we found that the destabilization of the protein structure resulted in an enhanced cytotoxicity of the ß-HpH-IL complexes in respect to the native ß-HpH. The effect is stronger, both concentration- and time-dependent for the complex of ß-HpH with cholinium tryptophanate.

Key words: *Helix pomatia* hemocyanin; cholinium amino acids; protein secondary structure; cytotoxicity assay

INTRODUCTION

Hemocyanins (Hcs) are large copper-containing oxygen-transporting proteins that are freely dissolved in the hemolymph of many mollusks and arthropods [1]. The interest of these proteins has increased markedly over the last decades due to their great potential for application in medicine. Many authors reported on immunostimulatory, anti-cancer and antibacterial properties of Hcs isolated from various sources [2–6]. Many studies have been focused on the elucidation of structure and factors that contribute or affect the stability of Hcs [1,7,8].

The hemocyanin isolated from the hemolymph of terrestrial snail *Helix pomatia* (HpH) consists of two alpha components (α_D-HpH and α_N-HpH) and one beta component (ß-HpH). Beta-hemocyanin is characterized with subunit homogeneity, and therefore in comparison to α_D-HpH and α_N-HpH, has been more frequently used for structural studies [9, 10]. HpH characterizes with relatively high carbohydrate content (ca 9%), which probably correlates with the protein immunogenicity tested in animal models but did not have an effect on the protein thermal stability [5,8,11]. The therapeutic effect of ß-HpH was shown in murine model of colon carcinoma as well as its adjuvant potential for microbial and viral antigens [5,12]. In addition, ß-HpH exhibits a phenoloxidase activity, which can be enhanced by detergent treatment and lyophylisation which results in conformational changes of the protein [13].

Ionic liquids (ILs) are salts that consist of an organic cation and/or organic anion that are characterized with melting temperatures below 100°C, low vapor pressure and tunable physic-chemical characteristics [14]. Biodegradable and biocompatible ILs are of great interest in view of their biotechnological application as reaction media for biocatalysis or isolation of proteins or plant metabolites, as additives aiming to enhance thermal or storage stability of proteins [15–18].

The aim of this paper is to follow the changes of the secondary structure of ß-HpH induced by four ILs containing cholinium cation and amino acid anion. All anions that are in the focus of the study are non polar amino acids, however their side-chains differ substantially in size and structure; therefore, we suppose that possibly differences in their interactions with proteins can be observed. In addition, we evaluated the cytotoxic effect of the

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modified ß-HpH on murine fibroblasts in respect to changes in its structure. Results are discussed in comparison to those obtained for the native ß-HpH.

EXPERIMENTAL

Materials

Beta-hemocyanin from *Helix pomatia* (ß-HpH) was isolated as described in previous study [10]. Cholinium glycinate [Chol][Gly], cholinium valinate [Chol][Val], cholinium methionate [Chol][Met] and cholinium tryptophanate [Chol][Trp] were synthesized as given in [19]. Murine embryotic fibroblast (3T3) cell line was purchased from American Type Culture Collection (ATCC). Thiazolyl Blue Tetrazolium Bromide (MTT) (98%) was obtained from Sigma. DMEM high glucose media, L-glutamine and sodium bicarbonate were purchased from PAN-Biotech GmbH, Aidenbach, Germany.

Fourier transform infrared spectroscopy (FTIR)

Prior to the measurements, 0.08 mL ß-HpH (16.4 mg/mL) were incubated for 60 min at room temperature with 0.02 mL of 1M solution of the corresponding [Chol][AA] dissolved in sodium phosphate buffer (pH 7.2, 5mM). FTIR spectra of the ß-HpH-[Chol][AA] complexes were recorded on Bruker Tensor 27 spectrometer, equipped with a detector of deuterated triglycine sulphate (DTGS). The FTIR spectra were collected by direct deposition of the samples on attenuated total reflectance (ATR) element, a diamond crystal, in the frequency region 4000 – 600 cm^{-1} with 128 scanning and at resolution of 1 cm^{-1}. The absorptions of the medium and ILs were taken into account. For each sample, the protein secondary structure content was estimated in few steps: 1) Fourier deconvolution using Opus software version 5.5 at band width of 14 cm^{-1}, 2.9 resolution enhancement factor, and Lorentzian lineshape; 2) obtaining the second derivative spectra by the Savitzky-Golay algorithm based on 25 smoothing points; 3) curve fitting according to the Local Least Squares algorithm as the initial bandwidth of all components was set to 14 cm^{-1} and the components were approximated by mixed Lorentzian/Gaussian functions and setting [20].

Cytotoxicity assay

Murine fibroblasts (3T3) were cultured in DMEM high glucose medium containing L-Glutamine, Penicillin-Streptomycin-Amphotericin B and 10% fetal bovine serum at humidified atmosphere, 37°C and 5% CO_2. Then, the cells were seeded in a sterile 96-well plate at 1x 10^4 cells per well and incubated for 24 hrs at 37°C and 5% CO_2 for obtaining adherent cell cultures and good cell spreading. Next, the cells were incubated for additional 24 or 48 hrs with ß-HpH or ß-HpH - [Chol][AA] complexes, at concentrations ranging from 100–700 µg/mL. The ß-HpH-[Chol][AA] complexes were obtained as described above. Cytotoxicity assay was performed as described in the literature using tetrazolium dye, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [21]. For each well, the conversion of MTT into formazan was monitored spectrophotometrically at 570 nm and was used to be estimated the percentage of the viable cells in respect to control experiment without added ß-HpH or ß-HpH-IL-complexes. Blank experiments containing only reaction medium were also performed. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

IL-induced structural changes

The effect of the four selected for this study choline amino acids on ß-HpH secondary were followed using ATR-FTIR spectroscopy. Analysis of the amide I adsorption band was done in order to be determined the secondary structure content for each sample. The observed peak positions in the spectral region 1700-1600 cm^{-1} are: α-helix (1660-1652 cm^{-1}), unordered structures or random coils (1951-1942 cm^{-1}), ß-sheets (1640-1624 cm^{-1}), ß-turns (1679-1668 cm^{-1}), anti-parallel ß-sheets (1693-1681 cm^{-1}) and side chains of the amino acid residues (1609-16003 cm^{-1}). The band area of each structural element was presented as a percentage of the total area and the changes of the conformation of the native ß-HpH in presence of [Chol][AA] can be seen in Fig.1. The most significant changes in the secondary structure were observed in presence of [Chol][Met]. For example, for this ß-HpH-IL complex we detected a complete loss of the α-helical structures at expense of the ß-structures. The 2-fold increase of the intensity of the absorption band that is assigned to the side-chain amino acid residues implies a denaturation of the protein in presence of the methionate, however, it seems that in this medium the protein aggregation is suppressed. In presence of the glycinate an increase in the α-helices is observed implying that ß-HpH occupies more folded conformation in this solution.

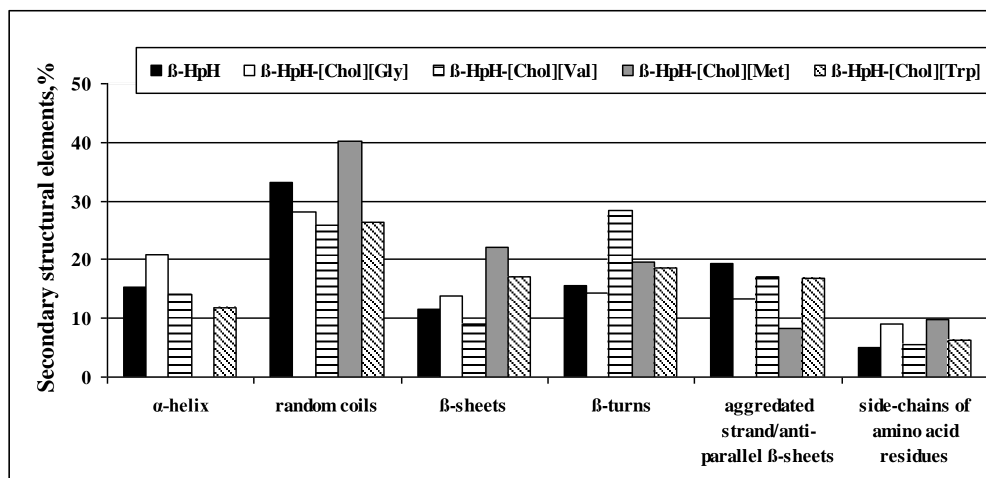

Fig. 1. Elements of the protein secondary structure (%) determined form deconvoluted ATR-FTIR spectra for native ß-HpH and its complexes with choline amino acids.

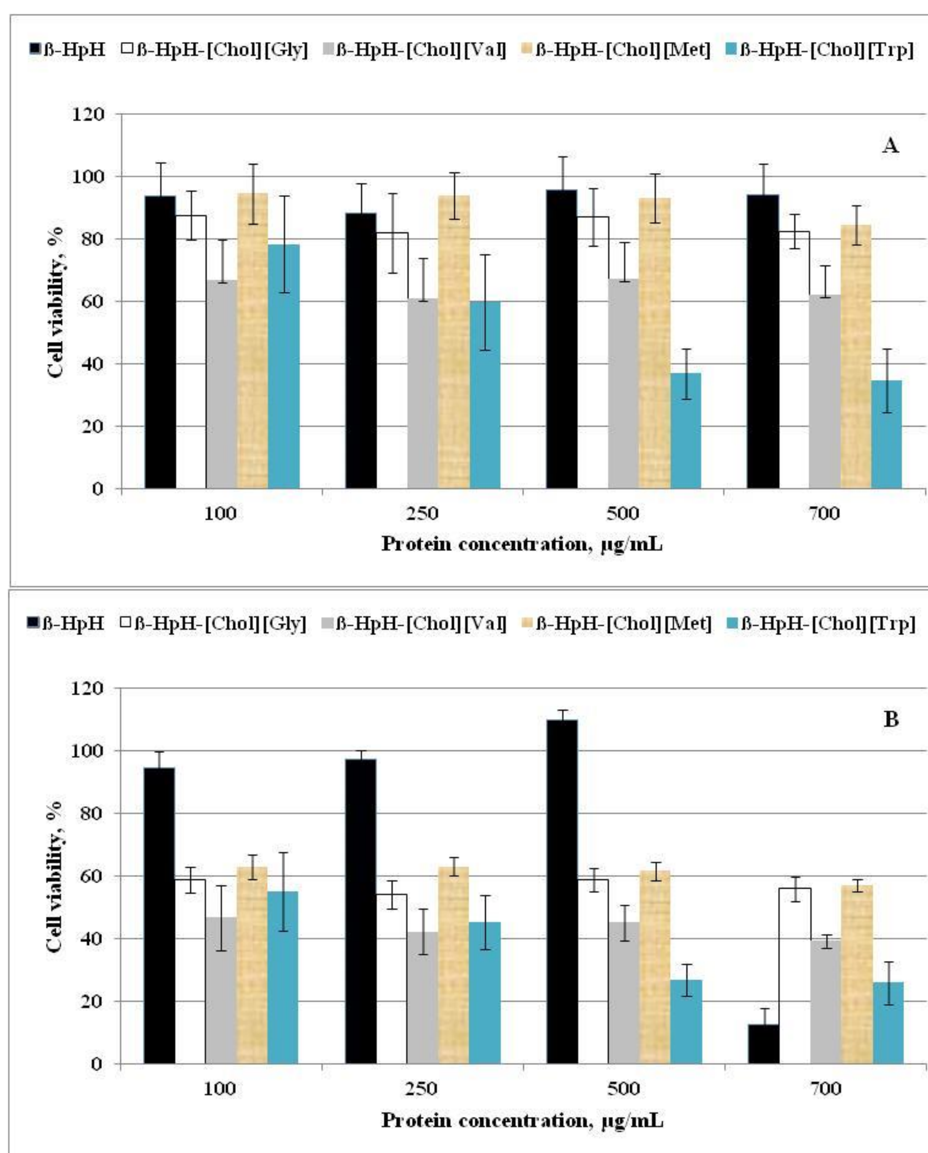

Fig. 2. Percentage of viable fibroblasts after incubation with ß-HpH and its complexes for 24 h (A) and 48 h (B).

In comparison to the native protein, ß-HpH-[Chol][Val] characterizes with an increase of the coil and unordered structures mostly at expenses of the ß-structures. Rearrangement in the protein molecule, but still close to the native conformation, was also observed in presence of the tryptophan-based IL.

3T3 cytotoxicity assay

Murine fibroblasts (3T3 cells) cytotoxicity assay is used as supporting identification or screening of substances with potential acute oral toxicity or skin irritation [22]. Viability of 3T3 cells in presence of ß-HpH and ß-HpH-IL was assessed at 24 and 48h after incubation. The native protein and its complexes were tested in a concentration range from 100 to 700 µg/mL. The exposure of 3T3 cells to a high concentration (> 500 µg/mL) of native ß-HpH for 48 h resulted to a moderate cell growth stimulation. However, tested at lower concentrations or after a short-tem incubation (24h) ß-HpH did not affect the viability of the fibroblasts. As can be seen in Fig. 2, ß-HpH became more cytotoxic to 3T3 cells after being modified with the four choline amino acids. The strongest negative effect which is both concentration- and time-dependent was observed for ß-HpH-[Chol][Trp]. A 24-hour exposure of fibroblasts to ß-HpH-[Chol][Met] do not reduce cell growth. However, longer exposure to this complex resulted in a decrease in cell viability by 40% which was observed for the whole concentration range (Fig. 2B). The effect of ß-HpH-[Chol][Gly] was concentration independent. For this complex, we observed a moderate (15-18%) to high (40%) decrease in cell viability after 24 and 48 h incubation, respectively. ß-HpH-[Chol][Val] exhibited stronger inhibitory effect on the 3T3 cell growth in comparison to that found for the glycinate complex, however it was also only time dependent.

CONCLUSION

Even added in low concentration to the reaction mixture, the four tested [Chol][AA] induce significant changes in the secondary structure of ß-HpH. The structural destabilization of the hemocyanin results in its enhanced cytotoxicity on fibroblasts. The observed effect is time-dependent for the whole series of the tested compounds. However, concentration dependence is found only for ß-HpH-[Chol][Trp].

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ДЕСТАБИЛИЗАЦИЯ И ПОВИШЕНА ЦИТОТОКСИЧНОСТ ВЪРХУ ФИБРОБЛАСТИ НА БЕТА-ХЕМОЦИАНИН ОТ *HELIX POMATIA* В ПРИСЪСТВИЕ НА ЧЕТИРИ ОРГАНИЧНИ СОЛИ НА ОСНОВАТА НА ХОЛИН И АМИНОКИСЕЛИНИ

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(Резюме)

Получени са четири комплекса на бета хемоцианин от *Helix pomatia* (ß-HpH) с йонни течности (ЙТ) на основата на холинов катион и аниони, остатъци от аминокиселини. Промените във вторичната структура на протеина индуцирани от ЙТ са проследени с помощта на ИЧ спектроскопия. Установено е, че холинил метионата оказва най-голям ефект върху структурата на ß-HpH. В ИЧ спектрите на този комплекс не се наблюдава абсорбционната ивица характерна за α-спирални структури, а интензивността на ивицата, отнасяща се за страничните вериги на аминокиселинните остатъци на протеина е увеличена до два пъти. Въпреки наблюдаваната значителна денатурация на ß-HpH в присъствие на холинил метионат, се вижда, че агрегацията на протеина в този разтвор е подтисната. В допълнение, беше установено, че структурната дестабилизация на ß-HpH води до повишаване на цитотоксичността на комплексите на ß-HpH-ЙТ спрямо фибробластни клетки. Ефектът е най-значителен за комплекса на ß-HpH с холинил триптофанат и зависи от концентрацията на ЙТ и времето за третиране.