

Scientific paper

Biophysical Properties and Cytotoxicity of Feruloylated *Helix Lucorum* Hemocyanin

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Abstract

For the first time *Helix lucorum* hemocyanin (HIH) has been feruloylated. Two HIH conjugates with 40- and 120- ferulic acid residues were prepared, denoted as FA-HIH-1 and FA-HIH-2. Expectedly, the feruloylation of HIH induced a rearrangement of the protein molecule, a decrease in the α -helical structure at the expense of β -structures was observed. Besides, the FA-HIH conjugates were more prone to aggregation, which is probably due to the stabilization of the partially unfolded protein molecules by non-covalent bonding. Interestingly, the thermal stability of HIH was not affected by the modification. The native and feruloylated HIH were not toxic to normal fibroblasts (BJ cells). We observed a decrease in cell viability of breast cancer MCF-7 cells to about 66% after a 48h exposure to 70 μ g/well of FA-HIH-2.

Keywords: Hemocyanin conjugates; ferulic acid; conformational stability; thermal stability; cytotoxicity

1. Introduction

Ferulic acid (FA) is a naturally occurring antioxidant, radical scavenging and photoprotective agent with huge therapeutic potential. It has been reported that FA has also strong hepatoprotective activity.¹ Much attention has been paid in the literature on the beneficial role of FA in the treatment and prevention of Alzheimer's disease, a serious neurodegenerative disorder.² In addition, FA inhibits melanin production, therefore can be involved in formulations for skin depigmentation.³ Interestingly, graft copolymers containing ferulic and hyaluronic acid exhibit wound healing properties and can be involved in medicines for the treatment of chronic and acute wounds.⁴ FA is also considered to have potential as an anti-inflammatory drug for curing endometritis. It has been shown that FA reduces the level of the lipopolysaccharide-induced pro-inflammatory cytokines released by bovine endometrial epithelial cells if they are pretreated with the compound.⁵ On the other hand, chitosan-FA conjugates are proposed as novel drug delivery system based,⁶ while FA-modified glycol chitosan exhibited remarkable neuroprotective activity and can be applied in functional resto-

ration of the traumatically injured spinal cord, a life-threatening trauma.⁷ Recently, Caia et al. have reported the preparation of functional foods based on FA grafted curdlans, water-insoluble linear beta-1,3-glucans.⁸ In addition, Liu et al. have shown that daily supplements of a sodium ferulate increased the relative abundance of *Lactobacillus* and *Parabacteroides* in mice model of transverse aortic constriction and the changes in the gut microbiota resulted in an improved cardiac function in the tested animals.⁹

Here we report the preparation of two FA-hemocyanin conjugates. Hemocyanins (Hc) are large complex respiratory proteins freely dissolved in the hemolymph of arthropods and mollusks.¹⁰ Hc isolated from *Keyhole limpet* (KLH) is commercialized and is applied as an immunotherapeutic in case of prostate cancer for several decades.¹¹ Currently, many other vaccines against non-Hodgkins lymphoma, cutaneous melanoma, breast and bladder cancer, that are based on KLH undergo clinical trials. In addition, the whole protein or its subunits are used protein carriers or adjuvants.¹¹ Many other Hcs have been isolated and structurally characterized, some exhibit anticancer, immunostimulatory, antiviral, antifungal activities in in vitro and in vivo assays.^{12,13,14} At present,

KHL- β -1,2-mannan-peptide conjugates are tested as anti-fungal vaccines against *C. albicans*, *C. tropicalis*, *C. lusitanae*, and *C. glabrata*.^f Based on KHL-conjugates are developed easy and fast ELISA methods for detection neurotoxins, vaccines against drugs of abuse and vaccines administrated by new routes.^{15,16,17} Hc from *Helix lucorum* (HIH) has been recently isolated and characterized.¹⁸ Lately, it has been reported that HIH and its structural subunits are cytotoxic to bladder carcinoma cells.¹⁹

The aim of the study is to prepare FA-HIH conjugates and to assess the effect of the feruloylation on the HIH conformation and thermal stability. Cytotoxic effect of the FA-HIH conjugates against fibroblasts (BJ cells), hormone-dependent breast cancer cells (MCF-7 cells) and hormone-independent breast cancer cells (MDA-MB-231 cells). Experiments with the native HIH were conducted for comparison.

2. Materials and Methods

2.1. Materials

Ferulic acid (>99%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (>98%) (EDC), N-hydroxysuccinimide (>98%) (NHS), thiazolyl blue tetrazolium bromide (98%, MTT), Dulbecco's Modified Eagle's (DMEM) low glucose medium, fetal bovine serum, 0.25% trypsin-EDTA solution and penicillin-streptomycin-amphotericin B antibiotic and disposable PD-10 columns were purchased from Sigma-Aldrich.

Hc was isolated from the hemolymph of *Helix lucorum* garden snails as described in.¹⁰ All other used organic solvents and salts were analytical grade.

The human breast carcinoma cell lines (MCF-7 and MDA-MB-231) and normal human skin fibroblasts (BJ cell line) were obtained from the American Type Culture Collection (ATCC).

2.2. Preparation of the FA-HIH Conjugates

At first, FA was stirred with EDC/NHS in 50 mM phosphate saline buffer (PBS)(pH 7.2) at a stoichiometric molar ratio of FA/EDC/NHS = 1:2:2 for 2 h at room temperature. Then, a 100-to 500-fold molar excess of the obtained NHS-ester of FA was added to the native HIH dissolved in PBS. The mixture was stirred at room temperature for 4 h, and then incubated at 4 °C overnight. The FA-HIH conjugates were purified on prepacked PD-10 desalting columns following the manufacturer's instructions.

The concentration of the native and feruloylated HIH was determined using the Bradford assay.²⁰

Absorbance spectra of the protein samples were recorded on Evolution™ 300 UV-Vis Spectrophotometer (Thermo Electron Corporation) equipped with a Peltier temperature control accessory with the highest resolution (1 nm). The degree of feruloylation was estimated using

the differences in the spectra of the native and the feruloylated HIH at 315 nm. The molar extinction coefficient of ferulic acid at 315 nm in PBS (pH 7.2, 50 mM) is 13 740 L · mol⁻¹ · cm⁻¹.

2.3. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectra were recorded on Bruker Vertex 70 spectrometer equipped with a diamond ATR accessory in the frequency region 4000–600 cm⁻¹ with 128 scanings at a resolution of 1 cm⁻¹. Protein samples were dissolved in PBS buffer (pH 7.2, 50 mM) at a concentration of 20 mg/mL. All spectra were obtained after subtraction of the buffer baseline and were strictly collected under the same conditions, after adjusting the subtraction baseline until a flat baseline was obtained in the 2000–1700 cm⁻¹ region. Second derivatives were obtained using the Savitzky-Golay algorithm based on 25 smoothing points. Curve fitting of the amide I band from 1700 to 1600 cm⁻¹, in water, was performed using Opus software version 5.5 according to the Local Least Squares algorithm. The initial bandwidth of all components was set to 12 cm⁻¹ and the components were approximated by mixed Lorentzian/Gaussian functions. Amide I band positions are assigned to the corresponding secondary structures according to the literature.²¹

2.4. Differential Scanning Calorimetry (DSC)

DSC scans were carried out on a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with a sensitivity > 0.017 mJ K⁻¹ and a noise level < ± 0.05 μW. The protein samples were dissolved in PBS (pH 7.2, 50 mM) at a concentration of 3 mg/mL. Heat capacity versus temperature profiles were obtained in the range 30–110 °C at scan rate 1K · min⁻¹. Sample scans were buffer-subtracted, concentration normalized and corrected with the progress baseline. DSC curves were analyzed using the ORIGIN (MicroCal Software) program. Experimental deconvolution of heat capacity curves into individual components by a successive annealing procedure was applied following the approach of Idakieva et al.²²

2.5. Cell Culture

BJ, MCF-7, and MDA-MB-231 cells were maintained in a complete growth DMEM low glucose medium supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin-amphotericin B antibiotic mixture under a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every other day.

2.6. Cell Viability Assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assays were carried out to evaluate the cytotoxic

effect of FA-HIH on BJ, MCF-7, and MDA-MB-231 cells.²³ The cells were seeded in 96-well plate at a density of 1×10^4 cells per well and cultured in complete growth medium for 24 h. Then, cells were incubated with 0–70 $\mu\text{g}/\text{well}$ of native or FA-modified HIH for 24 h and 48 h. The culture medium was changed to 100 μL serum-free medium containing 500 $\mu\text{g}/\text{mL}$ MTT and plates were incubated for 3 h at 37 °C and 5% CO_2 . The formazan salts were dissolved in 120 μL of dimethylsulfoxide (DMSO) and the absorbance was measured at 544 nm on FLUOstar Optima microplate reader (BMG Labtech, Germany). Samples containing only DMSO were used as a blank control. Control experiments containing untreated with FA-HIH or HIH cells were carried out. The survival of the cells, treated with HIHs was presented in percentages from the corresponding control. Two independent experiments were performed for each cell line.

2. 7. Statistical Analysis

Data were analyzed by one-way ANOVA followed by Tukey–Kramer post-hoc test. The values were considered to be significantly different if the p-value was < 0.05 .

3. Results and Discussion

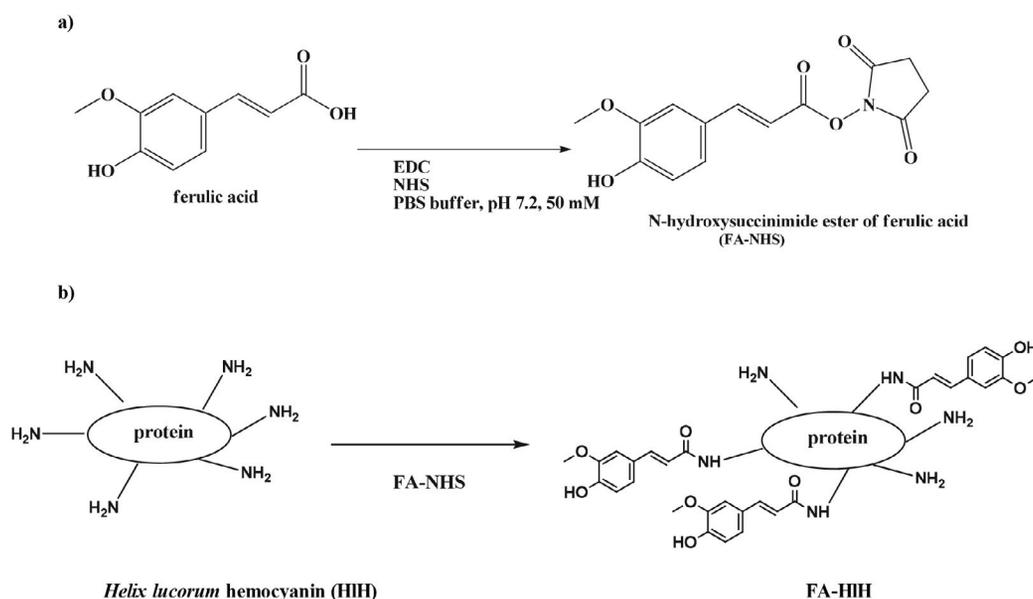
3. 1. Synthesis and Biophysical Characterization of the FA-HIH Conjugates

Ferulic acid was covalently linked to the accessible ϵ -amino groups of lysine residues and N-terminal amino groups of HIH via activated ester method (Scheme 1).

The reaction was conducted under mild reaction conditions in PBS buffer (pH 7.2), at which the primary amino groups are positively charged and exposed to the protein surface. The intermediate, NHS ester of ferulic acid was not isolated and synthesis was performed in one step. An excess of ferulic acid (100- to 500- fold) was used in order to obtain conjugates with a high degree of substitution. Two FA-HIH conjugates containing 40- and 120 ferulic acid residues were prepared and isolated, and were denoted as FA-HIH-1 and FA-HIH, respectively.

We applied ATR-FTIR spectroscopy to assess the effect of the modification on the HIH secondary structure. Spectra of FA-HIH-1, FA-HIH-2, and native HIH were compared in the Amide I band region (1700–1600 cm^{-1}), which is mainly due to C=O stretching vibrations (70–80%) of the amide groups and is known as conformationally sensitive.²¹ For the three protein samples, the original, the second derivative and the deconvoluted spectra scaled to an identical area under Amide I and Amide II (1700–1400 cm^{-1}) are given in the Supplementary (Fig. S1). The spectra were analyzed using 7 (native HIH and FA-HIH-1) or 8 (FA-HIH-2) bands having a centre within 1600–1615; 1615–1640; 1640–1660; 1660–1690; and 1690–1700 cm^{-1} , which are assigned to extended conformations or side-chains residues, β -sheets; α -helical, coiled or disordered structures; β -turns and antiparallel β -sheet structures.²¹ As obtained from the FTIR studies, the conformation of the native and the two feruloylated HIHs are reported in Table 1.

We observed a decrease in the α -helices at expense of the β -structures, which is an indication that HIH is partially unfolded due to the feruloylation. In the spectra of FA-HIH-1 and FA-HIH-2 are found the typical bands that are assigned to protein aggregates, which are probably addi-



Scheme 1: Steps in chemical modification of *Helix lucorum* hemocyanin with ferulic acid: a) activation of the carboxylic group of ferulic acid; b) Conjugation of ferulic acid and primary amino groups of the hemocyanin.

Table 1. Secondary structure band assignments for the native and ferulic acid-conjugated *Helix lucorum* hemocyanin in 50 mM PBS buffer (pH 7.2).

Assignment of the secondary structure components ²¹	HIH native		FA-HIH-1		FA-HIH-2	
	Band position (cm ⁻¹)	Relative area (%)	Band position (cm ⁻¹)	Relative area (%)	Band position (cm ⁻¹)	Relative area (%)
anti-parallel β -sheets	–	–	1609	11.31	1613; 1694	10.91 2.12
β -structures (sheets and turns)	1629; 1633; 1664; 1676 1690	0.15; 8.0; 11.76; 23.31; 7.90	1621; 1633; 1667; 1683	7.31; 14.0; 12.74 10.73	1624; 1632; 1666; 1682	10.28; 14.57; 15.80; 7.74
α -helices	1653	30.39	1651	20.79	1653	23.7
unordered structures/random coils	1642	18.4	1644	23.13	1642	14.57

FA-HIH-1 (40 ferulic acid residues); FA-HIH-2 (120 ferulic acid residues)

tionally stabilized by non-covalent interactions involving the aroma ring of the substituents.

Interestingly, despite of the observed conformational changes of the HIH induced by the modification, the thermal stability of the protein remained mostly unaffected. The DSC profiles of the native HIH, FA-HIH-1, and FA-HIH-2 are depicted in Fig. 1.

The experiment was conducted at a protein concentration of 3 mg/mL in PBS (pH 7.2, 50 mM), and heating rate – 1K/min.

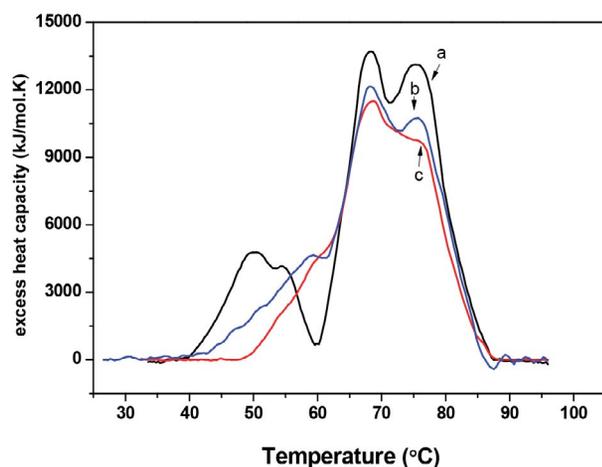


Fig. 1. DSC profiles of the thermal unfolding of native HIH (a) and conjugated with 40 and 120-ferulic acid residues, FA-HIH-1 (b) and FA-HIH-2 (c).

As seen the three DSC curves have an asymmetric shape, which is due to the complex multimeric structure of hemocyanins and is in agreement with the literature data on the thermal denaturation of another Hc in a phosphate-saline buffer.¹⁶ The thermal denaturation temperatures of the feruloylated HIH are slightly shifted toward the higher temperatures (by up to 5 °C) in comparison to denaturation temperatures of the native HIH. DSC curves were experimentally deconvoluted using a successive annealing procedure as previously described by Idakieva et al,²⁴ which allowed a more precise estimation of the thermodynamic parameters, transition temperature (T_{m_i}) and calorimetric enthalpy (ΔH_i) (Table 2). Apparently, the thermal stability of HIH was preserved after the modification.

3. 2. Comparison of the Cytotoxicity of the Native and the Feruloylated HIH

The effect of FA-HIH-1 and FA-HIH-2 on the cell viability of human fibroblasts (BJ cells) was tested in a concentration range between 25 and 200 $\mu\text{g}/\text{well}$ (Fig 2).

We observed a slight increase in proliferation or no effect after a 24h exposure of the BJ cells to 25 or 50 $\mu\text{g}/\text{mL}$ of the native or feruloylated HIH. For the same samples, not evidence of any sign of toxicity was observed even after longer incubation time. Reduction in BJ cell growth was observed after a 24h-treatment with 200 $\mu\text{g}/\text{well}$ ($18.4 \pm 5\%$, $P < 0.001$) of FA-HIH-1, a similar effect was produced

Table 2. The thermodynamic parameters of the native and ferulic acid-modified HIH thermal denaturation.

Sample	T_{m1} (°C)	ΔH_1 (kJ mol ⁻¹)	T_{m2} (°C)	ΔH_2 (kJ mol ⁻¹)	T_{m3} (°C)	ΔH_3 (kJ mol ⁻¹)	T_{m4} (°C)	ΔH_4 (kJ mol ⁻¹)	T_{m5} (°C)	ΔH_5 (kJ mol ⁻¹)	ΔH_{total} (kJ mol ⁻¹)	ΔH_{cal} (kJ mol ⁻¹)
native HIH	49.9	38080	55.2	10752	67.6	92858	75.7	99308	79.8	22263	263261	279337
FA-HIH-1			60.4	40802	68.3	79585	75.4	77372	80.4	20111	217870	214977
FA-HIH-2			58.9	72399	68.5	70882	75.5	67464	81.0	27466	239091	245351

FA-HIH-1 (40 ferulic acid residues); FA-HIH-2 (120 ferulic acid residues)

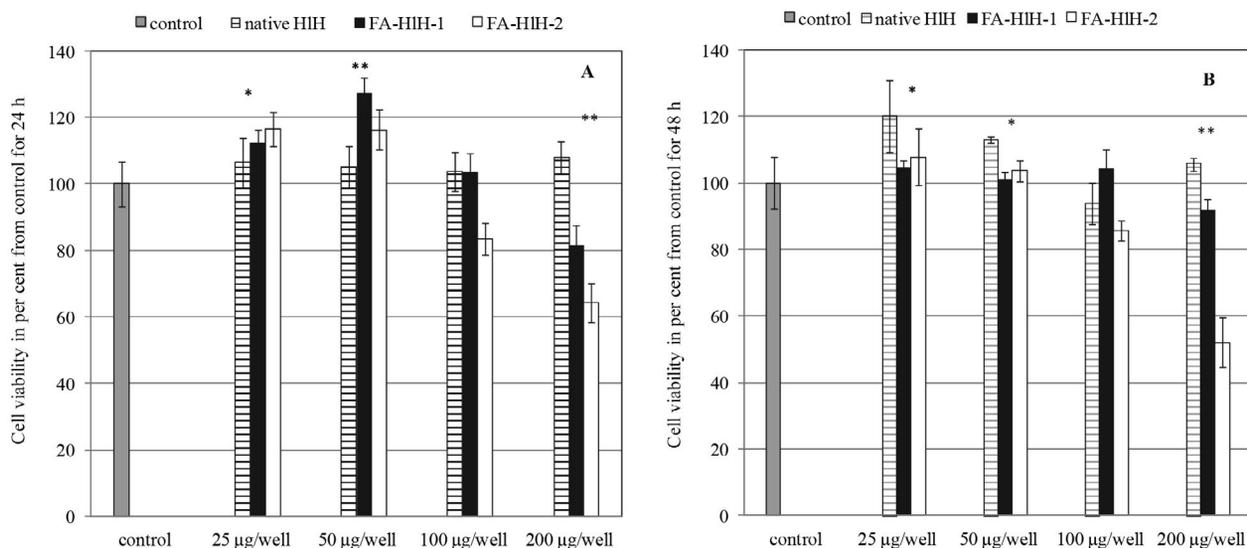


Fig. 2. Antiproliferative effect of native and ferulic acid conjugated HIH on normal fibroblasts (BJ cells) after 24 h (A) and 48 h (B) incubation. Data are presented as mean \pm SD, *($p < 0.05$), ** ($p < 0.01$), $n = 3$.

by 100 µg/well of FA-HIH-2. The lowest cell viability of the BJ cells, 64.1 and 51.1%, was detected after 24 and 48 h, respectively of incubation with 200 µg/mL FA-HIH-2.

Cytotoxicity of the feruloylated HIH against two breast cancer cell lines, a hormone-dependent (MCF-7) and a hormone-independent (MDA-MB-231), was esti-

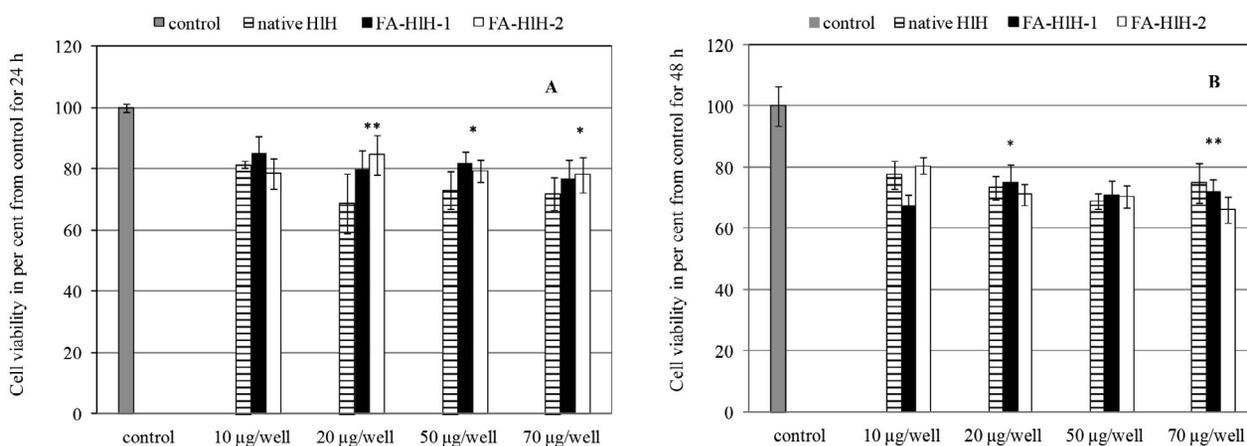


Fig. 3. Effect of native HIH and ferulic acid conjugated HIH on MCF-7 cell viability after 24 h (A) and 48 h (B) incubation. Data are presented as mean \pm SD, *($p < 0.05$), ** ($p < 0.01$), $n = 3$.

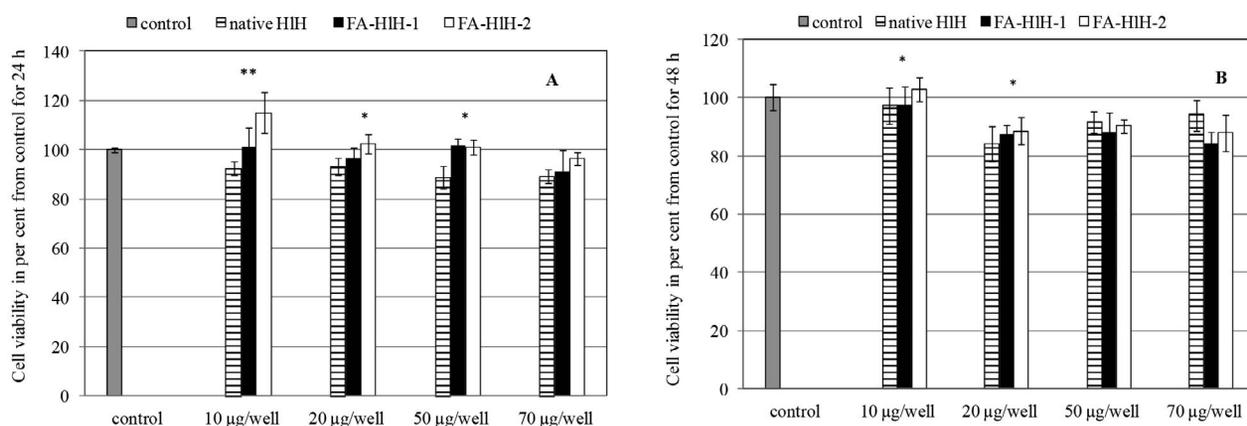


Fig. 4. Effect of native HIH and ferulic acid conjugated HIH on MDA-MB-231 cell viability after 24 h (A) and 48 h (B) incubation. Data are presented as mean \pm SD, *($p < 0.05$), ** ($p < 0.01$), $n = 3$.

mated at concentrations from 10 to 70 µg/well, which are not toxic for normal fibroblasts. The native and the two feruloylated HHs produce a similar effect within one and the same cell line. The effect was more pronounced for FA-HH-2, especially for the MCF-7 cells, for which we noticed concentration dependence (Fig. 3). The three Hcs had negligible cytotoxic effects on MDA-MB-231 cells, even at a concentration of 70 µg/well, the highest tested concentration (Fig. 4).

4. Conclusions

For the first time, two conjugates of *Helix lucorum* hemocyanins with ferulic acid have been successfully prepared. The modified Hcs is likely to adopt more extended β conformations and are apparently additionally stabilized by π - π stacking interactions that involve the ferulic acid residues and aromatic amino acid residues of the protein, which on the other hand explains their increased thermal stability in comparison to that of the native protein. At a concentration of 50 µg/well, FA-HH-1, the sample containing 40 ferulic acid residues, appears to stimulate the growth of the fibroblasts. The moderate to excellent activity and selectivity of the native and the feruloylated HH towards MCF-7 cells makes them promising for future studies because of their biopharmaceutical potential.

5. Supplementary Materials

The supplementary PDF file contains the original, second derivative and deconvoluted in the Amide I band region FTIR spectra of the native and the two feruloylated (FA-HH-1 and FA-HH-2) hemocyanins. A graphic illustration of the experimental annealing procedure for the DSC experiment is also given.

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Povzetek

Ferulacija hemocianina (HIH) *Helix lucorum* je opisana prvič. Pripravili smo dva HIH konjugata z 40-in 120-ostanki ferulične kisline, označena kot FA-hlh-1 in FA-hlh-2. Po pričakovanjih je ferulacija HIH povzročila preureditev beljakovinske molekule, opaženo je tudi zmanjšanje α -spiralne strukture na račun β -struktur. Poleg tega so bili konjugati FA-HIH bolj nagnjeni k agregaciji, ki je verjetno posledica stabilizacije delno neprepognjenih beljakovinskih molekul z ne-kovalentnimi povezavami. Zanimivo je, da spremembe niso vplivale na toplotno stabilnost HIH. Nativne in ferulirane HIH niso strupene za normalne fibroblaste (celice BJ). Opazili smo zmanjšanje preživetja celic raka dojke MCF-7 na približno 66 % po 48 h izpostavljenosti 70 $\mu\text{g}/\text{žep}$ FA-HIH-2.



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