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Elucidation of the effect of some cholinium amino acid ionic liquids on the thermal and the conformational stability of insulin

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ABSTRACT

The major concerns about protein-based drugs are their stability i.e. maintaining the protein in the folded state throughout processing and storage, as well as the preparation of novel formulation. Stabilization of the monomeric form of insulin (*In*) under the condition of low pH has been a recent challenge. In our earlier investigation, we found that 1-butyl-3-methylimidazolium-based ionic liquids (ILs) containing acetate, trifluoroacetate or dicyanamide anions enhance *In* thermal stability and prevent protein aggregation. In the present study, six non-toxic ILs containing biocompatible cholinium cation [Chol] and an anion charged amino acid (asparginyl (Asp), glutaminyl (Glu), lysinyl (Lys) and arginyl (Arg)) were synthesized using a two-step procedure. Their effect of the ILs on the *In* secondary structures was evaluated using FTIR spectroscopy. Rearrangement in the protein molecules, an increase in the beta-structures on behalf of the α-helices, partial denaturation but no aggregation was observed in all *In* solutions containing ILs. Differential scanning calorimetry was applied to elucidate the effect of ILs on thermal stability of *In*. Interestingly, in presence of [Chol][Glu] and $[Chol]_2$[Asp] the denaturation temperature of the *In* was shifted to higher temperatures with 9.6 and 4.1 °C, respectively.

1. Introduction

Insulin (In) is a peptide hormone secreted by the beta-cells of the pancreas, and regulates the glucose levels in blood. In is a small protein (5800 Da) and consists of two chains A-chain (21 amino acid residues) and B-chain (30 amino acid residues) that are linked by two disulfide bridges, additional intrachain cysteine bridge in A-chain stabilize the molecule [1]. The active form is the monomeric has low stability and is available at 20% acetic acid at pH 1.9. At other aqueous solution e.g. diluted HCl (pH 1.9), more concentrated solutions, higher pH (2–8) or in presence of small amounts of phenol exists as a diamer. In presence of bivalent ions (Zn^{+2}, etc.) dimmers associate in one of the three allosteric hexamers, which differ in their secondary structure [2].

Insulin is discovered in 1916 by Dr. Banting and Best, and in 1922 for the first time it was administrated to a young diabetic patient [1]. Hundred years later, insulin is the only alternative for patients with diabetes I type and applied in some cases of diabetes type II. In the eighties, insulin was obtained exclusively by extraction from pig or bovine pancreas, and subsequent enzymatic modification to convert it in the human analogue of insulin. Later on, progress in insulin production was made applying recombinant DNA techniques [3]. Nowadays, many novel insulin analogues present on the market e.g. very rapid-acting (onset of action <15 min, peak: 30–90 min, duration: 3–5 h), rapid acting (onset of action: 30–60, min., peak: 2–3 h, duration 5–8 h), intermediate-acting (onset of action: 1.5–4 h, peak: 4–10 h, duration: 10–16 h) long-acting (onset of action: 1.5 h, duration: 24 h) insulin analogues [1,4].

Still, the prescription correct dosage is a challenging task especially, having in mind that the pharmacokinetics of In depends on many factors e.g. metabolism of each individual and absorption rate, which depends on the injection site, injection depth, concentration, massage on the injection site. Application of higher In dosages may result in weight gain due to anabolic processes and increase the risk of hypoglycemia, while lower dosages may result in not effective control of the blood glucose levels and multiple organ failure [1]. On the other hand, in order to be avoided the traumatic effects of In injections, development of proper non-invasive In drug delivery systems is of current interest for the pharmaceutical industry.

Another challenge that faces the pharmaceutical industry is related to insulin stability upon isolation, purification and storage, because the correct folding and structure is a key factor for the biological activity of proteins. Insulin is prone to fibrillate and aggregate upon contact with hydrophobic surfaces i.e. Teflon, peristaltic pump tubing, under

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agitation at pH 7.0 and 37 °C, air-water interface, at sites of the injection (injection-localized amyloidosis) [5,6]. In molecules undergo dramatic structural changes upon freeze-drying, prolonged exposure to light, shaking or stirring, pressure, etc. [7,8]

Typically, In is stabilized in presence of phenol, m-cresol, methylparabene, glycerol in solution, and in presence of zinc chloride, sodium chloride, etc. as powder. Zhang et al. observed a suppressed proteolytic degradation of In in formulations containing cyclodextrine and casein (or protamine) [9]. In another research, Kraineva et al. have found that only weak interactions occur between cubic mesoporous lipid systems based on monoolein and In molecules, thus the structure of the encapsulated in the lipid matrix In is not significantly altered/changed [10]. Rasmussen et al. have shown that in comparison to polysorbate 80, sucrose and human serum albumin, α-crystalline prevents more effectively In from fibrillation induced by vigorous stirring [11].

Ionic liquids (ILs) are salts that are typically composed of organic cations and organic/inorganic anions; have melting points below 100 °C, low vapor pressure, and high thermal stability [12]. Their physicochemical properties can be tuned by modification or selection of an appropriate cation and anion, thus they are referred as designer's solvents. They have numerous applications in research and industry. Room temperature ionic liquids, particularly those consisting of biocompatible cations and anions, are of great interest for biotechnology and protein chemistry as reaction media for biocatalytic reactions aiming to enhance enzyme activity and selectivity; or as solvents aiming to improve protein solubility, crystallization and stability and to facilitate protein extraction and separation [13]. Yet, the potential of ILs in stabilization of protein and enzymes is not fully revealed although there are many research papers that have shown promising results. For example, Takekiyo et al. have found that 1-butyl-3-methylimidazolium thiocyanate ([bmim][SCN]), ethylammonium nitrate ([EAN][NO_3], and propylammonium nitrate ([PAN][NO_3]) prevent the formation of In amyloid aggregates, while [bmim][Cl] and [EAN][NO_3] are cryoprotectants for lysozyme [14,15]. Kumar and Venkatesu showed that In is stabilized in more folded conformation and its denaturation and self-aggregation is suppressed in presence of ILs containing trimethylammonium cation [16]. It is noteworthy to be mentioned that choline dihydrogen phosphate ([Chol][dhp]) enhance the storage stability of cytochrome C (>18 months at room temperature), enhance the thermal stability of lysozyme and stabilize the structure of human serum albumin [13,17,18]. In an in vitro experiment, Tanner et al. observed that IL based on choline and geranic acid (ratio 1:2) facilitated the transdermal delivery of In [19].

Conversely, many other researchers reported of detrimental effect of many ILs on protein stability e.g. imidazolium-and pyridinium based ILs accelerate the amylogenesis in for α-synuclein and α-lactalbumin, and triethylammonium-based ILs stimulate the fibrillation and aggregation of Aβ1-40 peptide [14], butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]) denatured human serum albumin [18], while ILs containing choline cations and bis(trifluoromethylsulfonyl)imide, hexanoate, trifluoromethanesulfonate, trimethyllactate anions decrease the thermal stability of lysozyme [17].

Interestingly, Rodrigues et al. observed for a choline-based series of ILs that some anions lactate or dihydrogen phosphate stabilize lysozyme only when added at high concentration (above 0.5 M, and 1 M, resp.), while lower tested concentrations destabilize the protein [17]. On contrary, 1-tetradecyl-3-methyl imidazolium bromide added at low concentrations (below critical micelle concentration) stabilizes bovine serum albumin, while higher concentrations of this IL denature the protein [13].

In summary, protein–IL interactions are complex and it is not possible to generalize the observed for one particular system and to transfer it to another. However, biocompatatible ILs have potential to be applied in preparation of drug delivery systems and formulations of biotechnological incl. protein-based drugs, thus worth to be further investigated.

Here, we report the synthesis of a series of ionic liquids containing a choline cation (Chol) and a charged amino acid (Asp, Glu, Arg, Lys) as an anion. Glutaminate and asparginate were obtained as dicholinium salts as well. The cytotoxicity of the ILs on fibroblasts was evaluated. Then, the effect of the six choline amino acids on the secondary structure and the thermal stability of In was evaluated.

2. Materials and methods

2.1. Materials

Insulin from porcine pancreas United States Pharmacopeia reference standard, (2-hydroxyethyl)trimethylammonium (cholinium) chloride ([Chol][Cl] (≥97%), Dowex Monosphere 550 A UPW (OH form) resin, thiazolyl blue tetrazolium bromide (MTT) (98%), Dulbecco's Modified Eagle's Medium (high glucose), L-Glutamine–Penicillin–Streptomycin solution were purchased from Sigma-Aldrich. L-glutamic acid, L-arginine, L-aspartic acid (≥99%) and L-lysine monohydrate (≥98.5%) were provided by Carl Roth.

Murine embryotic fibroblast (3 T3) cell line was purchased from American Type Culture Collection (ATCC).

2.2. Synthesis of the choline amino acids [Chol][AA]

Mono- and di- cholinium salts of amino acids were synthesized by two-step procedure (Scheme 1). In the first step, chloride anion in cholinium chloride was exchanged to hydroxide anion using the ion exchange resin. The content of chloride anions in collected eluates was checked with $AgNO_3$. The eluates without chloride were used in the second synthesis step. The concentration of cholinium cation was determined by titration method in biphasic system in the presence of two indicators (dimidium bromide/disulphine blue) and sodium lauryl sulfate (SLS) as titrant.

In the second synthesis step, amino acid (L-glutamic acid, L-arginine, L-aspartic acid or L-lysine) was dissolved in the aqueous solution of cholinium hydroxide. Molar ratio of cholinium hydroxide to amino acid was 1:1 for monosalts synthesis and 2:1 for di-salts. The mixture was stirred at room temperature for 24 h, subsequently. The water was evaporated at 60 °C under vacuum by using rotary evaporator. The obtained ionic liquids were dried in vacuum oven at 60 °C.

The compounds were identified by ^{1}H NMR. The NMR spectra were recorded in D_2O on a Bruker DPX-400 spectrometer operating at 400.13 MHz.

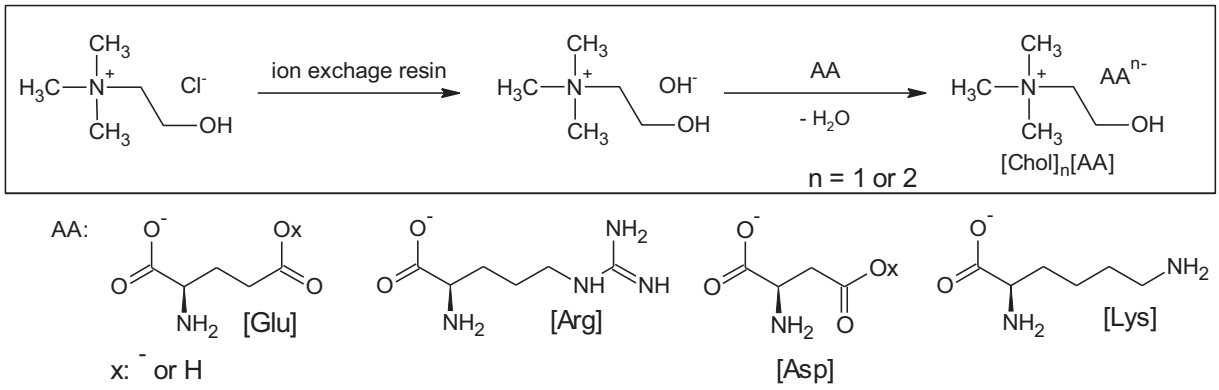
Thermal stability was determined based on thermogravimetric analysis carried out using a termomicrobalance TG 209 F1 Libra® NETZSCH within the temperature range of RT ÷ 800 °C at a constant heating rate of 10 °C·min^{-1}, in the air.

The specific rotation $[\alpha]_D^{20}$, for all of the amino acid ionic liquids reported here were measured in aqueous solutions on AUTOPOL IV Polarimeter (Rudolph Research Analytical). The concentrations were 0.01 g/cm^3.

2.3. Cytotoxicity of [Chol][AA] on fibroblasts

Murine embryotic fibroblast (3 T3) (ATCC) were cultured in DMEM high glucose medium containing L-Glutamine–Penicillin–Streptomycin and 10% fetal bovine serum. at humified atmosphere, 37 °C and 5% CO_2. Cells were seeded in a sterile 96-well plate at 1 · 10^4 cells per well and incubated for 24 h at 37 °C and 5% CO_2 for obtaining adherent cell cultures and good cell spreading. Then, the cells were incubated for additional 48 h with 0.05, 0.5, and 5.0 mmol/L [Chol][AA] and the effect of the ionic liquids on cell viability was assessed in a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye (MTT). All experiments were presented in triplicate [20].

One-way ANOVA with Tukey–Kramer post-hoc test was applied for the analysis of the experimental data (Graph Pad Software Inc.,



Scheme 1. Synthesis and structures of the cholinium-based amino acid ionic liquids $[Chol]_n[AA]$.

San Diego, USA). The values were considered to be significantly different if the *p* value was <0.01.

2.4. Differential scanning calorimetry (DSC)

The changes in the phase transitions of In in presence of the ILs were performed on DASM 4 (Privalov, BioPribor)-built-in highly sensitive calorimeter with cell volumes of 0.5 ml, a sensitivity >0.017 mJ/K and a noise level below 0.05 μW. An over-pressure of 2 atm dry nitrogen was kept constant over the samples in the cells throughout the scans to avoid any degassing during heating. A scan rate of 1 °C/min from 30 to 110 °C with equilibrium period of 30 min was used. Buffer or buffer-IL baseline was established prior to the analysis of the samples. Then, the transitions were corrected for the difference in heat capacity between the initial and final state by using the corresponding linear chemical baseline. The temperature at the maximum of the excess heat capacity curve was taken as the transition temperature (Tm). DSC measurements with In and In-ionic liquid complexes were carried out in 10 mM KCl/HCl buffer, pH 2.0 (20 °C). The In-IL complexes were prepared immediately before the measurements at a In/IL molar ratio of 1.5, the pH was adjusted if necessary. The concentration of insulin was kept constant (3 mg/mL, 0.517 mM) for all experiments. Assays with higher concentration of the ILs in this buffer resulted in decrease in the protein solubility and precipitation of the solutions. Molecular mass of 5800 Da for In was used in the calculation of its molar concentration. The van't Hoff enthalpy (ΔH_{VH}) was calculated using the following equation:

$$\Delta H_{VH} = \frac{RT_m^2 C_p}{\Delta H_{cal}},$$

where R is gas constant (KJ/K·mol); T_m is the estimated transition temperature (K); C_p is the specific head capacity (KJ/mol·K); and ΔH_{cal} is the calculated from the experimental curve enthalpy (KJ/mol). All experiments are conducted in triplicates and data are expressed as means ±the standard deviation (SD).

2.5. Infrared spectroscopy

FTIR spectra of the In (25 mg/mL) dissolved in KCl/HCl (pH 2.0, 10 mM) or in 10 mM aqueous solution of the ILs (in the same buffer) 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 (diamond crystal) in frequency region 4000–600 cm^{-1} (ATR) with 128 scanning and at resolution of 1 cm^{-1}. The spectra of the proteins were referenced to the respective spectra of 10 mM KCl/HCl (pH 2.0) or 10 mM solutions of the corresponding ionic liquid the same media in order to substact their absorptions.

The effect of the [Chol][AA] on In secondary structure was estimated comparing the obtained second-derivative FTIR spectra in the 1700–1600 cm^{-1} region (Amide *I* band) with the spectrum of In in IL-free medium. In order to describe quantitatively the changes in the In secondary structure induced by the tested choline amino acids, the ATR-FTIR spectra were treated in accordance with the established methods given in the literature [21]. ATR-FTIR spectra were Fourier deconvoluted by Opus software version 5.5 using band width of 14 cm^{-1}, 2.9 resolution enhancement factor, and Lorentzian lineshape. Second derivative spectra were obtained using the Savitzky-Golay algorithm based on 25 smoothing points. Then, the relative contribution of each band component of the Amide *I* band was determined by curve fitting following the procedure of OPUS program. 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 14 cm^{-1} and the components were approximated by mixed Lorentzian/Gaussian functions. The curve-fitting was performed according to the Local Least Squares algorithm. The assignment of the Amide *I* band positions to secondary structure was done according to [22].

3. Results and discussion

3.1. Synthesis and characterization of the ILs

The four mono- and the two dicholinium salts of charged amino acids i.e. (2-hydroxyethyl)trimethylammonium L-glutaminate [Chol][Glu], (2-hydroxyethyl)trimethylammonium L-asparaginate [Chol][Asp], (2-hydroxyethyl)trimethylammonium L-argininate [Chol][Arg], (2-hydroxyethyl)trimethylammonium L-lysinate [Chol][Lys], bis(2-hydroxyethyl)trimethylammonium L-glutaminate $[Chol]_2[Glu]$ and bis

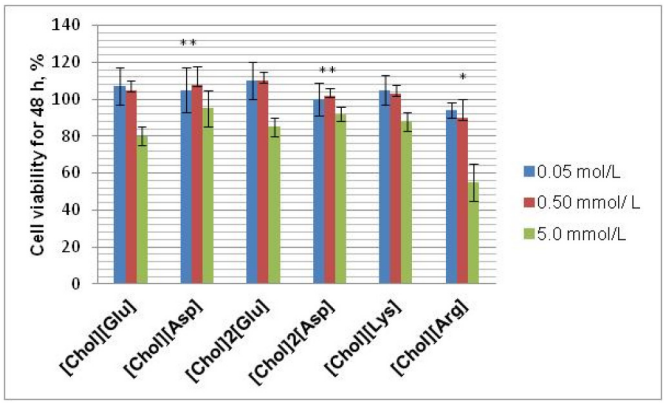


Fig. 1. Cell viability of 3T3 cells after 48-h incubation with $[Chol]_n[AA]$.

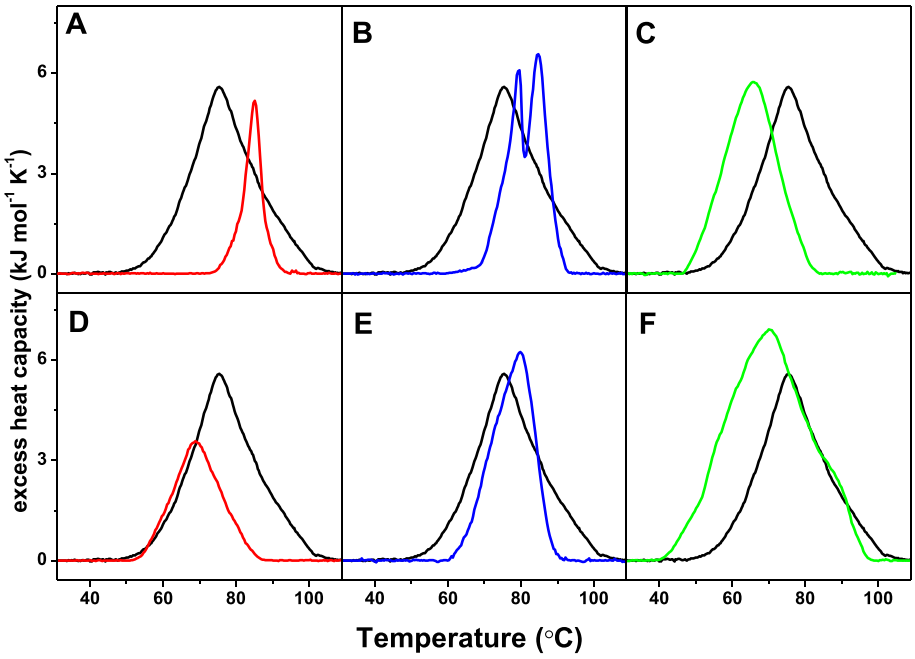


Fig. 2. DSC thermograms of the native In in KCl/HCl buffer (pH 2.0, 10 mM) (black line); In-ILs complexes: In-[Chol][Glu] (A); In-[Chol][Asp] (B); In-[Chol][Arg] (C); In-$[Chol]_2$[Glu] (D); In-$[Chol]_2$[Asp] (E); In-[Chol][Lys] (F). Experiments were performed at a heating rate of 1 °C/min. In concentration was 3 mg/mL. Molar ratio of In to ILs was 1:1.5.

(2-hydroxyethyl)trimethylammonium L-asparaginate $[Chol]_2$[Asp] were synthesized using the following scheme (Scheme 1). The characterization of the compounds is given as Supplementary.

3.2. Cytotoxicity of the ILs on fibroblasts

Choline is an essential micronutritient participating in many processes related to cell structure, synthesis of neurotransmitters, and other and choline-deficiency have a negative effect on liver structure and function, atherosclerosis and other neurological disorders [23]. On the other hand, amino acids are essential for protein synthesis, which are crucial for almost all biological processes in the living organisms [24]. Therefore, the ILs based on choline cation and aminoacid anions are considered biocompatible and possibly could find a biopharmaceutical application.

All ILs applied at concentration of 0.05 and 0.5 mmol/L had no effect on 3T3 fibroblast proliferation for 48 h (Fig. 1.). We found that the most of the tested cholinium amino acid salts produced weak effects on 3T3 cell growth even at 5.0 mmol/L. Interestingly, we observed a significant reduction of the cell growth (up to 50%) only with [Chol][Arg] and a moderate anti-proliferative activity (up to 25%) with [Chol][Glu] in the experiments with the highest tested concentration.

In addition, Hou et al. reported that [Chol][Lys], [Chol][Arg], [Chol][Asp] and [Chol][Glu] inhibit acetylcholinesterase with half maximal inhibitory concentration (IC50) values in the range from 3.4 to 3.8 mmol/L [25]. They also found that >87% of [Chol][Asp] and [Chol][Glu] are biodegraded for 28 days, while the biodegradability of arginate and lysinate is about 70% for the same period [25].

3.3. Effect of the ILs on insulin thermal stability

The effect of the ILs on thermal unfolding of In was monitored using DSC. We observed a non-two-state thermally induced unfolding of In with a Tm centered at 75.4 °C and Δ H_m 119.9 of kJ/mol in IL-free medium. As can be seen from the DSC curves (Fig. 2), in presence of the ILs the unfolding occurs as a single transition, except for the sample containing [Chol][Asp]. Interestingly, in this medium the thermal denaturation of In is a more complex process consisting of two endothermic events centered at 79.2 and 84.7 °C. For In-[Chol][Lys] complexes, the DSC curve has a relatively symmetric shape, which is an indication of existence of more than one structural unit. The results from the DSC studies indicate that the thermal denaturation temperature is lower in presence of [Chol][Arg], $[Chol]_2$[Glu] and [Chol][Lys], and the observed endothermic peaks are centered at 65.6, 68.6 and 70.0 °C, respectively. On the other hand, in presence of $[Chol]_2$[Asp] and [Chol][Glu] In denaturation transitions shift to higher temperature – 79.9 and 85.1 °C, respectively.

The transition parameters for In thermal unfolding in presence of choline-amino acid ILs are given in Table 1. Based on the experimental thermodynamic parameters, van't Hoff enthalpies (ΔH_{vH}) were calculated to evaluate the transition states during the thermal unfolding of In in presence of ILs. The effect of the media on the cooperatively of

Table 1
Thermodynamic parameters of the of the thermal unfolding of insulin in aqueous solutions containing $[Chol]_n$[AA].

In in presence of ionic liquids	Δ H, kJ/mol	C_p, kJ/mol·K	T_m, °C	$T_{1/2}$, °C	Δ H_{vH}, kJ/mol
IL-free medium	119.9 ± 1.3	5.6 ± 0.06	75.4 ± 0.2	19.0 ± 0.20	47.7
[Chol][Glu]	32.4 ± 0.3	5.2 ± 0.04	85.1 ± 0.3	4.9 ± 0.05	171.2
$[Chol]_2$[Glu]	57.0 ± 0.9	3.7 ± 0.04	68.6 ± 0.2	15.2 ± 0.20	63.0
[Chol][Asp]	76.6 ± 1.5	Cp1 = 6.1 ± 0.06	Tm_1 = 79.2 ± 0.3	11.8 ± 0.20	
		Cp2 = 6.6 ± 0.05	Tm_2 = 84.7 ± 0.2		
$[Chol]_2$[Asp]	86.7 ± 1.8	6.2 ± 0.04	79.9 ± 0.1	13.7 ± 0.15	74.1
[Chol][Arg]	101.9 ± 3.2	5.7 ± 0.03	65.6 ± 0.2	17.0 ± 0.20	53.4
[Chol][Lys]	194.4 ± 3.8	6.9 ± 0.07	70.0 ± 0.4	27.4 ± 0.30	34.7

In all experiments the final insulin concentration (3 mg/mL, 0.517 mM). Molar ratio of In to ILs is 1:1.5. IL-free medium contains only KCl/HCl (pH 2.0, 10 mM).

Table 2
Quantitative estimation of the main secondary structural elements of insulin in KCl/HCl (pH 2.0, 10 mM) in absence and in presence of choline-based amino acid salts calculated from their curve fitted FTIR spectra.

Secondary structural element	Sum of the estimated relative areas, %					
	IL-free media	Media containing the corresponding ionic liquid				
		[Chol][Glu]	$[Chol]_2$[Asp]	$[Chol]_2$[Glu]	[Chol][Arg]	[Chol][Lys]
Antiparallel β-structures	13.1	18.4	16.9	17.4	14.2	12.9
β-turns, β-sheets	32.1	34.2	39.0	32.0	32.8	29.9
Unordered structures and random coils	15.4	10.6	12.7	21.2	12.2	18.4
Helical structures (α-helices, 3_{10}-helices)	32.3	36.8	30.8	29.4	40.6	38.7
Side-chains residues of the amino acids	7.21	–	–	–	–	–

the unfolding transitions of In was evaluated based on the $\Delta H_{vH}/\Delta H_{cal}$ ratio so called size of the cooperative unit. Interestingly, only in presence of $[Chol]_2$[Glu] the transition occurs in a two stage model ($\Delta H_{vH}/\Delta H_{cal} \approx 1$), which indicates a sharp equilibrium transition from native to denature state. The highest value of the $\Delta H_{vH}/\Delta H_{cal}$ ratio (~5.3) was estimated for the In denaturation in presence of monocholine glutaminate, which suggests the formation of molecule associates (multimers), which probably is due to strong intermolecular interactions as well as possible partial aggregation. In IL-free medium and in presence of [Chol][Lys], [Chol][Asp], and $[Chol]_2$[Asp] the $\Delta H_{vH}/\Delta H_{cal}$ ratio was in the range between 0.18 and 0.85, which is evidence that in these media the In thermal unfolding proceeds with formation of one or several stable intermediates.

DSC is sensitive to the conformation of the molecule and any change in the conformation affects the position and shape of the curves. The results from the calorimetric studies suggest that the tested ILs, even added to the media at low concentration have a significant effect on the In structure.

3.4. Effect of [Chol][AA] on insulin secondary structure

Typically, the polypeptide chain gives rise of several characteristic infrared absorption bands amongst which the Amide *I* band (1700–1600 cm^{-1}) region is the most applied for protein conformational studies. Amide *I* band is due mainly to the stretching C═O vibrations and is very sensitive to changes in the backbone conformation, on the other hand, the band is typically characterized with higher intensity in comparison to Amide II and Amide III bands [22,26]. Furthermore, the frequencies of the amide I band components correlate closely to each secondary structural elements of protein. For the FTIR deconvoluted spectrum of In dissolved in KCl/HCl (pH 2.0, 10 mM) ten well-defined components in the Amide *I* band region are observed. (Fig. S1). The two bands at 1650 cm^{-1} and 1661 cm^{-1} are assigned to α-helices and 3_{10}-helices, respectively, while the band at 1942 cm^{-1} corresponds to random coils and unordered structures. The peaks for β-sheets (1626 cm^{-1}, 1634 cm^{-1}), β-turns 1673 cm^{-1}, 1683 cm^{-1}) and anti-parallel β-sheets or intermolecular aggregates (1616 cm^{-1}, 1690 cm^{-1}) are also clearly distinguished. Interestingly, a band centered at 1600 cm^{-1} that is due to Tyr and the other amino acid side-chains was observed only in the FTIR spectrum of the native In and we assume that in presence of ILs these residues are less exposed to the solvent. The analysis of the secondary structure of In show high consistency with the data reported in the literature for the secondary structure of In based on FTIR spectroscopy and crystallography [27,28].

The original, the second derivative and the deconvoluted FTIR spectra in the amide I region of In in KCl/HCl (pH 2.0, 10 mM) in presence of [Chol][AA] as well as the estimated peak positions and the relative band areas are given in supplementary (Fig. S1, Table S1). The percentages of the main secondary structural elements are summarized in Table 2. As can be seen, the Amide I band region is composed of six to nine components depending on the media composition. The approximate structural content is varying in the range as follows: 10.6–21.2% α-helix; 12.4–29.7% 3_{10}-helix; 10.6–21.2% random coils and unordered structures; and 30.0–39.0% β-structures, and 12.9–18.4% antiparallel β-sheets, which is clear evidence that the ILs induce conformational changes in the protein molecules. We faced a problem with recording of the FTIR spectrum of In in presence of [Chol][Asp], which we ascribe to the observed precipitation of the In-[Chol][Asp] sample or to an insufficient correction of the baseline in this medium.

In terms of decreasing total helical content (%) of the In samples, the effect of the IL series reads: [Chol][Arg] > $[Chol]_2$[Glu] > [Chol][Lys] > [Chol][Glu] > IL-free medium>$[Chol]_2$[Asp], which correlates with the observed effect of the ILs on thermal unfolding of In i.e. expectedly the molecules with the higher helical content were less thermostable.

Interestingly, the most compact structure of In is realized in presence of $[Chol]_2$[Glu], while the most unfolded structure is obtained in presence of [Chol][Glu]. In addition, the monocholine glutaminate seems to stimulate In fibrillation and aggregation, which is consistent with the observed from the DSC experiments.

In spite of some spectral variations, which are evidence for the rearrangements in the protein molecules, we observed similar patterns of the Amide I band, which suggests that almost in all media containing the tested ILs In is near the native conformation.

4. Conclusion

Despite the observed rearrangements in the In molecule in the presence of the tested cholinium-based ILs, it remains close to the native structure. The aggregation and fiblilation of the In was suppressed in presence of the lysinate and the arginate salts. The two ILs, however, exhibited the highest toxicity and decrease the thermal stability of the In. Choline-based ILs have potential to stabilize proteins, but better selection or modification of the anion should be done. The results of this study enrich the knowledge on the protein-IL interactions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2019.03.074.

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