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Thermal and conformational stability of insulin in the presence of imidazolium-based ionic liquids

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Abstract Stabilization of the monomeric form of insulin (*In*) under condition of low pH has been a recent challenge. This research aims to reveal the effect of a series of 1-butyl-3-methylimidazolium-based ionic liquids (ILs) on the stability of *In* dissolved in highly acidic solution (pH 2.0). Differential scanning calorimetry was applied to assess the thermal stability of *In* in the presence of these ILs. In addition, we monitored the IL-induced changes in the *In* secondary structure using Fourier transformed infrared spectroscopy. The peak of *In* thermal denaturation was shifted to higher temperatures in the presence of the tested acetate, trifluoroacetate and dicyanamide salts. At the same time, chloride and thiocyanate ILs had no effect on the thermal stability of the insulin, while the tricyanomethanide salt slightly destabilized the protein. The change in the *In* conformation affected not only the position but also the sharpness and the shape of the transition peak. As a whole, those ILs which were able to preserve or enhance helical structure of *In* produced stabilizing effect and those which stimulated the formation of unordered and random-coiled structures deteriorated its thermal stability. No aggregation of *In* in the presence of the imidazolium-based ILs was observed under the tested acidic media.

Keywords Insulin · Ionic liquids · Secondary structure · DSC

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Introduction

Insulin (*In*) is a peptide hormone (Mw 5800 Da), which is secreted from B-cells of the pancreas. It consists of A-chain (21 amino acid residues) and B-chain (30 amino acid residues) which are linked with two disulfide bridges. Additionally, the structure of *In* is maintained by an intramolecular disulfide bond between two cystein residues of A-chain [1]. At low concentration, *In* is in a monomeric form—the active one. However, the monomeric *In* is prone to aggregation, denaturation and/or fibrillation upon contact of the hydrophobic domains of the protein with hydrophobic surfaces. *In* is more stable at higher concentration and in the presence of excipients among which phenol and its derivatives, protamine, bovine α-crystallin, $ZnCl_2$, etc. that are the most commonly applied [2]. In liquid formulations the protein molecules are usually dimers, and in the presence of bivalent ions they are associated with three allosteric hexameric complexes, named T_6, R_3T_3 and R_6.

In the human body insulin regulates the metabolism of carbohydrates and fats, and conditions like reduced insulin production or insulin resistance are related to the metabolic diseases known as diabetes (type I or type II). This is a serious widespread disease, and according to the World Health Organization, its global prevalence in 2014 was estimated to be 9 % among adults. Furthermore, it is predicted that in the next decade diabetes will be seventh leading cause of death [3]. Thus, *In* has been a subject of considerable interest since its discovery in 1916. It is noteworthy to be mentioned that the first commercial *In* preparations became available for practice in 1922. Since then, much attention has been focused on development of new insulin formulations aiming at enhanced bioavailability, better solubility and longer stability of the protein

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drug [4, 5]. Still, the injectable insulin formulations are the most accessible; however, in the recent years, experiments with inhalable and oral *In* formulations have been in progress [6, 7].

Under the processing conditions (low pH, agitation, filtration, centrifugation, etc.) *In* can be easily unfolded and subsequently to form inactive fibrillar aggregates [8, 9]. Nowadays, the efforts of the scientists are directed to investigation of new excipients, methods for protein isolation, routes of the protein drug administration, etc. in order to minimize the production costs of *In* and to improve its effective application, i.e., to minimize the risk of insulin overdosing or its low bioavailability. In addition, *In* is very often used as a model protein in fundamental studies on the factors that are responsible for the correct protein folding. It is noteworthy to be mentioned that a century after *In* discovery the mechanism of its fibrillation is still disputable and is a subject of extensive investigations.

Recently, a great attention has been paid to ionic liquids (ILs) as potential stabilizers, i.e., suppressors to aggregation and hydrolysis, refolding additives, and appropriate solvents of proteins and enzymes [10–12]. In terms of Hofmeister series it has been found that small or multiply-charged ions with high charge density (kosmotropic ions) usually stabilize proteins but larger or single charged ions (chaotropic ions) are destabilizing for them [13]. In general, a lot of ILs containing both chaotropic cations and kosmotropic anions have beneficial effect on proteins and enzymes. In addition, hydrophobic ILs are more appropriate co-solvents for biocatalytic reactions than hydrophilic ones and there are many reports on enhanced enzyme activity in such reaction media [14]. However, it seems that many other characteristics of ILs such as their polarity, hydrogen-bond basicity and viscosity have strong influence on structure and stability of proteins and enzymes [13, 15].

Only few studies on the effect of ILs on insulin can be found in the literature. Recently, Kumar and Venkatesu have found that imidazolium-based thiocyanate, acetate, hydroxycitrate and halogenates denatured or even some of them hydrolyze the dissolved in sodium phosphate buffer (pH 7.0) *In* [16]. On the other hand, they have shown that in the presence of some ammonium-based ILs the thermal stability of *In* is enhanced and formation of protein aggregates is avoided [17]. In attempt to obtain insulin preparations suitable for oral delivery, Mahkam et al. [18] have found that soluble *In* can be successfully stabilized via encapsulation in ionic liquid silica functionalized nanoparticles.

Long-acting *In* formulations are still on demand because they offer less stressful treatment to patients than multiple daily insulin regimen. However, with many of these drugs arise problem with respect to their absorption by different individuals. Some of them are overcome with *Glargine*, a clear liquid *In* formulation with pH 4.0, which after injection form microprecipitates in tissues, and then is slowly and steadily absorbed [4]. Thus, stabilization of insulin at low pH is of great interest to pharmaceutical scientists and industrial biotechnologists. Hence, the aim of the research is to assess the effect of a series of imidazolium-based ILs $[C_4C_1im][X]$ on the stability of soluble *In* under acidic conditions. Differential scanning calorimetry (DSC) is applied to assess the thermal behavior of soluble *In* in the presence of the organic salts. In addition, the changes in the secondary structure of *In* induced by the tested ILs are monitored using Fourier transformed infrared spectroscopy (FTIR). The results on thermal stability of *In*-IL complexes are discussed with respect to their structural peculiarities.

Materials and methods

Materials

Insulin (pork) United States Pharmacopeia reference standard and 1-butyl-3-methylimidazolium acetate $[C_4C_1im][CH_3COO]$ were obtained by Fluka. 1-Butyl-3-methylimidazolium chloride $[C_4C_1im][Cl]$, 1-butyl-3-methylimidazolium trifluoroacetate $[C_4C_1im][CF_3COO]$, 1-butyl-3-methylimidazolium thiocyanate $[C_4C_1im][SCN]$ and 1-butyl-3-methylimidazolium dicyanamide $[C_4C_1im][N(CN)_2]$ were purchased from Aldrich. 1-butyl-3-methylimidazolium tricyanomethanide $[C_4C_1im][C(CN)_3]$ was supplied by Iolitec.

Methods

Differential scanning calorimetry

DSC experiments were performed on a high-sensitivity differential scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russia), with a sensitivity >0.017 mJ K^{-1} and a noise level $<\pm$ 0.05 μW. A scan rate of 1 °C min^{-1} from 30 to 110 °C with equilibrium period of 30 min was used. Buffer baseline was established prior to the analysis of the samples. The buffer–buffer baseline has been subtracted from each scan. Samples were scanned through two consecutive heating and cooling cycles in order to estimate the reversibility of the thermally induced transitions. The curves were plotted using the ORIGIN (MicroCal software) program package. The temperature at the maximum of the excess heat capacity curve was taken as the transition temperature (T_m). DSC measurements with *In* and *In*-ionic liquid complexes were taken in 10 mM KCl/HCl buffer, pH 2.0 (20 °C). The pH of the *In* solutions containing ILs was monitored, and where necessary, adjusted to pH 2.0

with 0.01 M NaOH or HCl. Molecular weight of 5800 Da for *In* was used in the calculation of its molar concentration.

Infrared spectroscopy

FTIR spectra of the *In* (17.8 mg mL^{-1}) dissolved in KCl/HCl (pH 2.0, 10 mM) or in 0.3 M aqueous solution of $[C_4C_1im][X]$ (in the same buffer) were recorded on Bruker Tensor 27 spectrometer, equipped with a detector of deuterated triglycine sulfate (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 0.3 M solutions of the corresponding ionic liquid the same media in order to subtract their absorptions.

In order to describe quantitatively the changes in the *In* secondary structure induced by the $[C_4C_1im][X]$, the ATR-FTIR spectra were treated in accordance with the established methods given in the literature [19]. ATR-FTIR spectra were Fourier deconvoluted by Opus software version 5.5 using bandwidth 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 [20].

Results and discussion

Effect of the tested series of imidazolium-based ILs on the thermal stability of *In*

The DSC profile of *In*, dissolved in KCl/HCl (pH 2.0, 10 mM), is characterized with an endothermic event with maximum centered at 75.4 °C (Fig. 1). The DSC curves of *In* in the presence of 0.05 M $[C_4C_1im][X]$ are depicted in Fig. 2. The corresponding values of the temperature of

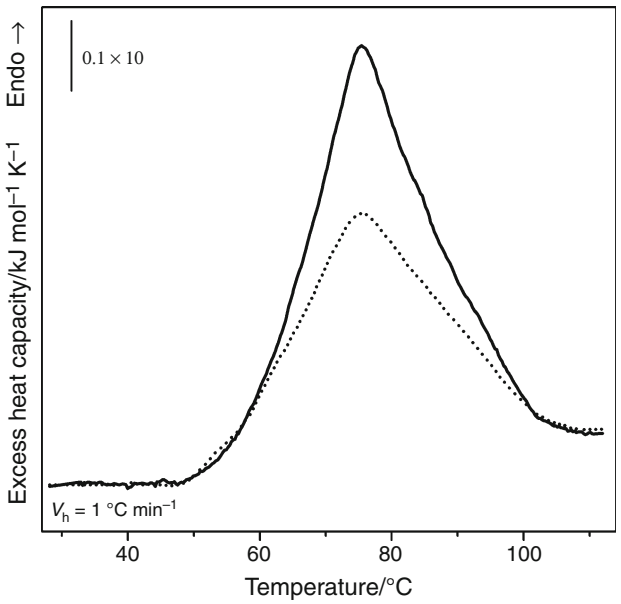

Fig. 1 DSC curves of insulin (0.43 mM) dissolved in 10 mM KCl/HCl, pH 2.0. Profile of DSC curve at heating to 110 °C (first scan; *solid line*); profile of DSC at heating to 110 °C, subsequent cooling to 30 °C, and repeated heating to 110 °C (second scan, *dash line*)

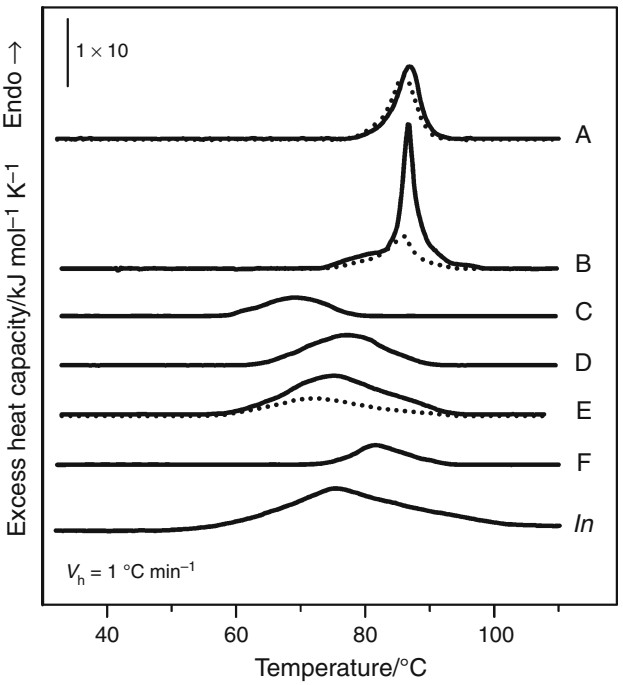

Fig. 2 Thermal denaturation profiles of insulin (0.43 mM) in KCl/HCl buffer (pH 2.0, 10 mM) with added $[C_4C_1im][CH_3COO]$ (*A*), $[C_4C_1im][CF_3COO]$ (*B*), $[C_4C_1im][C(CN)_3]$ (*C*), $[C_4C_1im][N(CN)_2]$ (*D*), $[C_4C_1im][SCN]$ (*E*), $[C_4C_1im][Cl]$ (*F*). Heating rate was 1 °C/min. The *solid lines* represent the first scans and the *dashed lines*—the second scans. The experimental conditions were identical to those given in Fig. 1

maximum heat capacity (T_m), the experimental enthalpy values (ΔH_{cal}) and the van't Hoff ratios ($\Delta H_{cal}/\Delta H_{VH}$) were calculated from the experimental curves (Table 1). As can be seen, regardless of the medium composition, the thermal unfolding of *In* appears as a single endothermic peak. The acetate and the trifluoroacetate imidazolium salts seemed to stabilize *In* significantly and in their presence the peak for *In* unfolding was shifted toward higher temperatures with 11.4 and 11.1 °C, respectively. We found that the three cyano-based ILs tested here produced weak effects on *In* thermal stability under the experimental conditions. The results from DSC studies indicate that the thermal denaturation temperature of *In* is shifted to lower temperatures in the presence of $[C_4C_1im][C(CN)_3]$ and $[C_4C_1im][SCN]$ by 6.2 and 0.5 °C, respectively. However, when $[C_4C_1im][N(CN)_2]$ was added to the dissolved in KCl/HCl (pH 2.0, 10 mM) *In*, a shift of its transition temperature with 1.7 °C toward higher temperatures was observed. A correlation between the stabilization/destabilization effect of the three cyano-based ILs and their tendency to interact with water molecules can be found. Batista et al. [21] have discovered that with increase in the number of cyano groups in the anion of imidazolium-based ILs, $(SCN < N(CN)_2 < C(CN)_3)$ decreases the strength of their interactions with water and the salts become more prone to aggregate. In the presence of these three ILs, the thermal

Table 1 Transition midpoints and enthalpies of unfolding of insulin (0.43 M) dissolved in KCl/HCl (pH 2.0, 10 mM) in the presence of 0.3 M imidazolium-based ionic liquids

Medium	T_m/°C	ΔH_{cal}/kJ mol^{-1}	ΔH_{VH}/kJ mol^{-1}	$\Delta H_{cal}/\Delta H_{VH}$	$T_{1/2}$/°C
IL-free first scan	75.43	120.2	185.9	0.65	19.0
(Second scan)	(75.5)	(108.5)			(27.4)
$[C_4C_1im][CH_3COO]$ first scan	86.85	53.6	869.2	0.06	4.1
(Second scan)	(85.88)	(50.6)			(4.5)
$[C_4C_1im][CF_3COO]$ first scan	86.59	88.8	1142.6	0.08	2.2
(Second scan)	(86.06)	(31.6)			(4.8)
$[C_4C_1im][C(CN)_3]$ first scan	69.21	32.8	333.7	0.10	11.2
$[C_4C_1im][N(CN)_2]$ first scan	77.13	65.9	280.1	0.24	13.8
$[C_4C_1im][SCN]$ first scan	74.93	105.3	224.2	0.47	17.4
(Second scan)	(72.27)	(47.2)			(16.5)
$[C_4C_1im][Cl]$ first scan	75.65	28.3	425.4	0.07	8.9

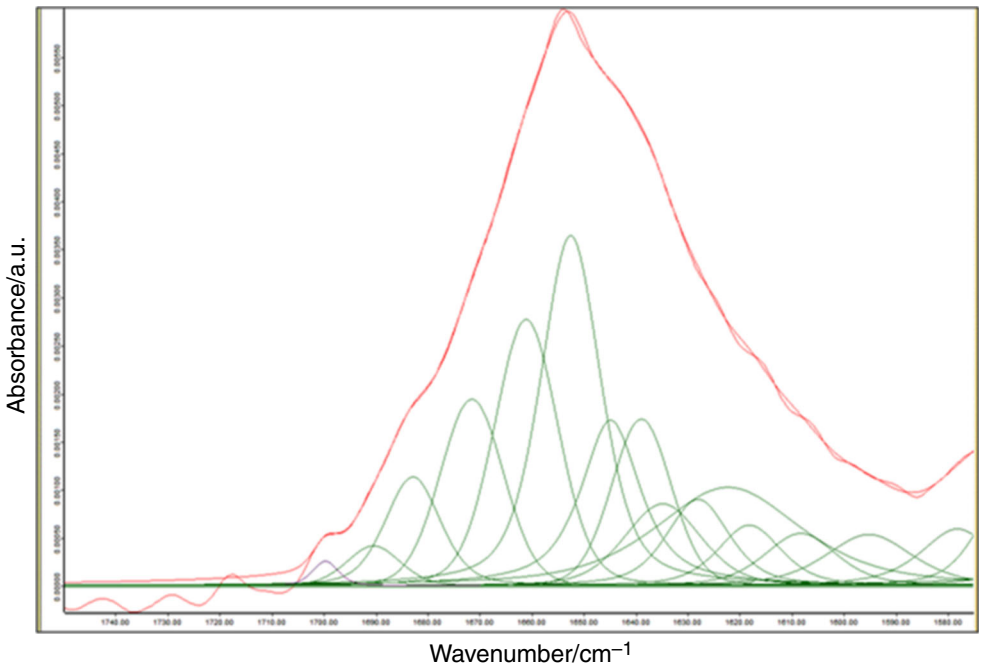

Fig. 3 Fourier-deconvoluted spectrum FTIR (ATR) in the Amide I region and reconstructed spectra after curve fitting of insulin in 10 mM KCl/Cl (pH 2.0)

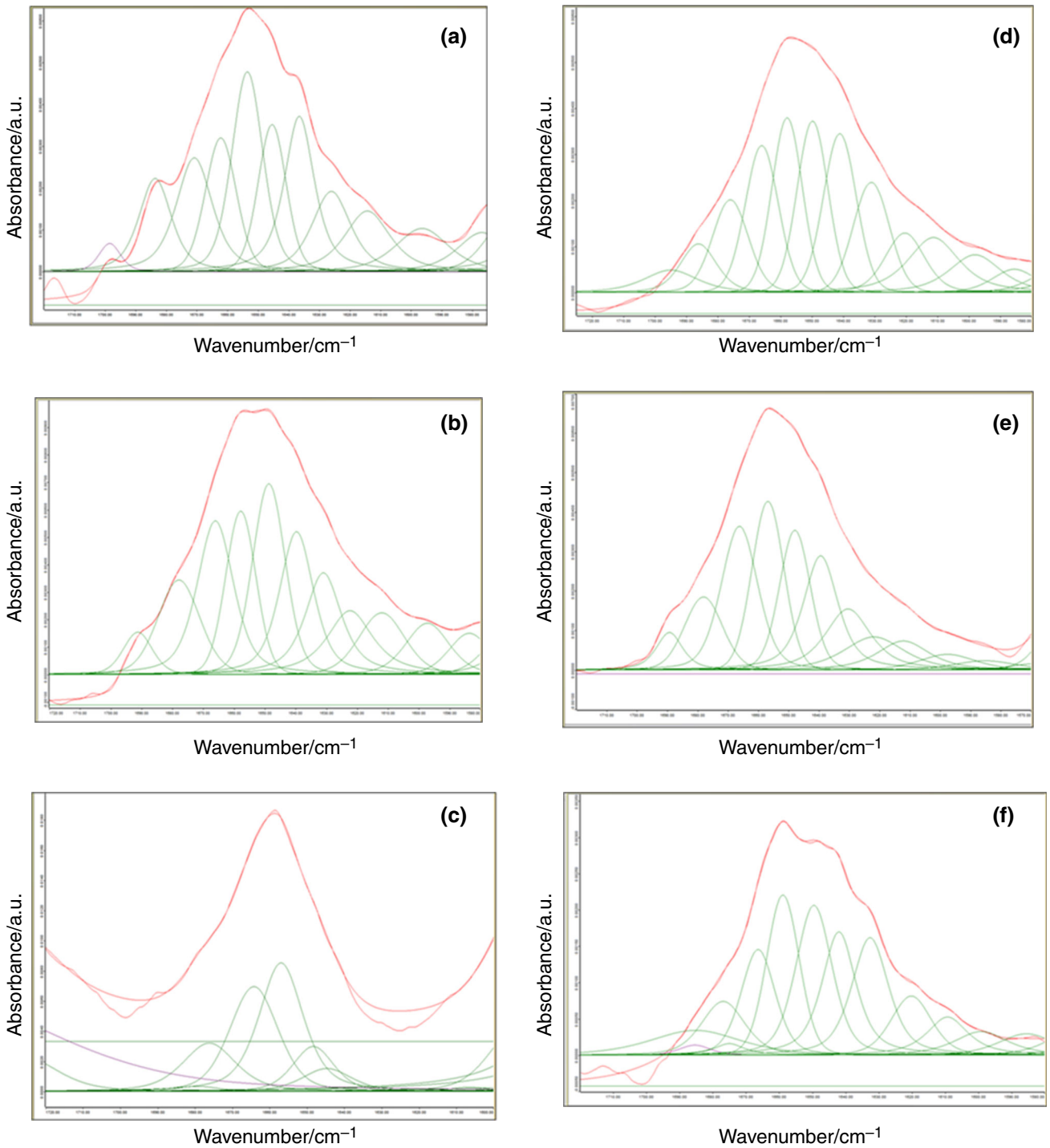

Fig. 4 Fourier-deconvoluted spectra FTIR (ATR) in the Amide I region and reconstructed spectra after curve fitting of insulin in 10 mM KCl/Cl (pH 2.0) in the presence of 0.3 M $[C_4C_1im][CH_3COO]$ (**a**), $[C_4C_1im][CF_3COO]$ (**b**), $[C_4C_1im][C(CN)_3]$ (**c**), $[C_4C_1im][N(CN)_2]$ (**d**), $[C_4C_1im][SCN]$ (**e**), $[C_4C_1im][Cl]$ (**f**)

stability of *In* decreased in the same order, i.e., with lowering the ability of the corresponding anion to establish hydrogen bonds with water molecules.

No changes in the transition temperature of *In* were observed in the presence of $[C_4C_1im][Cl]$ in comparison with the IL-free sample (Table 1). However, in the presence of the chloride and the tricyanomethanide IL, the measured heat capacity of the *In* was about two times lower than that estimated for the control sample (IL-free system). One possible explanation is that in these two media *In*

Table 2 Quantitative estimation of the secondary structural elements of insulin in the presence of imidazolium-based ionic liquids calculated from their curve fitted FTIR spectra

Band assignments (frequency interval)	Insulin dissolved in KCl/HCl buffer (pH 2.0, 10 mM) containing 0.3 M of the corresponding $[C_4C_1im][X]$						
	IL-free	$[X]=[CH_3COO]$	$[X]=[CF_3COO]$	$[X]=[C(CN)_3]$	$[X]=[N(CN)_2]$	[X]=[SCN]	[X]=[Cl]
Antiparallel β-sheet, extended side chains (1600–1615 cm^{-1})	3.49	9.17	7.35	1.39	9.59	5.95	4.57
β-Sheet (1615–1640 cm^{-1})	35.97	25.57	35.3	0.67	31.12	33.71	34.27
Unordered structures, random coils (1642–1650 cm^{-1})	10.24	11.34	15.92	17.69	14.06	13.79	22.94
α-Helices(1652–1658 cm^{-1})	19.41	17.89	12.0	34.75	14.04	16.40	14.83
3_{10}-Helices (1660–1663 cm^{-1})	13.89	11.22	16.04	–	–	–	–
Distorted, β-turns, loops (1666–1687 cm^{-1})	14.60	22.95	10.12	45.50	18.17	26.3	23.33
Antiparallel β-sheets(1690–1696 cm^{-1})	2.40	1.85	3.27		3.89	3.81	–

underwent phase transition and the system became more ordered [22]. On the other hand, lowering of the C_p can be due to increase in hydration of polar groups comprising the protein and decrease in the exposure of hydrophobic surface [23]. Decrease in heat capacity can also be ascribed to weakening of the protein–protein interactions in the presence of ILs or decrease in overall flexibility of the protein [22, 24].

The second heating scan of the *In* in IL-free medium showed about 90.0 % of reversibility. Interestingly, $[C_4C_1im][CH_3COO]$ seems to promote reversible folding and an increase in the area under the peak of the second scan by 4.4 % in comparison with *In* dissolved in KCl/HCl (pH 2.0, 10 mM) was observed. In contrast, in the presence of $[C_4C_1im][CF_3COO]$ and $[C_4C_1im]$ [SCN] the area under the peak of the second scan was reduced by approx. 64.4 and 55.2 % compared to the first scans, respectively. The thermally induced transition of *In*, however, was irreversible in the presence of ILs with $[C(CN)_3]$, $[N(CN)_2]$ or [Cl] anion.

Depending on the structure of the added IL, ΔH_{cal} ranged from 28.3 to 120.2 and from 47.2 to 108.5 kJ mol^{-1} at the first and the second scans, respectively. The highest ΔH_{cal} value was estimated for *In* in the absence of organic salts. In addition, in IL-free system, the effective van't Hoff enthalpy of *In* denaturation was found to be about 1.5-fold higher than calorimetric one (Table 1). In the presence of the imidazolium-based salts, however, the ratio $\Delta H_{cal}/\Delta H_{VH}$ deviates from 0.06 to 0.47, which implies strong intermolecular interactions that should be overcome to get from one state to another, aggregation or irreversibility. The lowest $\Delta H_{cal}/\Delta H_{VH}$ ratio was estimated for the *In* in the presence of the acetate salt; however, this result is not consistent with the observed high degree of reversibility of thermal denaturation.

Interestingly, the width of the transition at half height of the peak ($T_{1/2}$) values varies from 2.2 to 19.0 and from 4.5 to 27.5 °C for the first scan and the second scan, respectively. The highest $T_{1/2}$ value was estimated for the thermal unfolding of *In* in IL-free system (Table 1). The transition was highly cooperative in media containing the tested acetate or trifluoroacetate salts.

Changes in the secondary structure of *In* in the presence of $[C_4C_1im][X]$

To assess the effect of the tested ILs on *In* secondary structure we followed the changes in the Amide I region (1600–1700 cm^{-1}) of the infrared spectra of *In* recorded in the presence of these additives. The Amide I band is mainly due to the stretching vibrations of the carbonyl groups that constitute protein backbone (approx. 80 %), and the frequencies of each Amide I band component are closely correlated with the secondary structure composition. This band is highly sensitive to structural alternations. As can be seen in Fig. 3, the ATR-FTIR spectra of *In* in IL-free system consists of seven components observed at 13 well-defined frequencies. Similarly, six or seven structural elements were observed in the ATR-FTIR spectra of *In* taken in the presence of the imidazolium-based ILs (Fig. 4). For all samples, the percentages of β-structures were calculated by adding the areas of all bands typically assigned to the corresponding β-structural segment and the sum is expressed as a fraction of the total Amide I band area (Table 2). The estimated by us percentages of α-helical structures and β-structures for *In* in KCl/HCl (pH 2.0)

are in good agreement with the reported by Wei and co-authors secondary structure derived from FTIR and X-ray studies [25]. Introduction of $[C_4C_1im][X]$ into medium resulted in secondary structural rearrangements of *In*, which is evident from the observed differences in all ATR-FTIR spectra. As a whole the secondary structure of *In* is well preserved in the presence of the acetate salt. The later probably stabilizes the native state of the protein, which resulted in an enhanced thermal transition temperature and higher reversibility of *In* denaturation process in comparison with the values estimated for the *In* in IL-free system. Within all tested media, *In* occupies the most helical conformation in the presence of $[C_4C_1im][CF_3COO]$. The result is in agreement with the earlier reported stabilization of twenty-six gap junction proteins in a fluorinated co-solvent. Fort and Spray have reported that these transmembrane proteins are largely unstructured in aqueous media (buffer), while in 30 % trifluoroethanol they observed a presence of a considerable helical content (>40 %) [26]. Similarly, we assume that the trifluoroacetate salt stabilizes *In* inherent helical structures that are later unordered upon dissolution in aqueous medium. As can be seen in Fig. 4c, the most dramatic changes in *In* secondary structure occur in the presence of $[C_1C_4im][C(CN)_3]$, which is in agreement with the observed decrease in *In* thermal stability in this medium. The overlap of secondary structural components is significant even after mathematical enhancement. Thus, we are aware that more detailed interpretation of these results will be speculative.

The ATR-FTIR spectra of *In* in the presence of the dicyanamide, the thiocyanate and the chloride imidazolium salts were not identical; however, some distinct similarities were found. Indeed, for these three systems we observed a disappearance of the band indicative for 3_{10}-helices (1660–1663 cm^{-1}) and a decrease in the α-helical content in favor of the unordered, the coiled and the distorted structures. Interestingly, despite of the induced by $[C_4C_1im][N(CN)_2]$, $[C_4C_1im][SCN]$ or $[C_4C_1im][Cl]$ visible conformational changes of the *In* secondary structure, the later produced a weak effect on *In* thermal stability but the protein unfolding was irreversible in these media.

Conclusions

In terms of decreasing Tm values the ability of the tested ILs to stabilize/destabilize *In* followed the order: $[C_4C_1im][CH_3COO] > [C_4C_1im][CF_3COO] > [C_4C_1im][N(CN)_2] > [C_4C_1im][Cl] \approx$ IL-free medium $\approx [C_4C_1im][SCN] > [C_4C_1im][C(CN)_3]$. All five ILs altered the secondary structure of *In*. As a whole, those of the ILs which were able to preserve or enhance helical structure of *In* produced stabilizing effect and those which stimulated the formation of unordered and random-coiled structures deteriorate its thermal stability. In addition, no aggregation of *In* in the presence of the imidazolium-based ILs was observed under the tested acidic media.

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