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Optimization of the hydrochloric acid concentration used for trifluoroacetate removal from synthetic peptides

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Abstract: Trifluoroacetate (CF_3COO^-, or TFA) is almost always present in commercially synthesized peptides. Unfortunately, it has a strong infrared (IR) absorption band at 1673 cm^{-1}, significantly overlapping or even completely obscuring the amide I band of a peptide. In such cases TFA must be removed from the solution in order to be able to use IR absorption spectroscopy for peptide secondary structure determination. The most convenient and widely used procedure involves peptide lyophilization from a 0.1 M HCl solution. In our studies of the tryptophan-rich antimicrobial peptide indolicidin, we have found that caution should be taken when using this HCl concentration. High HCl concentrations (>10 mM in unbuffered solutions and >50 mM in buffered solutions) may modify the peptide structure and reduce its thermal stability, thereby interfering with subsequent structural investigations of the peptide. Our results indicate that HCl concentrations between 2 and 10 mM are adequate to remove essentially all TFA impurities without any modification of the peptide secondary structure. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hydrochloric acid; infrared spectroscopy; peptides; trifluoroacetate

INTRODUCTION

Over the past few decades Fourier transform infrared spectroscopy (FTIR) has been extensively used as a relatively fast and convenient tool for the determination of protein and peptide secondary structure [1–19]. Unlike protein crystallography or multidimensional NMR, this technique cannot provide a precise three-dimensional location of individual structural elements, but it is capable of determining the overall secondary structure of a protein or a peptide. Some of the obvious advantages of FTIR are its high sensitivity to structural and conformational changes of proteins, its relatively short acquisition and processing times, the relatively low sample requirements, the virtually unlimited molecular weight range of proteins that can be studied, and the possibility to work in various solvents (including aqueous solutions). Finally, it allows the study of various protein–ligand complexes, where the ligands can range from simple substances such as metal ions to complex biomolecules such as proteins, DNA, or lipid vesicles.

Because of its versatility, FTIR absorption spectroscopy has been widely applied to study both proteins and peptides. In most cases determination of the secondary structure of peptides is somewhat easier and more straightforward than that of large proteins owing to the presence of only one or two structural motifs (α-helix, β-sheet, turns, random coil, etc.) in peptides *versus* a combination of many secondary structure elements in proteins, producing sometimes poorly resolved and highly overlapping spectral features. However, because of the numerous steps and the various reagents involved in peptide synthesis and purification, there is a high probability of the presence of various organic counterions and/or residual impurities resulting from the chemical synthesis. Such ions are not normally present in protein preparations because these are usually prepared biosynthetically. Organic ion impurities might have a very strong IR absorption and even residual trace amounts of such substances can produce strong absorption bands in the peptide amide I band region (1700–1600 cm^{-1}), which might strongly overlap or even completely obscure the peptide amide I band, making any interpretation of the spectra impossible. We will focus here on one such organic counterion, trifluoroacetate (CF_3COO^-, or TFA), which is almost invariably present in commercially synthesized peptides.

Surewicz and Mantsch, while performing an IR absorption study of the peptide dynorphin A-(1–13), noticed the presence of a strong absorption band with a maximum centered at 1673 cm^{-1} [2]. This band was completely eliminated upon chromatography of the commercial peptide preparations on an Amberlite column. This finding indicated that the above band did not originate from the peptide itself but came from organic counterions or other impurities. Since TFA acid is routinely used as a solvent in the final stage of peptide purification, the authors suggested that residual TFA might be responsible for the strong absorption band.

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TFA may bind to certain amino acid side chains, which are particularly pronounced for peptides, comprising a large number of positively charged side chains. Indeed, Surewicz and Mantsch [2] obtained an IR absorption spectrum of sodium trifluoroacetate, which had a single strong band at 1673 cm^{-1}, arising from the antisymmetric COO^- stretching vibration of the TFA ion.

The presence of a TFA absorption band in the IR absorption spectra of synthetic peptides has also been discussed by a number of other authors [3–6,9,10,18–20]. It must be noted that some researchers do not pay enough attention to this issue and sometimes this band has been erroneously included as part of the amide I band of a peptide [21]. Gaussier *et al.* [7] have emphasized the necessity of TFA removal not only because it interferes with the amide I band in the IR absorption spectra but also because TFA binding to a peptide may modify its conformation. Formation of intermolecular hydrogen bonds between TFA and peptide groups may additionally modify the peptide spectra, complicating a reliable spectral analysis [22]. Moreover, TFA contaminants may hamper not only conformational analysis but also biological assays. It has been shown that TFA at the concentrations present in commercial peptides possesses significant biological activity and inhibits proliferation of osteoblasts and chondrocytes [23]. Therefore, whenever possible, its removal is always desirable. On the other hand, some authors have used the TFA peak as an internal concentration standard in IR absorption spectra, allowing quantitative spectral comparisons to be made [18].

Various procedures have been used to remove TFA from peptide samples, such as washing peptides in dialysis membranes, removing TFA by adding 2,2,2-trifluoroethanol (TFE), or by applying column chromatography [7]. However, most of these techniques are time consuming and significantly reduce the peptide yield; moreover, addition of TFE can induce the formation of helical structures in peptides [24]. Gaussier *et al.* [7] have proposed the replacement of TFA with HCl during peptide purification, which would eliminate the necessity of subsequent TFA removal. This might be a valid and efficient approach when peptides are synthesized directly in the research laboratories. However, as in most cases, commercially synthesized peptides are used and they already contain TFA; it should be removed anyway.

The most efficient way to remove TFA from peptides is to lyophilize the sample several times in the presence of HCl [1,3–7,20], directly replacing TFA counterions with chloride ions. The main advantage of this technique is its simplicity. In addition, there is essentially no loss of the sample in the lyophilization steps. Different authors report various HCl concentrations used in the TFA removal procedure. The most common concentration is 0.1 M HCl [3,4,6,15]. However, some authors have used lower HCl concentrations, for example 50 mM [20] or 10 mM [1,5]. Nevertheless, the influence of HCl or a low pH on the peptide structure has not been systematically assessed.

In the present work we have monitored changes in peptide conformation as well as peptide thermal stability upon TFA removal with different HCl concentrations. We have used the widely studied tryptophan-rich antimicrobial peptide indolicidin in these studies [25–33], which is amidated at the *C*-terminal end and has no His, Asp, or Glu residues, thereby rendering it stable against pH changes below pH 7.

MATERIALS AND METHODS

The commercially synthesized peptide indolicidin (ILPWKWPW-WPWRR-NH_2) was purchased from Anaspec (San Diego, CA). D_2O (99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA). TFA (99 + %, spectroscopic grade) was from Sigma-Aldrich (Oakville, ON, Canada). HCl (concentrated) and all salts were purchased from Fisher Scientific (Ottawa, ON, Canada). Double distilled water was used in all experiments.

The peptide was initially dissolved either in unbuffered double distilled H_2O or in phosphate buffer (50 mM phosphate and 100 mM NaCl) at a concentration of 1 mg/ml (5.2×10^{-4} M of peptide). Aliquots of the peptide solution were mixed with HCl solution to yield final HCl concentrations of 100, 50, 10, 5, 2, 1 and 0.1 mM. The samples were lyophilized overnight and repeatedly re-dissolved in a solution with the corresponding HCl concentration. The cycle of re-dissolving and lyophilization was repeated 3 times [1,4–7,20]. After the final lyophilization the peptide samples were dissolved in D_2O at a concentration of 2 mg/ml. The pH of the samples was controlled with an Accumet Research pH meter AR20 (Fisher Scientific), equipped with a PHR-146 Micro Combination pH electrode (Lazar Research Laboratories, Los Angeles, CA). The pH values for the unbuffered solutions varied between 3 and 6 depending on the HCl concentration used. The pH of the buffered solutions was 6.5 ± 0.2. The peptide sample (20 μl) was deposited on the attenuated total reflectance (ATR) crystal for FTIR measurements. An IR absorption spectrum of 0.05% (v/v) TFA solution in D_2O was also obtained for comparison with the peptide spectra.

The measurements were performed in the temperature range between 10° and 30°, in 4 °C steps. The temperature was regulated by a thermostated K10 water bath with a DC30 circulator (Thermo Haake, Karlsruhe, Germany) using the Protein software (Bruker Optics, Billerica, MA) with an accuracy of ±0.1 °C.

All FTIR absorption spectra were collected with an Equinox 55 FTIR spectrophotometer (Bruker Optics, Billerica, MA) with a Bio-ATR II attachment (Bruker Optics) equipped with a Silicon ATR crystal. A total of 256 scans were accumulated for each spectrum at 4 cm^{-1} resolution. ATR spectra were automatically converted to absorption spectra using the Opus 4.2 software (Bruker Optics). The ATR attachment was continuously purged with dry N_2 gas. In addition, residual water vapor traces were removed by the Atmospheric Water Compensation subroutine in the Opus 4.2 software. Solvent

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spectra obtained under the same conditions were subtracted from all sample spectra. Baseline correction and interpolation (with interpolation factor 4) were performed by the Opus 4.2 software. All IR absorption spectra were normalized to 2.0 a.u. (absorption units) for easier comparison.

For circular dichroism (CD) measurements, the same TFA removal procedure was used as for the FTIR experiments. Peptide samples were dissolved in double distilled H_2O with a final concentration of 0.25 mg/ml. 200 µl of the sample was used in a quartz rectangular UV cell with a path length of 1 mm. CD spectra were recorded using a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan). A total of three scans were accumulated and averaged for each spectrum at a resolution of 0.5 nm. The solvent spectrum measured at identical conditions was subtracted from all sample spectra. Noise reduction was performed using the supplied subroutine in the Jasco software. The necessary temperature was maintained by a thermostated RTE 111 water bath (Neslab Instruments, Portsmouth, NH) and controlled with an electronic thermometer with a thermocouple (Omega Engineering, Stamford, CT).

RESULTS AND DISCUSSION

IR absorption spectra of indolicidin purified with different HCl concentrations, and measured in unbuffered D_2O solution at 10 °C in the amide I region are compared in Figure 1. The IR absorption spectrum of TFA is presented for comparison with the peptide spectra.

The most intense band in the spectrum of the peptide not purified from TFA (no HCl) appears at 1672 cm^{-1} and clearly corresponds to the strong single band of the TFA spectrum. This band arises from the vibration of the carbonyl group in a TFA molecule, which might be involved in the intermolecular hydrogen bonds with peptide groups [22]. The second most intense absorption band with the peak centered around 1644 cm^{-1} is the amide I band of the peptide. Its position suggests a mainly random coil secondary structure for the peptide [2,7,11-15,17]. However, as the amide I band strongly overlaps with the TFA band, it is significantly broadened, and its peak position is hardly detectable, which undermine any reliable estimation of the peptide secondary structure.

Repeated lyophilization of the peptide with 0.1 mM HCl significantly reduces the content of TFA in the sample, which is seen from the decreased intensity of the TFA peak at 1672 cm^{-1}. The amide I peak position is more readily detectable in this spectrum and it is centered around 1642 cm^{-1}, now clearly indicating that the peptide conformation mainly corresponds to a random coil [2,7,11-15,17]. However, the relatively strong TFA band at 1672 cm^{-1} still overlaps with the amide I band and obscures any possible amide I structurally important bands around 1690–1650 cm^{-1}.

Increasing the HCl concentration to 1 mM virtually completely removes TFA from the solution, as seen from the absence of the TFA band in the peptide spectrum.

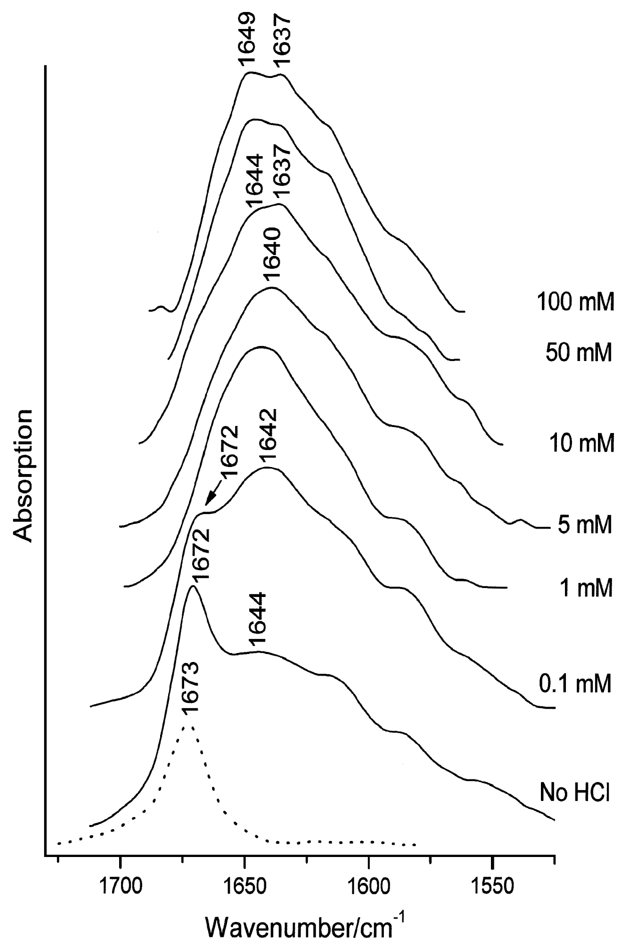

Figure 1 IR absorption spectra of indolicidin amide I region in D_2O at 10 °C with different concentrations of HCl used to remove TFA. The spectrum of TFA (......) is also shown.

The amide I band becomes usable for peptide secondary structure determination and application of resolution enhancement techniques [11–15].

Further increase of the HCl concentration during sample purification up to 10 mM does not change the amide I spectrum and the peak remains at around 1642–1640 cm^{-1}. However, at 10 mM HCl, the maximum of the amide I band splits into two peaks at 1644 and 1637 cm^{-1}. The splitting becomes more enhanced as the concentration of HCl increases, and at 100 mM HCl the peaks are clearly separated and appear at 1649 and 1637 cm^{-1}. Overall, the bandshape of the spectrum also becomes distorted. These spectral modifications indicate that the peptide conformation undergoes changes upon peptide treatment with HCl concentrations of 10 mM and above. It is known that tryptophan residue is sensitive to acidic treatments; tryptophan hydrolysis and complete destruction occurs in the presence of 6 M HCl [34], while formation of the tryptophan hydrochloride salt in the presence of 2 M HCl has been reported [35]. However, HCl concentrations used in our experiment are significantly lower and therefore

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tryptophan chemical modifications, which might induce the observed spectral changes, cannot be expected. Thus, we attribute the observed spectral modifications to the changes in the peptide secondary structure. The low-energy band at 1637 cm^{-1} appears in the range of β-sheet structure [2,7,11–15,17], which might indicate the appearance of the molecules with some extent of β-sheet conformation. The shift to lower wavenumbers might also occur owing to the higher extent of the intra- and intermolecular hydrogen bonding within the random coil structure. Disregarding the particular type of the structural changes occurring, such changes are not a desirable effect of the TFA removal procedure, particularly if further investigation of the secondary structure of the purified peptide is performed.

To check the validity of the obtained IR absorption data and confirm that the observed structural changes have a general character, we performed a similar set of experiments using CD spectroscopy. CD spectra for several HCl concentrations in unbuffered H_2O solutions measured at 10 °C are shown in Figure 2. The CD spectrum for 2 mM HCl displays a well-defined negative maximum located at 200 nm, suggesting that the peptide adopts a random coil conformation [7,36–38]. An additional shoulder is seen between 200 and 230 nm, centered at 218 nm, which is characteristic of some helical structure and may suggest a small number of molecules possessing an ordered secondary structure [7,36,38]. Upon increasing the HCl concentration, along with slight modifications of the spectral bandshape, the position of the negative maximum at 200 nm varies, and the intensity of the shoulder at 218 nm first decreases at 10 mM and then completely disappears at higher HCl concentrations. Therefore, the observed CD spectral changes indicate that the peptide secondary structure is modified at higher HCl concentrations, which agrees with the obtained IR absorption data.

In order to estimate the contribution of the low pH (3–5) on the spectral changes, we repeated the experiments in a buffer solution with pH 6.5. The corresponding IR absorption spectra are shown in Figure 3. As in the unbuffered solution, purification of the sample with 0.1 mM of HCl does not remove TFA ions from the solution completely and a shoulder at 1672 cm^{-1} is readily noticeable in the spectrum. The maximum of the amide I band appears at 1640 cm^{-1}, again indicating mostly random coil structure of the peptide [2,7,11–15,17]. Similar to the unbuffered solution, the addition of 1 mM of HCl during purification steps essentially removes TFA from the sample, as no absorption band or shoulder can be noticed around 1672 cm^{-1}. No spectral changes can be observed upon increasing the HCl concentration up to 50 mM. The amide I band peak position remains at 1640 cm^{-1}. The peptide structure appears to be more stable in buffered solution and spectra are almost identical in this HCl concentration range. However, when 50 mM of HCl was used, the amide I band broadens and its maximum starts to split into two peaks at 1650 and 1638 cm^{-1}, as in the unbuffered sample. Increasing the HCl concentration to 100 mM again further enhances the splitting and makes the two peaks more distinct, although the spectral changes are much less pronounced than in the unbuffered solutions. Therefore, the low pH plays an important role in the observed spectral modifications and, correspondingly, in structural changes of the peptide. Despite significant stabilization of the structure due to buffering of the solutions at neutral or near-neutral pH, some changes still take place at high HCl concentrations (50 mM and higher).

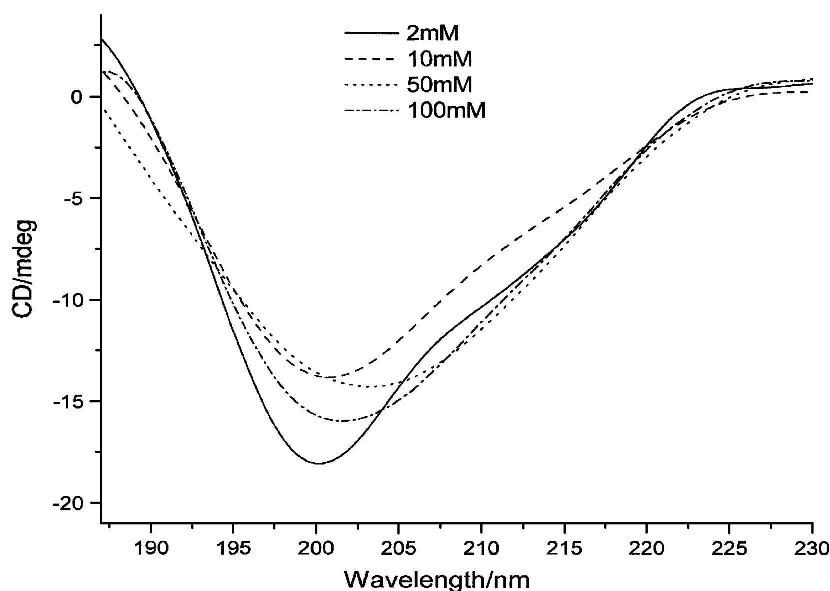

Figure 2 CD spectra of indolicidin in H_2O at 10 °C with different concentrations of HCl used to remove TFA.

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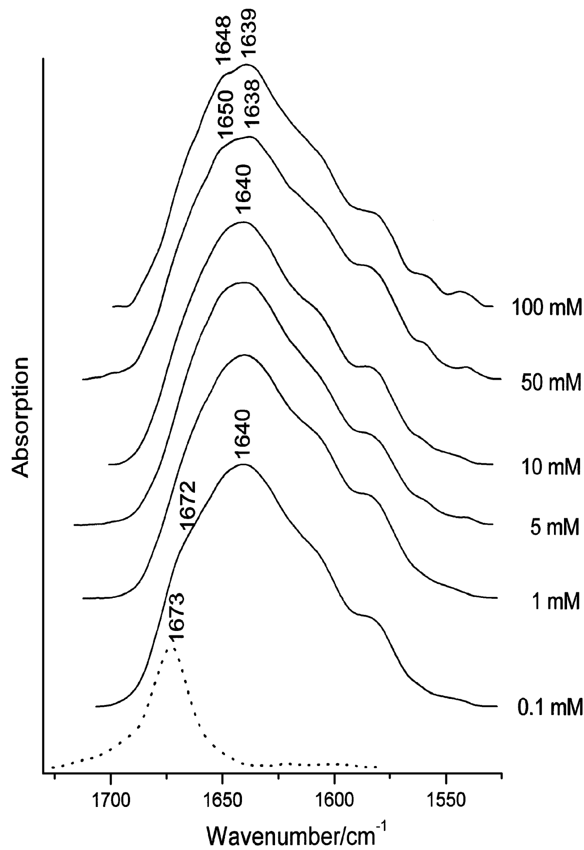

Figure 3 IR absorption spectra of indolicidin amide I region in 50 mM phosphate buffer at 10 °C with different concentrations of HCl used to remove TFA. The spectrum of TFA (......) is also shown.

In order to examine the influence of the high HCl concentration used in the TFA removal procedure on the thermal stability of the peptide structure, we compared the IR absorption spectra of the peptide amide I region between 10 °C and 30 °C for 1 mM and 100 mM HCl in Figure 4 (unbuffered solution) and Figure 5 (phosphate buffer). In unbuffered solution, the spectra of the peptide appear to be virtually identical at 10 °C and 30 °C when 1mM HCl was used (Figure 4), indicating high stability of the peptide structure and absence of any noticeable structural changes. However, the spectra differ at 10 °C and 30 °C, if 100 mM HCl is used, suggesting a decrease of the peptide stability and some structural changes. In the case of the buffered solution, while the peptide spectra at 10 °C and 30 °C are virtually identical with 1mM HCl, some noticeable differences can be seen for 100 mM HCl (Figure 5). These results show that, while buffering of the peptide solutions to neutral pH significantly increases the thermal stability of the peptide at a high HCl concentration, it does not completely eliminate the effect of high concentrations of HCl (>10mM) on the structure of the peptide.

In addition to indolicidin, we have tested low HCl concentrations for TFA removal on a number of other antimicrobial peptides (tritrpticin and its analogs) (data not shown). The TFA ions were essentially completely removed with 2 mM HCl for all the peptides tested.

CONCLUSIONS

Structural stability and variations of the peptide indolicidin, induced by different concentrations of HCl used

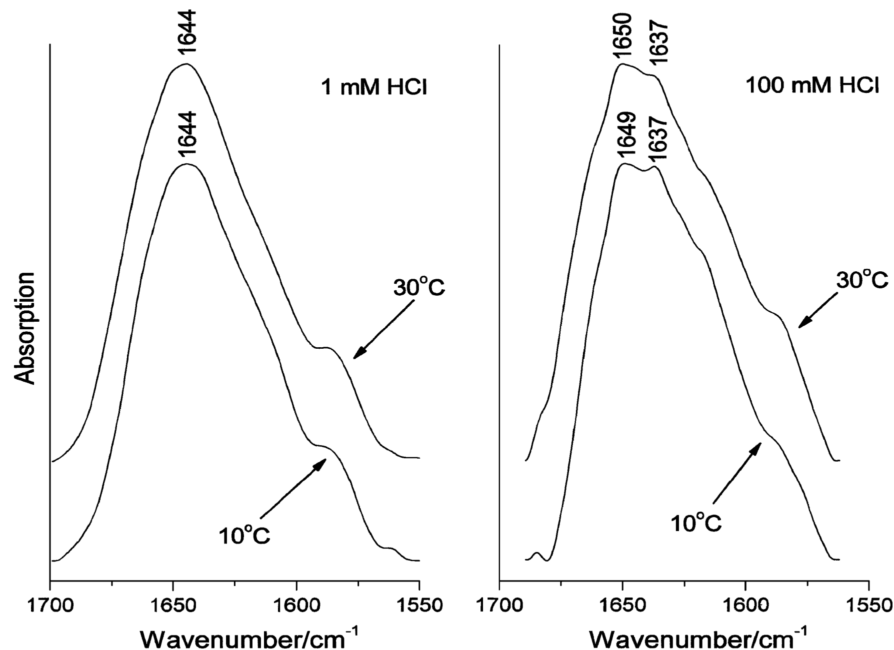

Figure 4 IR absorption spectra of indolicidin amide I region in D_2O at 10 °C and 30 °C with 1 mM HCl (left) and 100 mM HCl (right).

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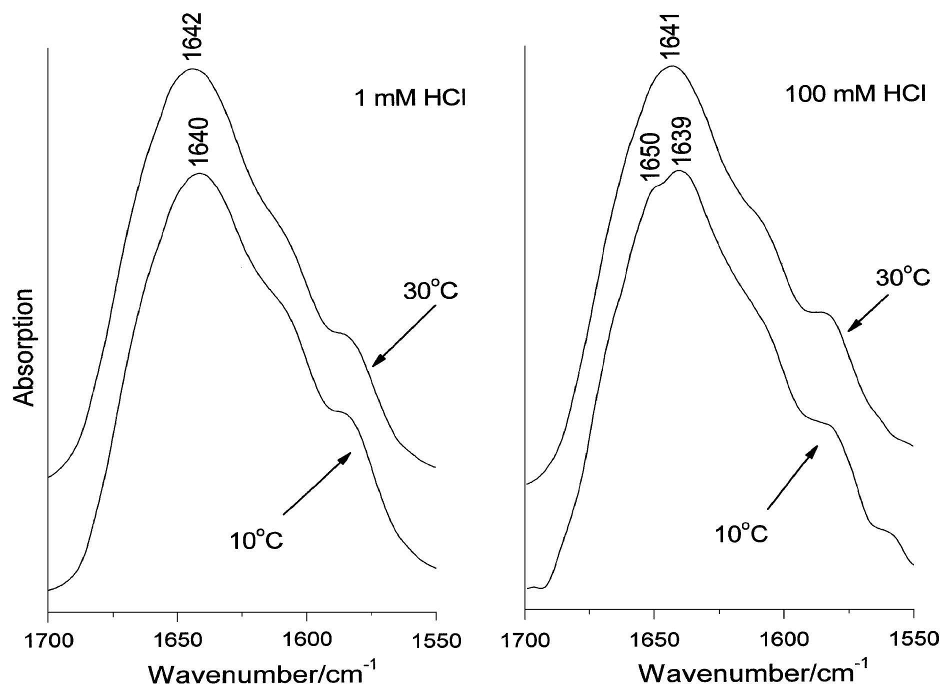

Figure 5 IR absorption spectra of indolicidin amide I region in 50 mM phosphate buffer at 10 °C and 30 °C with 1 mM HCl (left) and 100 mM HCl (right).

in the TFA removal procedure were studied by FTIR absorption and CD spectroscopy. It was shown that commonly used HCl concentration of 0.1 M may induce significant modifications in the peptide structure and, therefore, hinder the correct interpretation of the results obtained in subsequent studies of the peptide interaction with ligands. High HCl concentrations also reduce peptide thermal stability, facilitating even more pronounced changes of its secondary structure, which might interfere with subsequent thermal studies of the peptides. The destabilizing effect of HCl is significantly reduced if the peptide solution is buffered to a neutral or near-neutral pH.

We have also shown that the most widely used and relatively high HCl concentration of 0.1 M is not always required to completely remove TFA from the samples. Although 0.1 M HCl probably can be used if peptides are characterized by very high stability, sufficient TFA removal can be achieved by as low as 1 mM HCl by repeating the procedure three or more times. In our study the safest and most efficient range of HCl concentrations was between 2 and 10 mM with three or more repeats of the removal procedure. We suggest that this procedure might be also applicable to other peptides. However, it would be useful to estimate the minimum concentration of HCl necessary and sufficient for each particular peptide or group of peptides.

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