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Conformational studies of alanine-rich peptide using CD and FTIR spectroscopy

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Abstract: The circular dichroism (CD) and Fourier transform infrared (FTIR) methods were applied to the conformational studies of alanine-rich peptide Ac-K-$[A]_{11}$-KGGY-NH_2 (where K is lysine, A is alanine, G is glycine and Y is tyrozyne) in water, methanol (MeOH) and trifluoroethanol (TFE). The analysis of CD-spectra of the peptide in water at different concentrations revealed that the secondary structure content depends on the peptide concentration and pH of the solution. The increase of the peptide concentration causes a decrease of α-helix content and, simultaneously, an increase of β-sheet structure, while the unordered structure is the predominant one. Additional elements are discovered in MeOH and TFE but α-helix and β-turns predominate. Moreover, in these solutions the percentage content of the secondary structure does not depend on the temperature. FTIR measurements, carried out at higher peptide concentration (about one order of magnitude) than these CD measurements mentioned above, revealed that in water solution the solid state β-sheet, and aggregated structures, dominate. However, in TFE the most abundant are α-helix and β-turns structures. The thioflavine T assay showed the tendency of the studied peptide for aggregate. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: alanine-rich peptide; conformational analysis; circular dichroism (CD); FTIR spectroscopy; differential scanning calorimetry

INTRODUCTION

Many papers have appeared concerning the influence of peptide length [1] and/or residue replacements [1–6] on the helical stability. The alanine-based peptides are interesting because of their ability to form stable monomeric helices in water, discovered by Baldwin and coworkers [7]. They can also be used as models in the studies of the helix-random coil transition [6,8,9] and in the role of the solvent in helix stability [10]. Although alanine-rich peptides have been studied extensively for years, both experimentally [11–13] and theoretically [14–16], there are still some divergences in interpretation of conformations of these peptides in solution. Baldwin *et al.* [7,17] and Kallenbach *et al.* [18] assigned the high α-helix content of alanine-based peptides to a large intrinsic propensity of alanine (Ala) residues to form helices in water. However, Scheraga *et al.* [4,19] showed, by means of conformational calculations, that Ala is not a strong helix-forming residue and the helix-coil equilibrium of a peptide containing only Ala residues can be shifted toward structures such as the α-helical conformation in two different ways: (i) by introducing charged or highly soluble polar residues into the sequence and (ii) by lowering the dielectric constant of the solvent. Thus the roles of electrostatic and hydrophobic interactions for the stability of α-helical structures are still poorly understood.

Moreover, recently the advanced spectroscopic [20,21] and theoretical [22] studies indicated that a substantial fraction of residues in unfolded polyalanine peptides exist in polyproline II (PPII) conformation, and that the helix-coil transition is an order-to-order transition, where the unfolded state is mostly PPII [23]. However, this finding was countered by Makowska *et al.* [24] who demonstrated, based on spectroscopic and theoretical studies that the PPII conformation is a local conformation of Ala residues in alanine-rich peptides, and not the global fold of the entire chain.

In this paper we report the results of our experimental conformational studies of Ac-K-$[A]_{11}$-KGGY-NH_2 in water, methanol (MeOH), trifluoroethanol (TFE) and MeOH/TFE mixtures by means of circular dichroism (CD) and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. Additionally, the conformational studies have been completed by the aggregation studies based on a thioflavine T (ThT) fluorescence assay.

MATERIALS AND METHODS

Chemicals

TentaGel R RAM resin was purchased from RAPP Polymere; Fmoc amino acids from Peptides International; 1-hydroxybenzotriazole (HOBt) and *N*-methylpyrrolidone (NMP)

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from Fluka; *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), piperidine, TFE from Lancaster; *N*,*N*-diisopropylcarbodiimide (DIPCI), *N*-acetylimidazole, triisopropylsilane, MeOH, Quinolin Yellow and phenol from Aldrich, ThT from Sigma; Triton 100-X from Merck; *N*,*N*-dimethylformamide (DMF) and acetonitrile from LabScan; diethyl ether and dichloromethane (DCM) from Polish Chemicals (POCh).

Peptide Synthesis and Purification

The peptide Ac-K-$[A]_{11}$-KGGY-NH_2 was synthesized as previously described [25]. The crude peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Kromasil C_8 column (20 × 250 mm, 5 μm) and a linear gradient of 7.5–20% acetonitrile/0.1% TFA with a flow rate of 15 ml/min. The purity of the peptide was confirmed by analytical HPLC and by MALDI-TOF analysis.

CD Spectroscopy

The spectra were measured on a Jasco J-20 spectropolarimeter with 1 mm quartz cuvettesin. The spectra were recorded from 193 to 260 nm, using a sensitivity of 5 mdeg/cm and a scan speed of 2 cm/min. CD measurements were made at 0.05 mM in MeOH (from 20 to 60 °C, with a step of 10 °C), in TFE (from 20 to 70 °C, with a step of 10 °C), in phosphate buffers (pH = 6, 7, 8 and 11.6, $T = 20$ °C) and in mixtures MeOH/TFE ($T = 20$ °C), respectively. In water, sample concentrations were 0.2, 0.1, 0.05, and 0.025 mM, respectively and all spectra were recorded at $T = 20$ °C. The CD spectra were plotted as a mean ellipticity Θ (degree × cm^2 × $dmol^{-1}$) *versus* wavelength λ (nm). The secondary structure content was calculated using the self-consistent method (SELCON 3 software [26,27]) and data were converted and transferred into input files as outlined in the DicroProt documentation.

Thioflavine T (ThT) Fluorescence Assay

Fluorescence measurements were performed using a Perkin-Elmer LS-50B spectrofluorimeter. The spectra were recorded at 0.2 and 2 mM peptide concentration with addition of ThT water solution (0.3 mg/ml). The excitation wavelength was set at 430 nm and the emission spectrum was recorded in the range from 430 to 650 nm (slit widths 15 nm). Fluorescence measurements were made in the time interval from 1 to 23 days.

FT - IR Spectroscopy

Infrared spectra were recorded for the peptide solution in H_2O, TFE, as well as for the peptide in the solid state, using a Bruker IFS-66 spectrometer in the range 400–4000 cm^{-1} with a resolution of 2 cm^{-1}.

A sample for measurements in the solid state was prepared by dissolving peptide in MeOH, placing the solution on the CaF_2 plate, and evaporating the solvent (64 scans were averaged).

ATR-FTIR were recorded for the peptide solution in H_2O and TFE. The internal reflection element was a zinc selenide ATR plate (ATR Gateway, Specac LTD). The ATR FT-IR experiment were measured at 4 mg/ml peptide concentration in water in the range of temperature from 20 to 70 °C, with a step of 10 °C, and in TFE at $T = 20$ °C. The spectra of both solvents were obtained under the same conditions. Data processing was performed using GRAMS/32 5.22 software (Galactic Enterprises). A nine-point Savitzky-Golay smoothing function was applied to calculate the second derivative. Deconvolution of the spectra was done in the spectral range 1500–1800 cm^{-1}, using γ-factor 2.5. The deconvoluted spectra were fitted with Gaussian band profiles. The positions, and number of the components, which were used as an input file for the curve-fitting function, were obtained from both the second derivative and the deconvoluted spectra. The quality of the fitting was estimated by standard deviation. The assignment of absorption bands of the secondary structure was made as described in literature [28–34].

Differential Scanning Calorimetry

The differential Scanning Calorimetry (DSC) experiments were carried out on a VP-DSC (MicroCal), at scanning rate of 0.5 °C/1 min and 0.2 mM peptide concentration in water and TFE, respectively. The cell volume was 0.5 ml. The obtained data were analyzed with the Origin 7.0 software package.

RESULTS AND DISCUSSION

CD Spectroscopy

The CD spectra of the studied peptide in water, MeOH and TFE with concentration of peptide 0.05 mM are presented in Figure 1, while the Figure 2 shows the CD spectra recorded in water, at four different values of concentrations. The percentage content of secondary structures of the peptide at different concentrations, and pH value in water, are presented in Table 1, whereas those in TFE and MeOH at different temperatures in Tables 2 and 3, respectively. Moreover, in Table 3 are also collected data for MeOH/TFE mixtures, where the peptide concentration is 0.05 mM.

The increase of the peptide concentration from 0.05 to 0.2 mM, at pH 6 and 7 in water at room

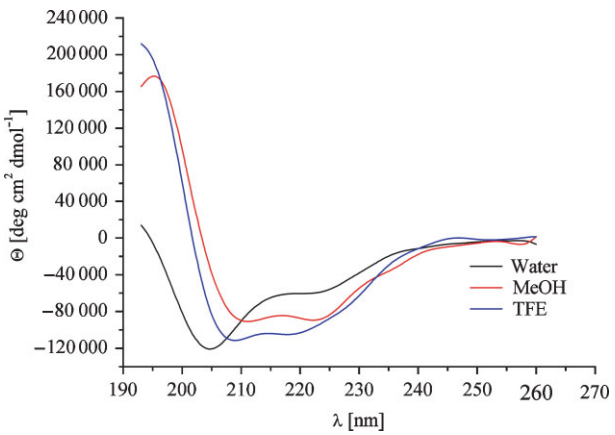

Figure 1 The CD spectra of the peptide ($c = 0.05$ mM) in water, MeOH and TFE at 20 °C.

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Table 1 The percentage content of secondary structures of the peptide at different concentrations and different pH in water at 20 °C revealed from CD measurements

Structure	pH = 6			pH = 7				pH = 8	pH = 11.6
	0.2 mM	0.1 mM	0.05 mM	0.2 mM	0.1 mM	0.05 mM	0.025 mM	0.1 mM	0.05 mM
α-helix	6	16	25	5	23	38	9	16	10
β-sheet	30	15	17	27	27	14	23	30	23
β-turns	12	10	14	14	13	11	8	10	10
PPII	7	7	5	6	11	4	6	8	7
random coil	45	52	39	48	26	33	54	36	50

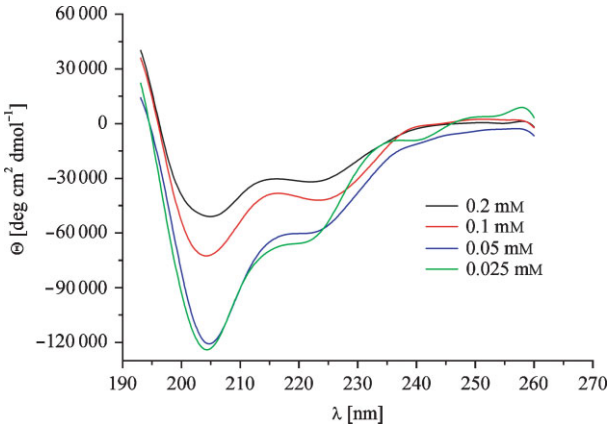

Figure 2 The CD spectra of the peptide measured at different concentration of peptide (0.2, 0.1, 0.05 and 0.025 mM) in water at 20 °C.

Table 2 The percentage content of secondary structures of the peptide ($c = 0.05$ mM) in TFE and MeOH revealed from CD measurements

Structure	20 °C	30 °C	40 °C	50 °C	60 °C	70 °C
			TFE			
α-helix	34	32	31	32	31	29
β-sheet	13	14	13	10	11	15
β -turns	13	13	14	14	14	14
PPII	7	7	6	7	7	6
random coil	33	34	36	37	37	36
			MeOH			
α-helix	32	31	25	28	25	—[a]
β-sheet	20	25	34	29	32	—
β -turns	11	9	7	9	8	—
PPII	7	7	4	5	5	—
random coil	30	28	30	29	30	—

[a] Boiling point of methanol equals to 64.5 °C.

temperature caused drop the α -helix content from ~40% to about 5–6%. The α-helix content depends on the pH and increases with it, however, for high pH (about 12) the α-helix content decreased to about 10%. For 0.1 mM peptide concentration, the α-helix content does not depend on pH in narrow pH range (from 6 to 8). The β-sheet content increases with the peptide concentration for all studied pH while the β-turns, as well as PPII structure, are almost independent of the peptide concentration and pH. The unordered structure does not show the regular dependence on peptide concentration and pH (Table 1).

Table 3 The percentage content of secondary structures of the peptide ($c = 0.05$ mM) in MeOH, TFE and MeOH/TFE mixtures at 20 °C revealed from CD measurements

Structure	MeOH	MeOH/TFE 8:2	MeOH/TFE 6:4	MeOH/TFE 4:6	MeOH/TFE 2:8	TFE
α-helix	32	10	19	19	21	34
β-sheet	20	18	22	25	23	13
β-turns	11	17	14	14	14	13
PPII	7	7	6	6	6	7
random coil	30	48	39	36	36	33

It was observed, that MeOH and TFE generate partially folded states of proteins by helix induction [30–32]. TFE does not induce secondary structure indiscriminately, rather, the region in the polypeptide or protein chain that is helical in the native state, or has a strong sequence propensity to adopt helical conformation, is stabilized preferentially [33]. Also, the studied peptide in TFE, at room temperature adopted predominantly the ordered structures: α-helix (about 30%) and β-turns (13%) with a small contribution of the PPII (7%) (Table 2). However, for higher temperatures, the most populated is an unordered structure. The content of secondary structure in MeOH at room temperature is similar to the TFE solution, except for the β-sheet, which is nearly twice than in TFE. Moreover, the different thermal stabilities of the secondary structure in each solution are observed. The increase of temperature from 20 to 60 °C for MeOH caused a growth of β-sheet structure from 20% to more than 30%, and a simultaneous drop of the remaining

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ordered structures, except for the unordered one (Table 2). In TFE the content of the secondary structure does not depend on temperature. The analysis of the CD spectra of the peptide in the MeOH/TFE mixtures revealed, that the TFE in MeOH caused substantial decrease of the α-helix content, whereas the reverse is observed for β-sheet and unordered structure. The content of the PPII and β-turn remained constant (Table 3). Taking into account that the contributions of the particular secondary structure contents were a little scattered, the influence of the TFE in the MeOH/TFE on the content of a particular secondary structure should be treated as preliminary data. The lack of a well defined peak on the DSC run of the studied peptide, at 0.2 mM concentration in buffered water (data not shown), confirms the data obtained from CD spectroscopy on the relatively small conformational changes due to temperature.

FTIR Spectroscopy

Structural information in the FTIR method is predominantly derived from analysis of the so-called amide band, particularly the amide I band (1600–1700 cm^{-1}). This band comprises mainly of the peptide backbone C=O stretching vibrations, and usually has a broad contour that is composed of several overlapping bands due to various protein segments with different secondary structures [28,29]. Deconvolution and differentiation of the amide band makes it possible to distinguish between the individual component types [30–33].

The FTIR spectra of the amide I band, of the peptide studied, measured in water and TFE solution are presented in Figures 3, 4 and 5, respectively. Additionally, the FTIR spectrum of the peptide in the solid state was also measured (Figure 6). As can be seen from Figure 3, the FTIR spectrum of amide band I of the peptide studied in water, consists of a dominant peak

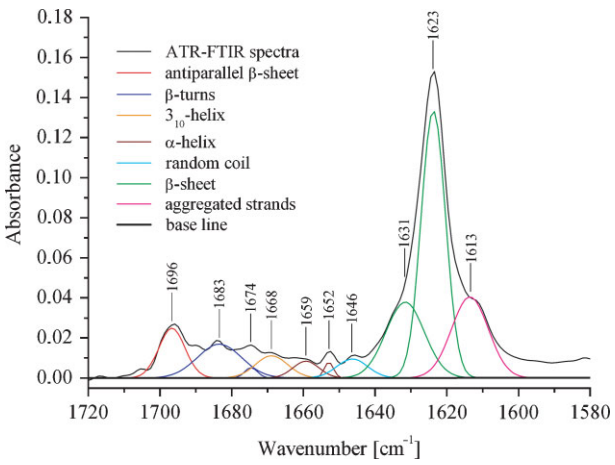

Figure 3 The ATR-FTIR spectra of the peptide ($c = 4$ mg/ml) in H_2O at 20 °C.

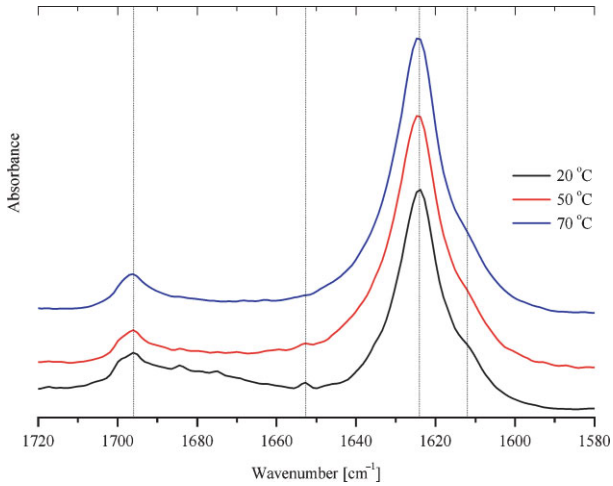

Figure 4 The ATR-FTIR spectra of the peptide ($c = 4$ mg/ml) in H_2O at 20, 50 and 70 °C.

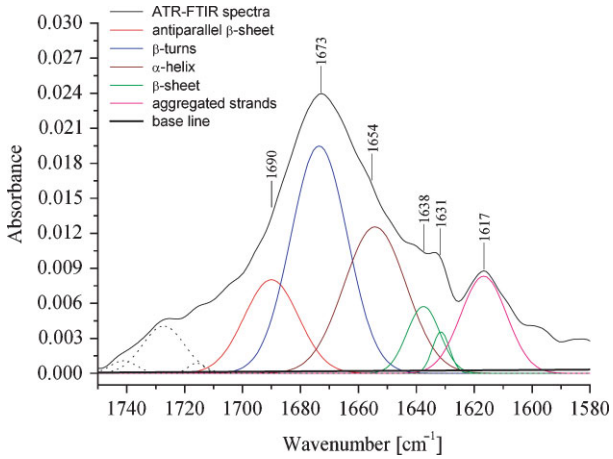

Figure 5 The ATR-FTIR spectra of the peptide ($c = 4$ mg/ml) in TFE at 20 °C (bands marked as dotted lines were not taken into account in calculations of the percentage content of secondary structures).

located at 1620 cm^{-1} and small peaks in the range of 1700–1600 cm^{-1}. The percentages at different types of secondary structure, obtained by analysis at the amide band, offer deconvolution as summarized in Table 3. The most populated secondary structures are β-sheet (parallel and antiparallel 62%) and aggregated strands (17%) with a small contribution of remaining structures (unordered 3%, β-turn 9% and α-helix 9%). The shape and position of the amide spectrum does not change much with temperature increases and the dominant peaks are those attributed to β-sheet and aggregated strands (Figure 2). A very similar FTIR spectrum was recorded by Khurana and Fink for KLEG peptide; the strong band, at 1623 cm^{-1} in water, was assigned to the intramolecular β-sheet with a very strong hydrogen bond. A minor peak, at position 1692 cm^{-1}, was also associated with β-structure [25]. Also in our case, the band shape in the range of 1640–1700 cm^{-1} did

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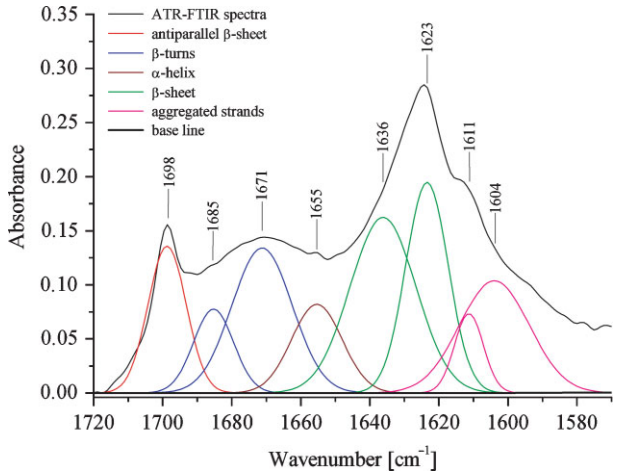

Figure 6 The ATR-FTIR spectra of the peptide in the solid state.

not change significantly with temperature (Figure 2). It could be connected with additional conformational change of β-structures (turn/sheet).

ATR FTIR spectroscopy of alanine-rich peptides was also applied to the TFE solution (Figure 5); characteristic frequencies of the amide I band, together with the percentage content of the secondary structure, are presented in Table 4. As could be expected for such a helix-inductive solvent [25,35,36], apart from the secondary structure revealed in water solution a significant increase of α-helix (27%) and β-turns were revealed; simultaneously, there was a significant content of β–sheet. Thus, at higher peptide concentration the TFE strongly forces the helical structures than at lower peptide concentrations, which come from CD spectra.

Lyophilization induced reversible changes in the secondary structure of proteins [37]. However, according to [38,39] the content of the secondary structure, the peptide or protein in the solid state is similar to that determined in solution from which the solid state was obtained. Comparing the FTIR spectra recorded in water, in TFE and in the solid state, one can notice that the solid peptide spectrum is more similar to that recorded in TFE than in water. Also, the data presented in Table 4, confirm this statement, though in the solid state the lower content of α-helix (8%) and β-turn (23%), and much higher content of β-sheet (39%) were observed. Contrary to water, in TFE solution the dominant are β-turn (37%) and an α-helical (27%) structures (Table 4). Such differences in the secondary structure content, between TFE solution and solid state obtained after evaporation of MeOH, could be rationalized by taking into account that increase of the peptide concentration during MeOH evaporation favors aggregation.

Thioflavine T Fluorescence Spectroscopy

ThT is widely used in the study of aggregation process of peptide or protein because of an increase of its fluorescence intensity upon binding to more hydrophobic products [40,41]. In the Figure 7, the increase of ThT fluorescence intensity (ΔI) in time for studied peptide is presented. For both peptide concentrations, a growth of fluorescence intensity is observed up to about 2 weeks, and then it gradually decreases, which is probably connected with some degradation process of the peptide or ThT. The more rapid increase of ThT fluorescence intensity for more concentrated peptides indicates that the alanine-rich

Table 4 Characteristic frequencies in the amide I region of ATR-FTIR spectra of Ac-K-$[A]_{11}$-KGGY-NH_2 in H_2O (20 °C), TFE (20 °C) and in the solid state

Assignment	H_2O		TFE		Solid state	
	Frequency (cm^{-1})	Relative intensity [a] (%)	Frequency (cm^{-1})	Relative intensity [a] (%)	Frequency (cm^{-1})	Relative intensity [a] (%)
Antiparallel β-sheet	1696	8	1690	15	1698	11
β-turns	1683	9	1673	37	1685	23
	1674				1671	
3_{10}-helix	1668	5	—	—	—	—
α-helix	1659	4	1654	27	1655	8
	1652					
Random coil	1646	3	—	—	—	—
β-sheet	1631	54	1638	8	1636	39
	1623		1631		1623	
Aggregated strands	1613	17	1617	13	1611	19
					1604	

[a] Relative intensity means the percentage of a component band in the total integrated area of the amide I region.

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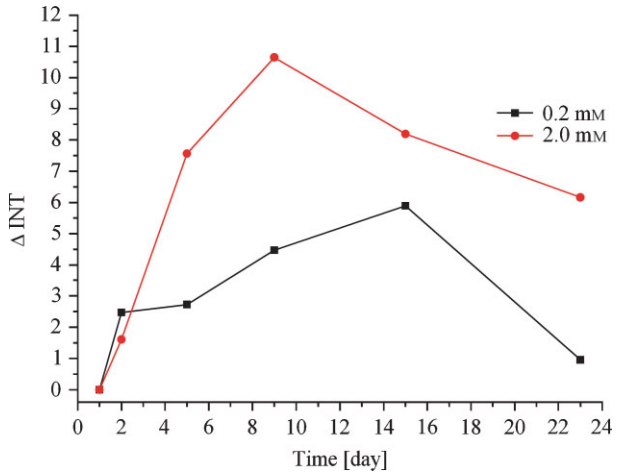

Figure 7 The increase of fluorescence intensity of thioflavine T in time for two concentrations of peptide (ΔINT, difference in the fluorescence intensity).

peptide studied possesses propensity to aggregation, which is consistant with FTIR data.

CONCLUSIONS

The Ac-K-$[A]_{11}$-KGGY-NH_2 peptide exhibits a small tendency to adopt an α-helical structure in water solution at low concentration of peptide, which is consistent with calculated structures using AMBER force-field [27]. For higher concentration of peptide in water, propensity to aggregation caused the predominant structure to be β-sheet while in TFE, at higher peptide concentration, the dominant structures are α-helix and β-turns.

The FTIR- is complementary to the CD-spectroscopy method according to the conformational studies of peptides and proteins. Therefore sometimes they may be used simultaneously [42]. However, in the FTIR spectroscopy in water solution to have a good signal-to-noise ratio, the concentrations of the peptide is usually from 4 to 30 mg/ml and it should be marked, in current studies for CD-spectroscopy the concentration of peptide is relatively low (~0.2 mM). Thus, for peptides or proteins with propensity for aggregation, the results obtained using those two methods are not always compatible.

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