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Characterization of triple-helical conformations and melting analyses of synthetic collagen-like peptides by reversed-phase HPLC

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Abstract

There is a confusion in the application of circular dichroism (CD) spectroscopy in analyzing collagen's structure for the overlapping of the spectral shapes and positions of the collagen triple helix and poly(proline-II)-like structure. The unique repetitive sequence of the collagen triple helix is susceptible to misalignment during the spontaneous assembly. Such misaligned structures are usually difficult to be characterized by CD or NMR spectroscopy. Here, RP-HPLC was developed as a conformational characterization technique for synthetic collagen-like peptides based on the different hydrophobicities exhibited by the triple-helical and unassembled peptides. RP-HPLC was also used to study thermal transitions and to measure melting point temperatures (T_m) of the collagen-like peptides.

Keywords: Collagen; Triple helix; RP-HPLC; Melting analysis

1. Introduction

Collagen can be distinguished from other extracellular matrix proteins by its unique tertiary structure, which is a triple helix. It consists of three extended left-handed poly(proline-II)-like chains, which are arranged in a parallel pattern with a one-residue register shift, intertwined to assemble into a right-handed triple helix. This assembly is a direct consequence of collagen primary structure characterized by the repetition of X-Y-Gly triplets, where position X is frequently occupied by a proline residue (Pro) and position Y is usually a hydroxyproline (Hyp). The native triple-helical conformation can accommodate only *trans* peptide bonds. In a transition from the unfolded state to the triple helix all *cis* peptide bonds have to be converted to *trans* via *cis–trans* isomerization of numerous prolines/hydroxyprolines [1,2]. The triple helix is terminated by disulfide covalent bonds, which interlink the three polypeptides chains. The bridging by disulfide knots is essential for proper chain registration and triple helix nucleation.

Mimicry of collagen structurally can help to elucidate its unique triple-helical conformation and provide an insight into the engineering of novel collagen-like biomaterials. Synthetic collagen-like peptides are frequently used as model peptides in the folding and conformational studies of natural collagen [3–9]. Many monodisperse polypeptides composed of tandem repeats of Gly-X-Y, such as Gly-Pro-Pro and Gly-Pro-Hyp triplets [9–12], have been synthesized to mimic collagen-like triple-helical structure. Various characterization techniques have been reported and widely used for the structural studies of the synthetic collagen model peptides. Among all, circular dichroism (CD) spectroscopy is the most broadly used method [3,13]. However, there has been confusion in the application of CD spectroscopy. The most vexing problem arises from the fact that the triple-helical structure and the individual poly(proline-II)-like chain possess similar CD spectral shapes and band positions for some synthetic polypeptide collagen models [14]. The presence of a triple-helical conformation in solution can also be established by nuclear magnetic resonance (NMR) spectroscopy [15]. The unique repetitive sequence of the collagen triple helix is susceptible to misalignment during the spontaneous assembly of the triple-helical conformations [16,17]. Such misaligned structures are usually difficult to be characterized by CD or NMR spectroscopy.

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RP-HPLC is a common analytical and preparative tool in the downstream process of peptide synthesis [14,18]. We aim to develop it as an alternative conformational characterization technique as well for synthetic collagen-like peptides based on the different hydrophobicities exhibited by the triple-helical and unassembled structures of the same collagen analog. Characterization of assembled, differently aligned and unassembled molecules by RP-HPLC is possible for its superior separation capability. This method gives a better separation between the triple-helical conformation and poly(proline-II)-like structure of the same collagen-like peptides as compared to the spectroscopic technique. We believe that this application can be subsequently extended to characterize natural collagen after purification and further optimization. In this study, (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ were used as the collagen model peptides while (Pro-Hyp-Gly)$_5$ and a control peptide (CP) were used as the control samples in our study. The biophysical characteristic of these peptides were assessed by CD and NMR spectroscopy, UV absorbance measurements, and RP-HPLC. The triple helicity of these peptides was studied as a function of temperature. This study may aid in advancing the structural and thermodynamic studies of collagen-like peptides and similar chemistry compounds and facilitating the breakthrough in the characterization techniques of the triple-helical structures.

2. Materials and methods

2.1. Materials

All peptide synthesis chemicals and solvents were of analytic reagent grade or better. All amino acids and resin were of L-configuration and purchased from Novabiochem (San Diego, CA, USA). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were purchased from Advanced Chemtech (Kentucky, USA). Chemical solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany).

2.2. Peptide synthesis

(Pro-Hyp-Gly)$_5$, (Pro-Pro-Gly)$_{10}$, (Pro-Hyp-Gly)$_{10}$ and a control peptide (CP), an unordered peptide of non-regular peptide sequence PPHypPGPGHypHypGPHypGPHyp-GHypPGHypGHypPGPHypGHypG, where G: Gly, P: Pro, Hyp: hydroxyproline, with a ratio of Pro:Hyp:Gly of 9:10:10, were synthesized in-house by solid phase peptide synthesis on an automated MultiPep peptide synthesizer (Intavis AG Bioanalytical Instruments, Köin, Germany). All peptide chains were assembled on Fmoc-Gly-Wang resin (substitution level = 0.66 mmol/g resin) at a synthesis scale of 50 μmol. Stepwise couplings of amino acids were accomplished using double coupling method with five-fold excess of amino acids, equivalent activator reagents, HBTU and HOBt, and two equivalents of base, *N*-methylmorpholine (NMM). The removal of Fmoc protecting group of N_α amino was completed by using 20% piperidine in dimethylformamide (DMF). Fmoc residues and deprotection chemicals were then washed away with DMF. Cycles of deprotection, washing, double couplings, and washing were repeated until the desired chain length was achieved. The dried peptidyl-resin was cleaved by a cocktail solution composed of 95% trifluoroacetic acid (TFA), 2.5% DI water, and 2.5% (v/v) triethylsilane (TES). The reaction was allowed to proceed for 3 h with occasional shaking. The peptide was collected by precipitation in cold ether by centrifugation and was washed at least three times with excess of cold ether to remove any residual scavengers. The final precipitate was re-dissolved and lyophilized. The crude peptide was purified by utilizing an Agilent 1100 semi-preparative HPLC (Agilent Technologies, Santa Clara, CA, USA). The purification was performed on a Agilent Zorbax 300SB-C18 reversed phase semi-preparative column (5 μm particle size, 300 Å pore size, 25 cm × 1.0 cm) with a linear gradient of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitrile) from 10% B to 45% B in 30 min for (Pro-Hyp-Gly)$_5$, (Pro-Hyp-Gly)$_{10}$, and the control peptide and 15–30% B in 40 min for (Pro-Pro-Gly)$_{10}$ at a total flowrate of 4 ml/min. Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) was done on a Bruker AutoFlex II MALDI-TOF MS (Bruker, Bremen, Germany) and was used in conjunction with an Agilent 1100 analytical HPLC to analyze the peptides. All peptide samples were of purity greater than 90% as determined by RP-HPLC. Purification of (Pro-Hyp-Gly)$_{10}$ was repeated using the same conditions and methods to obtain a peptide of purity greater than 99%.

2.3. MALDI-TOF mass spectroscopy

Samples were dissolved in ultrapure water at about 1 μg/ml. The matrix, α-cyano-4-hydroxy-cinnamic acid (CHCA) (Bruker), was dissolved in acetonitrile/water (50:50, v/v) containing 0.1% TFA to saturation. Equal volume (0.5 μl) of sample and matrix was mixed thoroughly, spotted on the MTP384 matt steel target plate (Bruker), and air-dried. Data was acquired using reflector detector operating at voltage 1614 V, low laser intensity, and 50 shots per acquisition (frequency 25 Hz). Matrix suppression was turned on to suppress the matrix signal below 800 *m/z*.

2.4. CD spectroscopy

CD measurements were performed on Jasco Model J-810 spectropolarimeter (Jasco, Great Dunmow, Essex, UK) using a quartz cylindrical cuvette (Hellma, Müllheim, Germany) with a path length of 0.1 mm. The cuvette was filled with 150 μl of samples for each measurement. The CD spectra were obtained by continuous wavelength scans (average of three scans) from 260 to 180 nm at a scan speed of 50 nm/min. All samples were dissolved in water, unless otherwise stated, and stored at 4 °C for at least 3 days prior to the test to allow for proper equilibration of triple-helical conformation. The samples were equilibrated for at least 3 h

at the desired temperatures before the CD spectrum was acquired.

2.5. NMR spectroscopy

NMR spectroscopy was done on a Bruker Avance DRX500 500 MHz spectrometer (Bruker). NMR samples were prepared in H_2O/D_2O (2:3, v/v) with a peptide concentration of about 0.10 mg/ml and stored at 4 °C for at least 3 days and equilibrated for another 1 h at specified temperature before data acquisition. 1D NMR spectra were recorded with a spectral width of 8012.820 Hz at 15 °C.

2.6. UV absorbance measurements

UV absorbance measurements [13,19] were performed on a Cary 50Bio UV Spectrophotometer (Varian, Palo Alto, CA, USA) equipped with a peltier temperature controller (Quantum Northwest, Spokane, WA, USA). Prior to any measurements, all samples were equilibrated at the initial temperature for at least 24 h. The samples were allowed to equilibrate for at least 15 min until the UV absorbance was time-independent at each subsequent temperature point. Data were collected at 225 nm.

2.7. RP-HPLC experiments

Analytical RP-HPLC was performed on an Agilent 1100 HPLC equipped with auto-sampler (with temperature controller) and column temperature controller using an Agilent Zorbax 300SB-C18 RP analytical column (5 μm particle size, 300 Å pore size, 25 cm × 0.46 cm) with the same gradient flow as that for the semi-preparative RP-HPLC at a flowrate of 1 ml/min, unless otherwise stated. The injection volume for each analysis was 20 μl. The delay time is 2.5 min. The diode array detection was set at 225 nm. The RP-HPLC system was allowed to equilibrate at each temperature point for at least 15 min prior to each measurement. All samples were prepared at 0.25 mg/ml in water, unless otherwise stated, and stored at 4 °C for at least 3 days prior to the test to allow for proper equilibration of triple-helical conformation. The samples were equilibrated for at least 24 h at initial temperature before the RP-HPLC analysis was performed. The samples were allowed to equilibrate for at least 15 min for each subsequent temperature point. The identity of peaks of RP-HPLC profiles was confirmed by MALDI-TOF MS. Peak fractions was collected using a time-slice mode, freeze-dried overnight, re-dissolved in water and examined by MALDI-TOF MS and analytical RP-HPLC. RP-HPLC sample preparation was similar as mentioned above and re-injected to RP-HPLC for analysis. A blank run was performed prior to any RP-HPLC analysis to confirm that the peaks observed on the chromatograms are from the samples but not the solvent system. The effect of the column pressure and shear forces on the peak shape was checked by running the same sample at same conditions except by changing the total flowrate. Result showed that all peaks observed are arisen from the samples and the effect of the total flowrate on the peak shape is negligible.

3. Results and discussion

3.1. Determination of triple-helical conformation in solution using CD and NMR spectroscopy

The collagen-like triple-helical conformation exhibited by the samples was verified using CD spectroscopy. The CD spectra of the collagen-like peptides were compared to that of native calf-skin collagen (Fig. 1d) to demonstrate the presence of the collagen triple helix. It is readily seen from Fig. 1a and b that both $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ exhibited CD spectra features characteristic of collagen-like triple helix, including a positive peak around 225 nm and a large negative trough near 200 nm. The establishment of triple-helical conformations by CD spectral band positions was also supported by comparing the CD spectra of the synthetic collagen-like peptides at low and increased temperatures. At elevated temperatures (60 °C), the positive peak signal at 220 nm, which is definitive for the presence of a triple-helical conformation, completely disappears indicating a thermal transition from the folded to unfolded state during the denaturation process.

In contrast, the CD spectra of the control peptide (CP) as displayed in Fig. 1c exhibited a strong broad negative band at 205 nm and a positive peak at 226 nm characteristic of a poly(proline-II)-like (PPII) helical conformation. PPII structure is stable under the experimental conditions and no transition was observed for the PPII conformation at elevated temperature by CD spectroscopy.

The presence of the triple-helical conformation in the peptide solution can also be established by NMR spectroscopy. The assembly of a triple-helical structure results in the appearance of a new set of NMR resonances which cannot be observed for the unassembled structures [20–22]. Among the resonances of the assembled triple-helical set, the signal of Pro $C_\delta H$ at 3.1 ppm is well resolved and not overlapped by any resonance of the unfolded structure sets. The resonance at 3.1 ppm can therefore be used unambiguously to identify the triple-helical structure [20]. It can be seen from Fig. 2 that $(Pro\text{-}Hyp\text{-}Gly)_{10}$ displayed a strong peak signal at near 3.1 ppm. This is consistent with the previous CD and UV melting curve analyses that the collagen analog adopt stable triple-helical conformation in solution. $(Pro\text{-}Hyp\text{-}Gly)_5$ which is known to be non-triple-helical was used as a counterpart of $(Pro\text{-}Hyp\text{-}Gly)_{10}$. The result showed that the set of triple-helical resonance at near 3.1 ppm is absent for $(Pro\text{-}Hyp\text{-}Gly)_5$.

3.2. Melting studies by using UV spectroscopy

Triple-helical conformations can be distinguished from the poly(proline-II)-like and nonsupercoiled structures based on the thermal melting characteristic [7]. The thermal transition curves are shown in Fig. 1e–h. Calf-skin collagen displayed a cooperative melting curve with a transition point at 38 °C, close to the reported value (39 °C) [23]. It can be seen that both $(Pro\text{-}Hyp\text{-}Gly)_{10}$, and $(Pro\text{-}Pro\text{-}Gly)_{10}$ dissolved in H_2O at varying concentrations also exhibited cooperative transition curves with a large transition magnitude, denoting the presence of a sig-

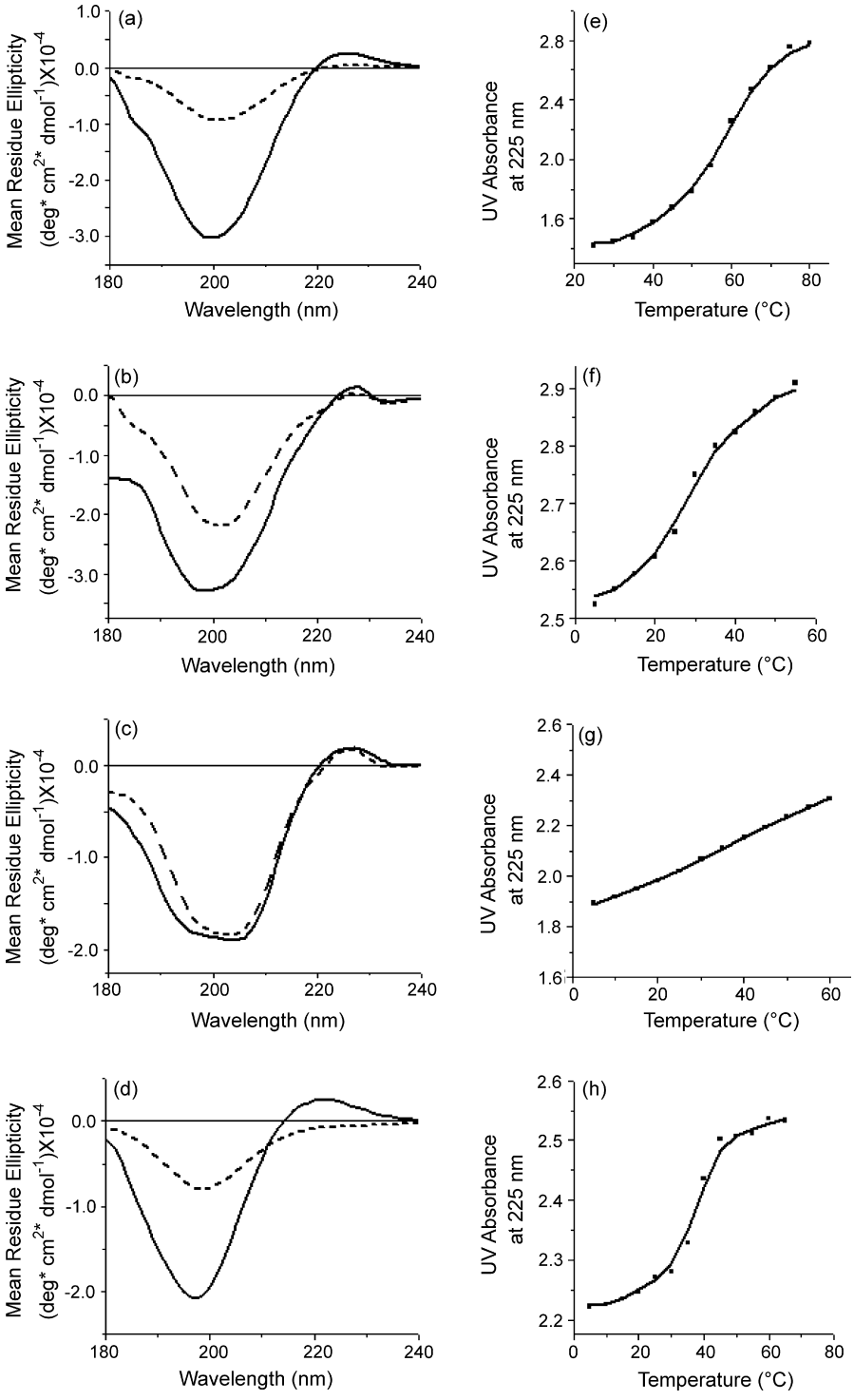

Fig. 1. CD spectra were obtained at 25 °C (solid line) and 60 °C (segmented line) for (a) $(Pro\text{-}Hyp\text{-}Gly)_{10}$ (0.20 mg/ml), (b) $(Pro\text{-}Pro\text{-}Gly)_{10}$ (0.15 mg/ml), (c) control peptide (CP) (0.40 mg/ml), and (d) calf-skin collagen (0.50 mg/ml) in H_2O. The positive peak of the CD spectra for all samples, except the CP, vanished at high denaturation temperature (60 °C) indicating a conformation transition. Thermal melting curves of (e) $(Pro\text{-}Hyp\text{-}Gly)_{10}$ (0.20 mg/ml), (f) $(Pro\text{-}Pro\text{-}Gly)_{10}$ (0.15 mg/ml), (g) control peptide (0.40 mg/ml), and (h) calf-skin collagen (0.50 mg/ml) in H_2O were established by UV absorbance measurements at 225 nm. Control peptide (CP) is an unordered peptide of non-regular peptide sequence PPHypPGPGHypHypGPHypGPHypGHypPGHypGHypPGPHypGHypG, where G: Gly, P: Pro, Hyp: hydroxyproline.

nificant percentage of triple-helical conformations in solution [14]. No transition was observed for CP in H_2O. An R value of 0.993 was obtained by a linear fitting, indicative of a smooth linear line. The results of the thermal denaturation experiments are consistent with those obtained from CD and NMR spectroscopy. Cooperative thermal transition curves were observed for the collagen-like peptides which exhibited similar CD spectra to that of the native collagen. Melting point temperature (T_m)

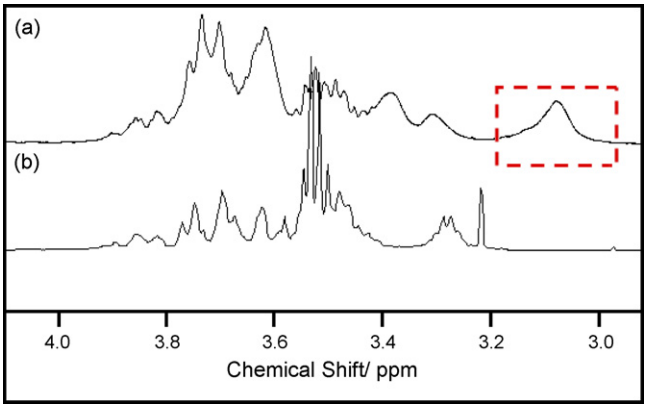

Fig. 2. 1D ^{1}H NMR spectra of (a) (Pro-Hyp-Gly)$_{10}$ and (b) (Pro-Hyp-Gly)$_5$. The boxed spectral regions contain a peak signal representative of the assembled Pro C_δH signal at 3.1–3.0 ppm. All spectra were acquired at 15 °C.

was obtained from the mid point of the transition and the results were presented in Table 1. The melting point temperatures of (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ in water were found to be 57 and 28 °C, respectively, close to the literature data (57.3 °C [12] and 28 °C [24], respectively).

3.3. Characterization of triple-helical conformations using RP-HPLC

The separation process on RP-HPLC is based on the different molecular weights, structures, and hydrophobicities possessed by various different molecules. The packing of RP-HPLC is hydrophobic and therefore it has a higher affinity for hydrophobic compounds. The hydrophobicity of synthetic collagen-like (X-Y-Gly)$_n$ peptide is determined by X and Y residues, in which the side groups of X and Y residues are projected outward of the triple helix core, with the second residue being more exposed to the solvent [25].

The RP-HPLC profiles of (Pro-Hyp-Gly)$_{10}$ at different temperatures are given in Fig. 3a–c. The multiple peaks representative of triple-helical conformations were denoted as THP 1, THP 2, THP 3, THP 4, and THP 5 while the single strand

Table 1
Melting points temperatures (T_m) obtained from UV absorbance$_{225\,nm}$ measurements and RP-HPLC melting studies

Peptides	T_m (°C) obtained from RP-HPLC	T_m (°C) obtained from UV spectroscopy
(Pro-Hyp-Gly)$_{10}$	42.0	57.0[a], 40.0[b]
THP 1 of (Pro-Hyp-Gly)$_{10}$	40.0	40.0[b]
THP 4 of (Pro-Hyp-Gly)$_{10}$	40.0	40.0[b]
(Pro-Pro-Gly)$_{10}$	27.0	28.0[a], 29.0[b]
Control peptide (CP)	No transition	No transition

Each sample was prepared in water or 20% acetonitrile and was allowed to equilibrate for at least 3 days at 4 °C. UV absorbance at 225 nm was measured as a function of temperature for each sample. Each sample was allowed to equilibrate for at least 15 min at each temperature point prior to the measurement.
[a] Sample was equilibrated in water.
[b] Sample was equilibrated in 20% acetonitrile.

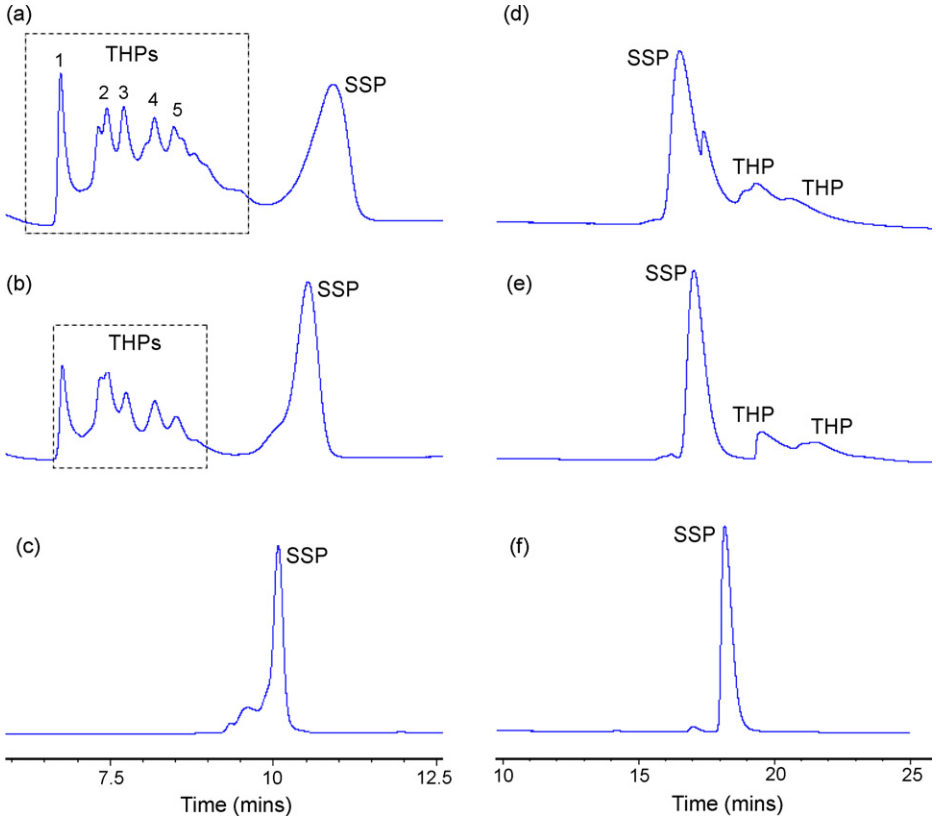

Fig. 3. RP-HPLC profiles of (Pro-Hyp-Gly)$_{10}$ at (a) 15 °C, (b) 30 °C, and (c) 60 °C were obtained by gradient flow from 10% B to 45% B in 30 min at 1 ml/min. RP-HPLC profiles of (Pro-Pro-Gly)$_{10}$ at (d) 10 °C, (e) 20 °C, and (f) 40 °C were obtained by gradient flow from 15% B to 30% B in 40 min at 1 ml/min. Each sample was allowed to equilibrate for at least 3 days in water at 4 °C prior to RP-HPLC analysis.

peptide peak was denoted as SSP. The shorter retention time for THPs than for the SSP is consistent with the properties of the triple helix structure and the packing of the collagen analogs. The Hyp residue at Y position has a polar side group and thus the triple-helical $(Pro\text{-}Hyp\text{-}Gly)_{10}$ is more hydrophilic than the unassembled structures. Therefore, THPs were eluted before the SSP.

The triple-helical characteristic of the THP peaks of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was further verified by the thermal denaturation studies using RP-HPLC. From Fig. 3a–c, it is clearly seen that THPs of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ disappeared completely at elevated temperature while the intensity of the SSP peak increased significantly (intensity scale not shown), demonstrating the transition of the triple helix ↔ single strand peptide transition. The small shoulder seen in Fig. 3c is due to the small amount of the deleted peptides as detected by MALDI-TOF MS (see Fig. 4). When the peptides were further purified and analyzed with RP-HPLC, the RP-HPLC profile as shown in Fig. 3 is reproducible (see Fig. 7) and there is no peak shoulder observed for this further purified sample when the temperature is increased to 60 °C (result not shown). Contents of THP 1, THP 4, and SSP 1 were fractionally collected and examined through independent experiments. Temperature titration of THP 1 and THP 4 by RP-HPLC gave a highly cooperative thermal melting curve as displayed in Fig. 10b and c indicating a conformation transition. MALDI-TOF MS result as displayed in Fig. 4 revealed that THP 1, THP 4, and SSP have a same constituent of molecular weight of 2692 $[M+H]^+$, which is consistent with the mass of $(Pro\text{-}Hyp\text{-}Gly)_{10}$. THPs and SSP of same origin are separated on C-18 chromatography column mainly due to the difference in their hydrophobicities. The difference of the hydrophobicity arises from the different conformations possessed by the peptides. As discussed above, the triple-helical conformation of $[(Pro\text{-}Hyp\text{-}Gly)_{10}]_3$ is more hydrophilic than the unassembled one and thus eluted before the SSP on the C-18 hydrophobic column. The SSP, which is eluted much later than and isolated from the THPs, is the single strand of $(Pro\text{-}Hyp\text{-}Gly)_{10}$. Each collected fraction was then analyzed through RP-HPLC. It is clearly seen from Fig. 5 that the chromatogram of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was reproducible in the independent RP-HPLC analyses of THP 1, THP 4, and SSP. This is a counteraction of an equilibrium system against perturbation. The triple-helical conformations are always in equilibrium and co-existing with the single strand monomers. Isolation of either one by RP-HPLC will perturb the two-state equilibrium system and subsequently cause the refolding or unfolding of the collagen-like peptides to achieve new equilibrium. Therefore, the RP-HPLC profile of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ as given in Fig. 3 was reproducible as shown in Fig. 5. The freshly prepared $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was examined by CD spectroscopy, melting curve analysis, and RP-HPLC analysis to study the changes that occur during the equilibration process. The freshly prepared $(Pro\text{-}Hyp\text{-}Gly)_{10}$ exhibited collagen-like CD spectrum similar to the equilibrated sample, except with a lower positive peak intensity (Fig. 6a). The freshly prepared sample exhibited a cooperative melting curve with a similar melting point temperature (57 °C) as that of the equilibrated $(Pro\text{-}Hyp\text{-}Gly)_{10}$ but with a much smaller transition magnitude (Fig. 6b), indicative of the low per-

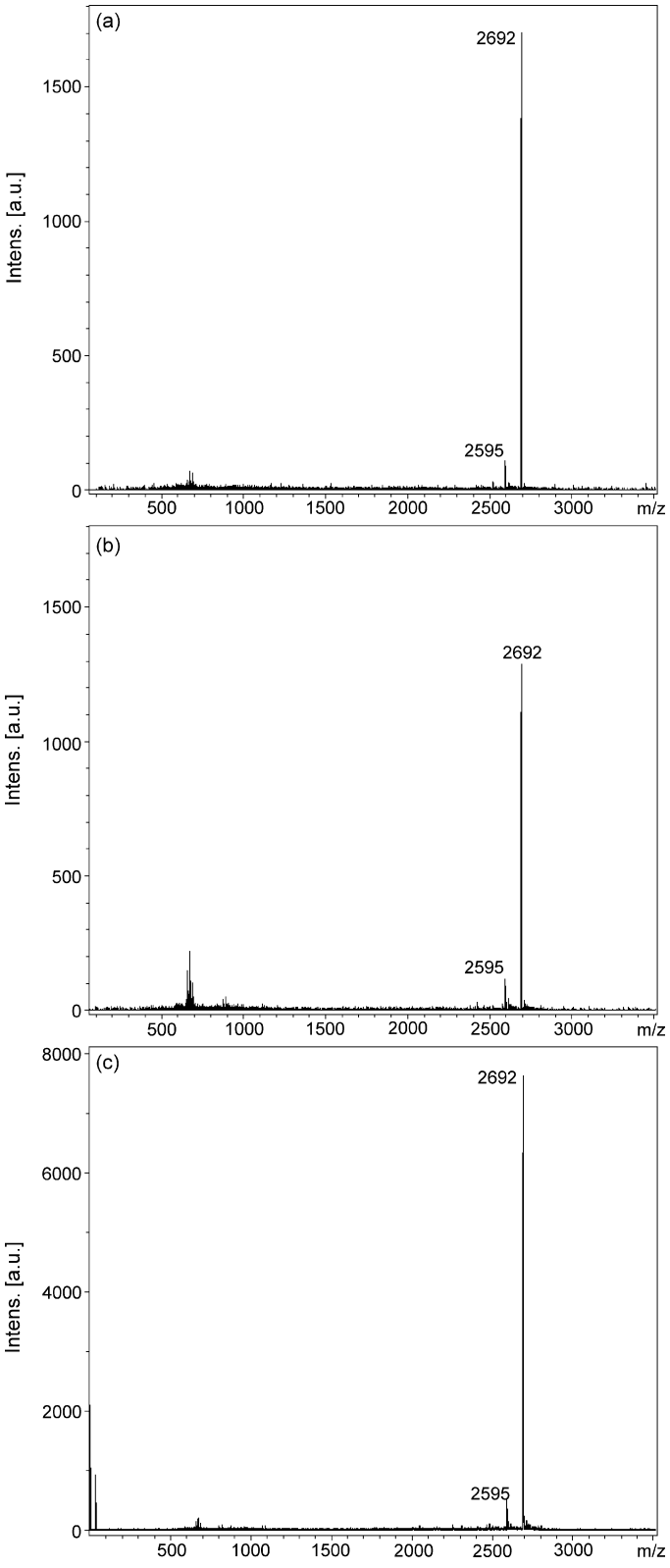

Fig. 4. MALDI-TOF MS spectra of (a) THP 1, (b) THP 4, and (c) SSP of $(Pro\text{-}Hyp\text{-}Gly)_{10}$. All samples have a same constituent of molecular weight of 2691, which is the mass of $(Pro\text{-}Hyp\text{-}Gly)_{10}$.

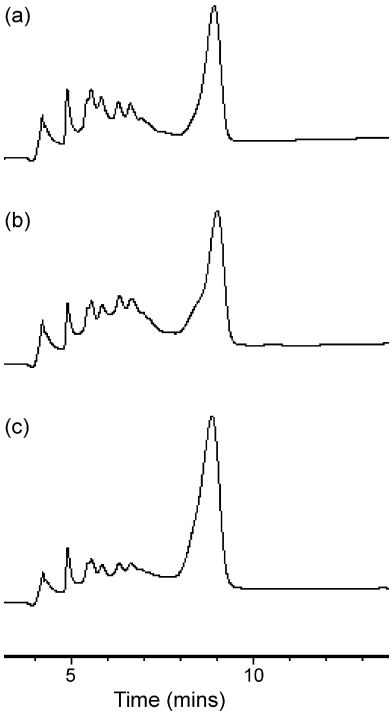

Fig. 5. RP-HPLC profiles of contents of peak THP 1 (a), THP 4 (b), and SSP (c) at 30 °C. Flow conditions: 10–45% B in 30 min at 1 ml/min total flowrate. The RP-HPLC profile of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was reproducible in three independent analysis of THP 1, THP 4, and SSP at same running conditions.

centage of triple helices in the solution. The RP-HPLC analysis of the freshly prepared $(Pro\text{-}Hyp\text{-}Gly)_{10}$ resulted in a similar profile as that of the equilibrated sample obtained at the same conditions and temperature, but with greatly lower THP peaks, denoting the presence of low percentage of the triple-helical peptides (Fig. 6c). These analysis of the freshly prepared $(Pro\text{-}Hyp\text{-}Gly)_{10}$ demonstrated that most of the collagen-like peptides are in unassembled form and are slowly folded into higher order structure to reach the equilibrium. The independent mass spectroscopy and a series of RP-HPLC studies confirmed that the multiple THP peaks as displayed in Fig. 3a contains triple-helical conformations of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ while the SSP peak is definitive for the unassembled structure of $(Pro\text{-}Hyp\text{-}Gly)_{10}$.

The observation of multiple THP peaks is probably best explained by the misalignment within the triple helix structure during the self-assembly process. Collagen triple helix is composed of three closely packed peptide chains. X-ray studies have deduced that the three closely packed chains, staggered by one residue from each other, form a right-handed triple-helical structure [26]. The unique primary structure of collagen allows for out-of-register alignment during the folding process [16]. The folding of the triple helix is nucleated at the C-terminal Gly-Pro-Hyp-rich sequence and propagated from the C- to the N-terminal in a zipper-like manner [27]. For synthetic unlinked collagen-like peptides, nucleation may happen at many sites and the trimerization can undergo sliding. The only option to correct a triple helix assembled by mismatched strands is a complete dissociation into three coiled chains, which can again undergo nucleation with the possibility of correct reg-

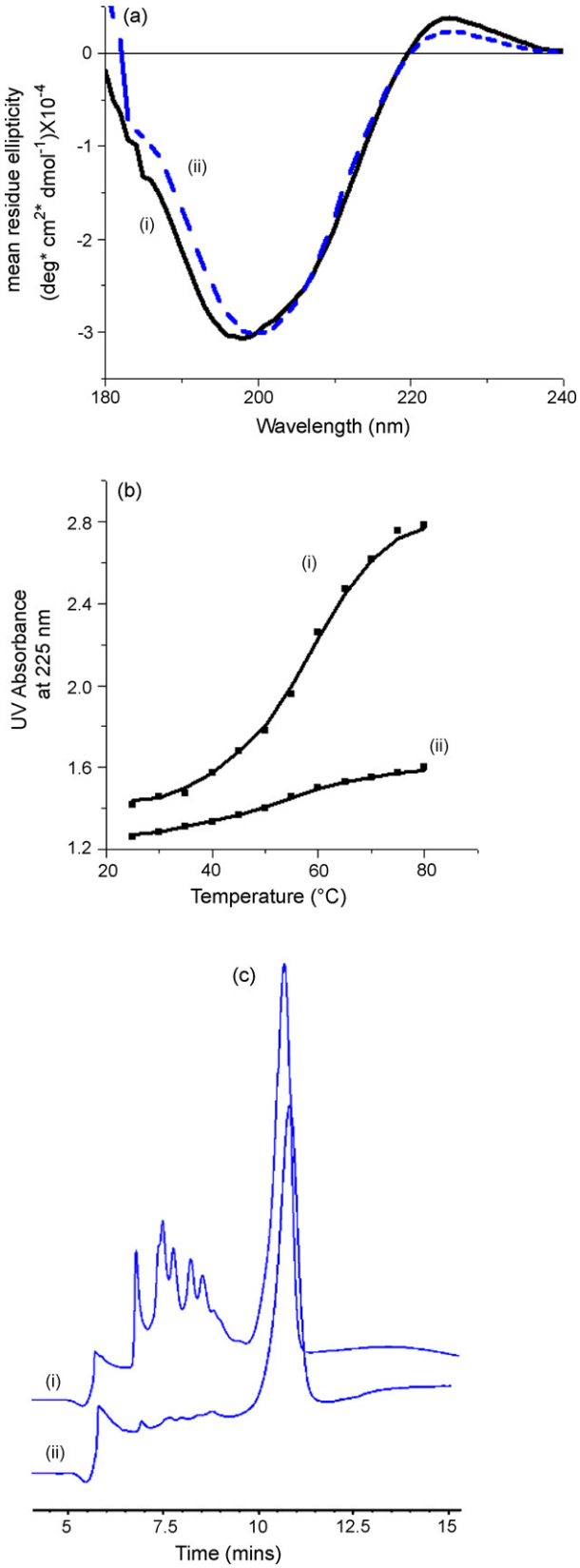

Fig. 6. CD spectra (a), thermal melting curves (b), and RP-HPLC profiles (c) of the equilibrated (i) and freshly prepared (ii) $(Pro\text{-}Hyp\text{-}Gly)_{10}$ (0.20 mg/ml) in water. RP-HPLC profile was obtained by using gradient flow from 10% B to 45% B in 30 min at 1 ml/min.

ister [17]. Alternatively, the misalignment can be overcome by bridging the three polypeptide chains closely together either by a template [3,18,28] or by a built-in cysteine-knot [29]. Template-assembled collagen mimetics have been shown to facilitate and stabilize the helix folding [3,18,28].

The production of the multiple THP peaks can also arise from the partially folded peptides. Both melting studies of (Pro-Hyp-Gly)$_{10}$ (dissolved in RP-HPLC solvent) by UV absorbance measurement and by RP-HPLC thermal denaturation gave a lower T_m value (see Table 1). The result indicated that the RP-HPLC solvent may partially destabilize the triple helix and thus resulted in a lower T_m value. The loosen triple-helical structures, even though is less stable, are still more hydrophilic and thus may be eluted earlier than the single stranded peptides but in different orders, depending on the degree of the partial unfolding.

As the helix nucleation may happen at many sites, the helix formation between the correct and deletion peptides is possible. The spontaneous assembly of these peptides may also lead to the occurrence of the multiple peaks. A deletion peptide with one proline deleted (Mr = 2595) was detected on the MALDI-TOF MS (see Fig. 4) giving a mixture of desired (Pro-Hyp-Gly)$_{10}$, and a small percentage of the deletion peptide (Pro-Hyp-Gly)$_m$-Hyp-Gly-(Pro-Hyp-Gly)$_n$, where $m + n = 9$ and $m = 0, 1, 2, \ldots, 9$. In order to verify the participation of the defective peptides in the triple helix assembly, these peptides were further purified to a purity greater than 99% (see Section 2.2). Mass spectrum of the purified sample was given in Fig. 7. The RP-HPLC profile in Fig. 3b was reproducible using the purified (Pro-Hyp-Gly)$_{10}$ at 30 °C (Fig. 7). Thus it is proposed that the participation of the defective peptide in the production of the multiple THP peaks is small and not significant.

The THP peaks and SSP peak have the same constituent. In addition, it is previously reported that the elution of proline-rich peptide on the reversed-phase HPLC may result in two partially resolved peaks at specific gradient flow and conditions [30].

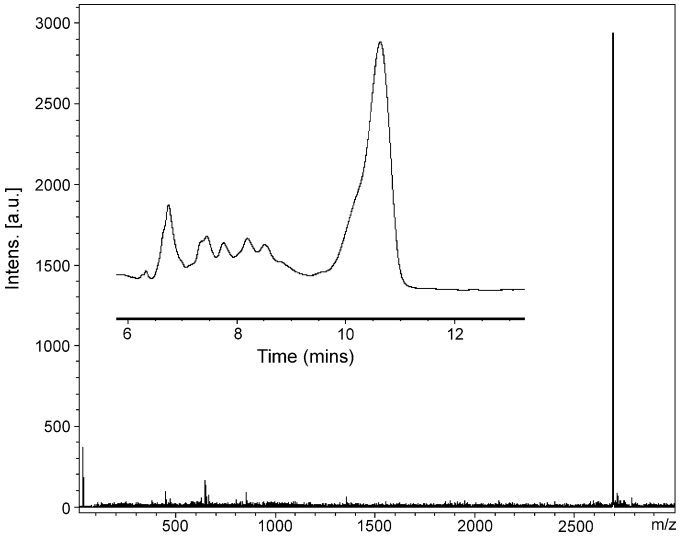

Fig. 7. MALDI-TOF MS spectrum of the purified (Pro-Hyp-Gly)$_{10}$. The profile attached in the MS spectrum is the RP-HPLC chromatogram of pure (Pro-Hyp-Gly)$_{10}$ obtained by gradient flow from 10% B to 45% B in 30 min at 1 ml/min at 30 °C.

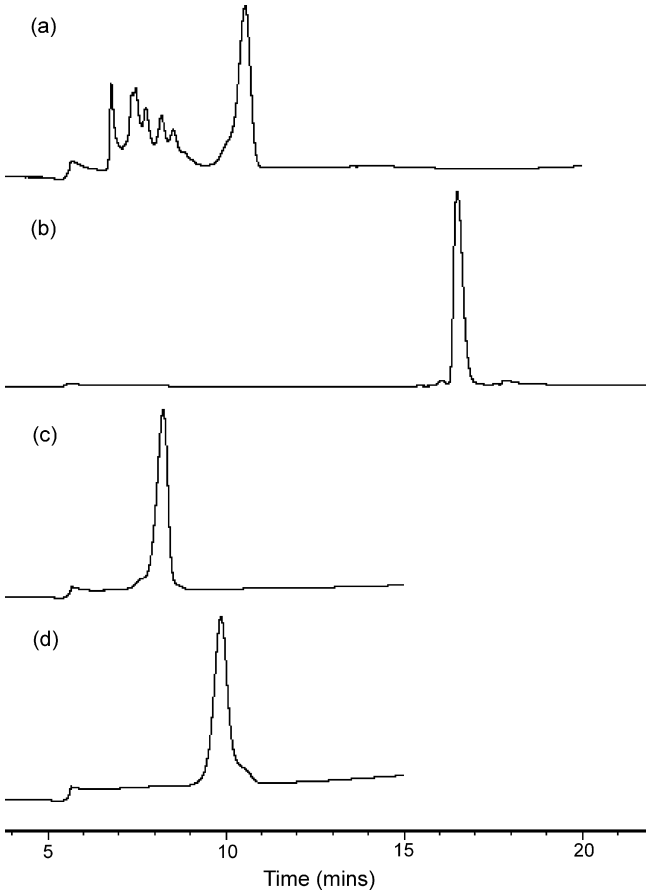

Fig. 8. RP-HPLC profiles of (a) (Pro-Hyp-Gly)$_{10}$, (b) (Pro-Pro-Gly)$_{10}$, (c) (Pro-Hyp-Gly)$_5$, and (d) control peptide (PPHypPGPGHypHypGPHypG-PHypGHypPGHypGHypPGPHypGHypG, where G: Gly, P: Pro, Hyp: hydroxyproline) at 35 °C at 1 ml/min from 10% B to 45% B in 30 min. All samples have numerous prolines and hydroxyprolines residues and there was only a single peak observed in their respective RP-HPLC profiles, except for (Pro-Hyp-Gly)$_{10}$, indicating that the multiple THP peaks arise not from *cis–trans* isomers.

Thus, it is very important in this analysis to verify the participation of *cis–trans* isomers in the production of the multiple THP peaks. The contribution of the *cis–trans* isomerization could be small and limited. This is because the analyses of proline-rich (Pro-Hyp-Gly)$_5$, (Pro-Pro-Gly)$_{10}$ and CP at same conditions as for the analysis of (Pro-Hyp-Gly)$_{10}$ resulted in only a single peak instead of multiple peaks (Fig. 8). The analyses temperature (35 °C) is higher than the T_m for both (Pro-Hyp-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$, therefore no additional peaks were to be observed except a single peak indicative of the unassembled structure.

Besides the hydrophobicity, there are other combined factors which can contribute to the overall retention mechanism, such as bulk steric effect, differences in the surface and volumes of the molecules of varying conformations, and the different adsorption–desorption kinetics, and these effects are likely to be responsible for the differences in the peak shapes in different HPLC runs. The misaligned, partially folded, and properly assembled peptide molecules differ from each other by not only the hydrophobicity but also the volume and the surface. The

differently folded (Pro-Hyp-Gly)$_{10}$ have varying conformations and thus dissimilar volume and bulk steric effects. The different arrangement and positioning of the hydroxyl side groups of the hydroxyprolines on misaligned, partially folded, and properly assembled molecules not only change the hydrophobicities but also the molecular surface, which may result in different interactions between the peptides and the stationary phase and thus adsorption–desorption kinetics. Generally, our results showed that (Pro-Hyp-Gly)$_{10}$ exhibited a reproducible RP-HPLC profile characterized by the multiple THP peaks and a single SSP peak.

The RP-HPLC profiles of (Pro-Pro-Gly)$_{10}$ were shown in Fig. 3d–f. RP-HPLC analyses of (Pro-Pro-Gly)$_{10}$ at temperatures lower than the T_m showed at least an additional broad peak indicative of the triple-helical conformations of (Pro-Pro-Gly)$_{10}$ (denoted as THP). The broad peak is probably due to the overlapping of peaks definitive of the misalignment products during the assembly of the triple-helical conformation. Or it can be due to the partial denaturation of triple helix structure by the solvent. It is interesting to note that the intensity of THP peaks of (Pro-Pro-Gly)$_{10}$ are not as high as those of (Pro-Hyp-Gly)$_{10}$ at stable temperature (<20 °C). This is mainly due to their different conformation stability. Furthermore, the UV absorbance of triple-helical conformation is known to be much lower than the single stranded peptide. Though the (Pro-Pro-Gly)$_{10}$ is stable in triple-helical form at 10 °C, there are always some co-existing unassembled peptides and thus the intensity of the multiple THP peaks will not be very significant as compared to that of SSP peak relatively. The same reason can be used to explain why the THP peaks of (Pro-Hyp-Gly)$_{10}$ are not significantly higher than the SSP peak at low temperatures. As both THP and SSP were detected on the same RP-HPLC chromatogram and there is significant difference in their UV absorbance, the ratio of SSP to THP peaks may not reveal the actual ratio of unfolded to folded structures without a proper calibration. The longer retention time for the elution of the triple-helical (Pro-Pro-Gly)$_{10}$ is consistent with the primary structure of this model peptide as well as the packing of the triple helix structure. (Pro-Pro-Gly)$_{10}$ contains no hydrophilic side groups at X and Y positions and thus the triple-helical conformation of (Pro-Pro-Gly)$_{10}$ is more hydrophobic than the single strand peptide and therefore leads to a longer retention time than the unassembled structure (denoted as SSP). The triple-helical characteristic of the THP peak was also examined by the denaturation experiments using RP-HPLC. The interconversion between the THP and SSP peaks as seen in Fig. 3d–f is due to the denaturation of the triple helix structure during the temperature titration process. The intensity of the THP peak decreases at elevated temperatures accompanied by the increase of the intensity of the SSP peak. Results from the CD spectroscopy and UV absorbance measurements indicated that (Pro-Pro-Gly)$_{10}$ adopts a stable triple-helical conformation at low temperature and the triple helix begins to dissociate with increasing temperature. The results were consistent with the RP-HPLC analyses and thus the THP peak must arise from the triple-helical conformations of (Pro-Pro-Gly)$_{10}$.

RP-HPLC analyses of the control peptide (CP) resulted in a single peak. No changes were observed between the analysis at low and high temperatures (see Fig. 9) indicating that the CP is stable at only one conformation in the temperature range of the study, which is not triple helix but most likely the poly(proline-II)-like conformation. Even though the control peptide was constituted by a considerable number of prolines and hydroxyprolines residues, no multiple peaks were observed at both low- and high-temperature RP-HPLC analyses again suggesting that the *cis–trans* isomerization of prolines/hydroxyprolines contributes to very small extent to the occurrence of the multiple peaks as found in the analyses of (Pro-Hyp-Gly)$_{10}$.

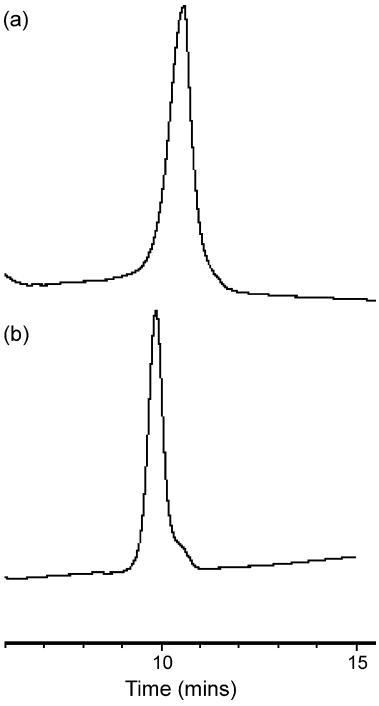

Fig. 9. RP-HPLC profiles of control peptide (CP) (PPHypPGPGHypHypG-PHypGPHypGHypPGHypGHypPGPHypGHypG, where G: Gly, P: Pro, Hyp: hydroxyproline) at different temperatures: (a) 5 °C and (b) 40 °C. Flow conditions: 10–45% B in 30 min at 1 ml/min.

3.4. Melting studies using RP-HPLC

To study the thermal transition and determine the melting point temperature (T_m) of the triple helix conformation, we plotted the peak intensity for the SSP peak as a function of temperature. We have also plotted the integrated peak area against the temperature and a similar curve was obtained. The principle is similar to that applied by the UV absorbance measurement except that this technique provides an additional advantage to the thermal melting curve measurement, which is the separation between the triple-helical conformations and the unassembled structure and also observation of possible misalignment products during the assembly of the triple-helical conformation.

Triple helix structures can be distinguished from the unassembled poly(proline-II)-like conformations based on the thermal denaturation behavior [7] in which the triple-helical conformations melt in a greatly cooperative way. A thermal transition curve was established for each collagen-like peptide by RP-HPLC denaturation studies and the results are shown

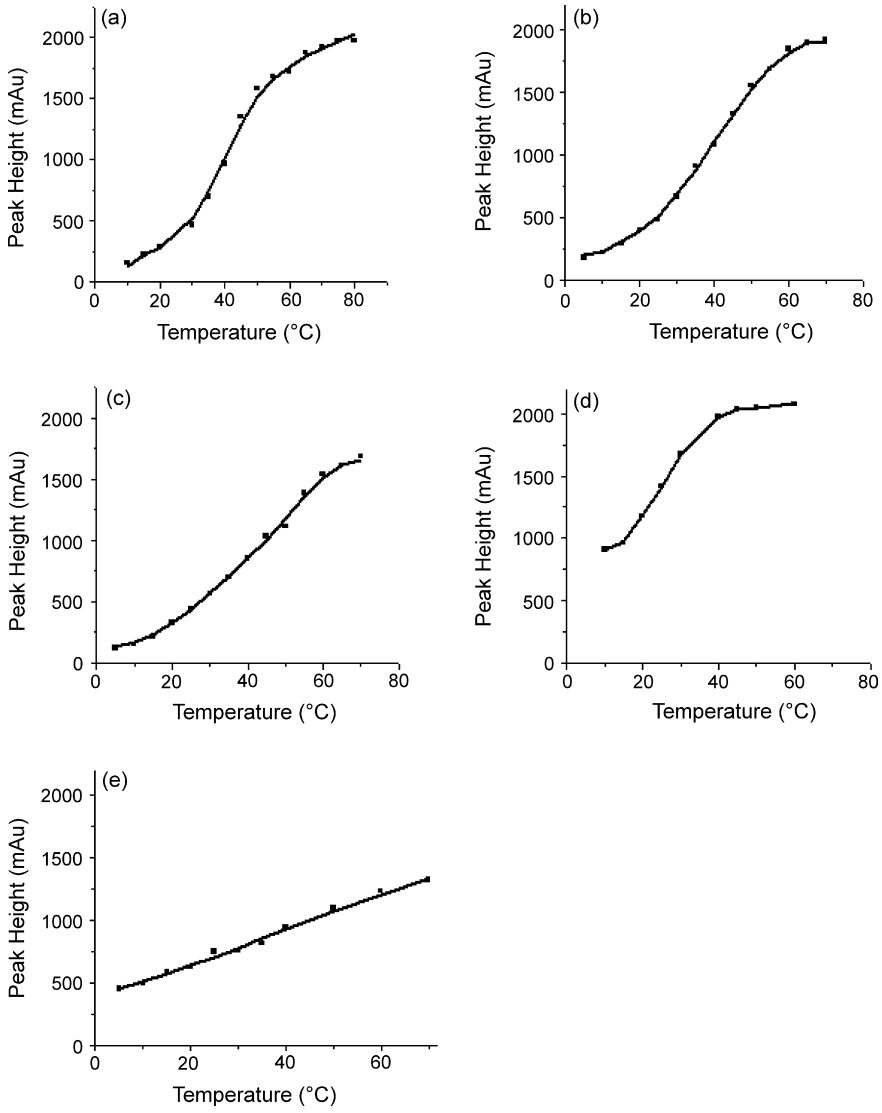

Fig. 10. Thermal transition curves of (a) $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (b) THP-1 of $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (c) THP-4 of $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (d) $(Pro\text{-}Pro\text{-}Gly)_{10}$, and (e) control peptide (PPHypPGPGHypHypGPHypGPHypGHypPGHypGHypPGPHypGHypG, where G: Gly, P: Pro, Hyp: hydroxyproline) obtained by RP-HPLC melting studies. Each sample was allowed to equilibrate for at least 3 days at 4 °C prior to RP-HPLC analysis. RP-HPLC analysis was performed as a function of temperature. Each sample was allowed to equilibrate for at least 15 min at each temperature point prior to the measurement.

in Fig. 10. All of the T_m values obtained from the RP-HPLC denaturation studies were tabulated in Table 1. It is readily seen that the T_m values obtained from the RP-HPLC melting studies are close to those obtained by UV absorbance measurement for samples prepared in 20% acetonitrile. The melting curves of both $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ (in 20% acetonitrile) obtained by UV absorbance measurement at 225 nm were given in Fig. 11. On the other hand, the T_m values obtained from the RP-HPLC melting studies deviated from those attained from the UV absorbance measurements for samples dissolved in water. The result suggests that the deviation is possibly due to partial denaturation of the triple-helical conformation during the elution by solvent. The interaction of the triple helix with the stationary phase may also lead to some degrees of destabilization. It is interesting to note that the T_m value of $(Pro\text{-}Pro\text{-}Gly)_{10}$ obtained from RP-HPLC thermal denaturation study is much closer to that obtained from the UV absorbance measurements as compared to the case for $(Pro\text{-}Hyp\text{-}Gly)_{10}$. It is possibly due to the slower unfolding rate of $(Pro\text{-}Pro\text{-}Gly)_{10}$, which has a lower T_m, at low-temperature measurements and thus the conformational denaturation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ is not significant within the time scale of the RP-HPLC analysis. It has been previously reported that the unfolding rate of collagen and collagen peptides is highly dependent on temperature [31,32]. Unfolding rate of collagen triple helix decreases exponentially at lower temperature [31]. On the other hand, the denaturation of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ is more rapid at higher temperatures and is within the time course of chromatography analysis. Moreover, collagen-like $(Pro\text{-}Hyp\text{-}Gly)_{10}$ has enhanced thermal stability as compared to $(Pro\text{-}Pro\text{-}Gly)_{10}$ and thus the percentage of triple helices in the solution is much higher. Consequently, the degree of denaturation of the triple-helical conformations of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ is much larger and more obvious as compared to $(Pro\text{-}Pro\text{-}Gly)_{10}$.

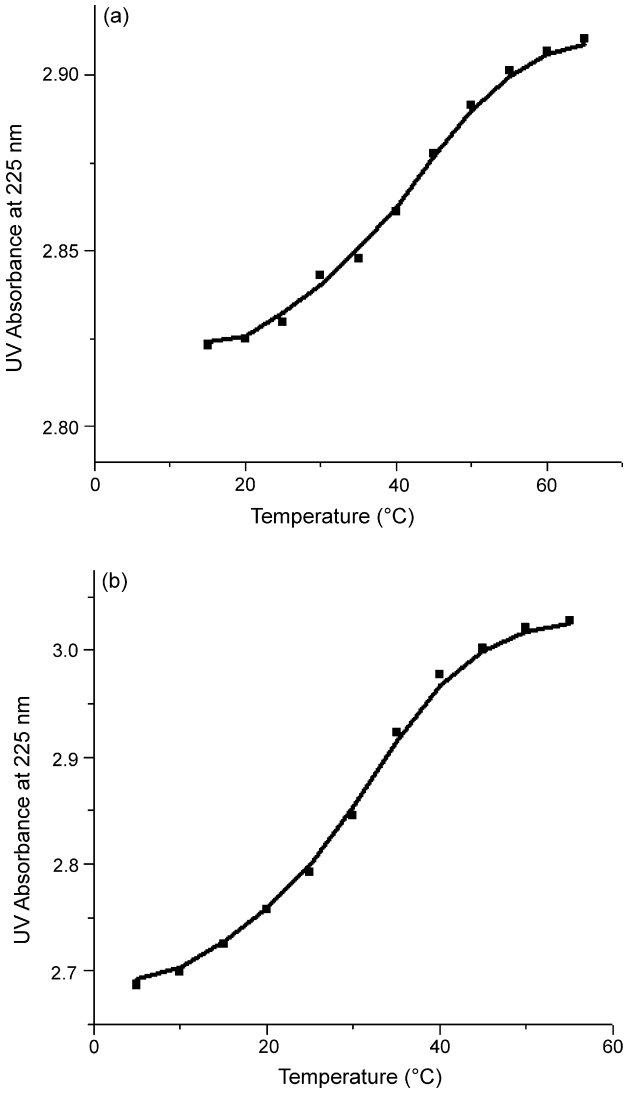

Fig. 11. Thermal transition curves of (a) (Pro-Hyp-Gly)$_{10}$ (0.20 mg/ml) and (b) (Pro-Pro-Gly)$_{10}$ (0.15 mg/ml) dissolved in RP-HPLC buffer obtained by UV absorbance measurement at 225 nm. The melting curves are similar to those obtained from RP-HPLC melting studies.

It can be seen from Fig. 10 that both (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ exhibited highly cooperative transition curves with a transition midpoint at 42 and 27 °C, respectively. The results were consistent with the CD spectroscopy and UV absorbance measurements that both (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ assembled into triple-helical conformations similar to that of the native collagen. On the contrary, no transition was observed in the melting curve measurement of the control peptide (CP), indicating that the CP is not folded into a triple helix structure but most likely into a poly(proline-II)-like conformation. This result is in agreement with the above CD and UV measurements. In order to verify the triple-helical characteristic of the THP peaks of (Pro-Hyp-Gly)$_{10}$, the fraction of THP 1 and THP 4 as seen in Fig. 3a were examined independently by the RP-HPLC melting studies. Varying the temperature of THP 1 and THP 4 by using the RP-HPLC resulted in a highly cooperative transition curve similar to that of (Pro-Hyp-Gly)$_{10}$ (see Fig. 10a–c). Combined with the independent control experiments such as MALDI-TOF MS and RP-HPLC, it is clear that the multiple THP peaks must arise from the triple-helical conformations of (Pro-Hyp-Gly)$_{10}$.

4. Conclusion

An alternative characterization technique for structural and melting studies of synthetic collagen-like peptides was established in this study. It has been demonstrated in a series of experiments that the characterization of the assembled, misfolded, and unassembled conformations of the collagen-like peptides has been achieved. The triple helices melted on the RP-HPLC in a highly cooperative way and the melting point temperature can be obtained from the midpoint of the transition. The triple-helical conformations can be well distinguished from the unassembled structure of the same collagen analogs based on the different hydrophobicities, volumes, and surfaces exhibited by the different conformations. This technique can be helpful when there is a confusion in the application of CD spectroscopy for the overlapping of the spectral shapes and band positions of the triple helix and poly(proline-II)-like structure. This technique can serve as a potential analytical tool for biophysical chemist as well as biologist in their studies of the importance of triple-helical conformations vis-à-vis the unassembled structure.

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