Investigation of the applications of reverse-phase high performance liquid chromatography in the structural studies of collagen-like model peptide

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Abstract

Various characterization techniques, such as circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy, have been reported and widely used for the structural studies of the triple helical twists of collagen. In this study, the potential applications of reverse-phase high performance liquid chromatography (RP-HPLC) in the structural studies of collagen triple helix were investigated. RP-HPLC analyses of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ resulted in numerous peaks indicative of triple helical conformations (denoted as THP) and a single peak representative of the unfolded single strand peptide (denoted as SSP). This characterization technique by RP-HPLC is based on the different hydrophobicities possessed by the folded and unfolded conformations. The triple helical characteristic of the THP peak was confirmed by several independent control experiments, including temperature dependency analyses. A new approach to study the cooperative thermal transition between the folded and unfolded conformations as well as determination of melting point temperatures (T_m) was also introduced in this study. HPLC denaturation experiments were performed based on the interconversion between the THP and SSP peaks at different temperatures. The T_m values of $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Hyp\text{-}Gly)_{10}$ dissolved in HPLC buffer were obtained from the midpoints of the cooperative transition curves as 26.0 ^oC and 42.0 ^oC respectively.

Introduction

Collagen is composed of three extended left-handed polyproline-II like chains arrayed in parallel with a one-residue register shift and intertwined to assemble into a right-handed triple helix. The assembly is a direct consequence of collagen primary structure characterized by the repetition of X-Y-Gly triplets, where position X is frequently a proline residue (Pro) and position Y is usually hydroxyproline (Hyp). 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. Various characterization techniques, such as CD and NMR spectroscopy, have been widely used for the structural studies of the triple helical twists of collagen[1]. Several investigations of thermal transition of the triple helical structure to an unfolded state have been reported by measuring temperature-dependent optical rotations[2], ellipticities[3], heat capacities[4] and NMR spectra[5] of collagen-like peptide solution as a function of temperature. In these structural and thermodynamic studies, short synthetic collagen-like peptides were used as collagen models. $(Pro\text{-}Pro\text{-}Gly)_n$ with n = 5, 10, 15, 20[5] and $(Pro\text{-}Hyp\text{-}Gly)_n$ with n = 5-10[6] have been widely used in the study of the triple helix conformation. The unique repetitive sequence of the collagen triple helix is susceptible to misalignment during the spontaneous assembly of the triple helical conformations[7]. Such misaligned structures are usually difficult to characterize by CD or NMR spectroscopy. As RP-HPLC is a common analytical and preparative tool in the peptide works[8], we present an intriguing application for the structural as well as thermodynamic studies of collagen-like model

peptides. Characterization of denatured, differently aligned and misfolded molecules by RP-HPLC is possible for its superior separation capability. A new approach to establish thermal melting curves as well as to determine the melting point temperatures (T_m) of collagen-like peptides using RP-HPLC is introduced. This study will aid in advancing the structural and thermodynamic studies of collagen and similar compounds, enabling a better understanding on the interactions between the RP-HPLC system and the triple helical collagen-like peptides and facilitating the breakthrough in the characterization techniques of the triple helical structures of collagen.

Experimental

Collagen-like peptides, including $(Pro\text{-}Hyp\text{-}Gly)_5$, $(Pro\text{-}Hyp\text{-}Gly)_{10}$, $(Pro\text{-}Pro\text{-}Gly)_{10}$ and a control peptide (CP), were synthesized using Fmoc solid phase synthesis method. CD spectroscopy and melting curve analysis were used to verify the presence of the triple helices. HPLC characterization was done by injecting samples into a C18 column at different temperatures at constant flow rate of 1ml/min and linear gradient from 10% B to 45% B (buffer A and B are 0.1% TFA in water and acentonitrile respectively) in 30 minutes.

Result and discussion

The CD spectra of collagen-like peptides were compared to that of the native one (calf skin collagen) as given in Figure 1. Collagen has a unique CD spectrum with a small positive peak at around 220 nm and a large negative peak at 197 nm. Both $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ displayed similar CD patterns as the native one. In contrast, the CD spectrum of the control peptide (CP) showed characteristic of a polyproline II-like (PPII) helical conformation which is stable even at elevated temperature. All peptides, except the CP, underwent thermal transition from folded to unfolded states and their T_m values are given in Table 1.

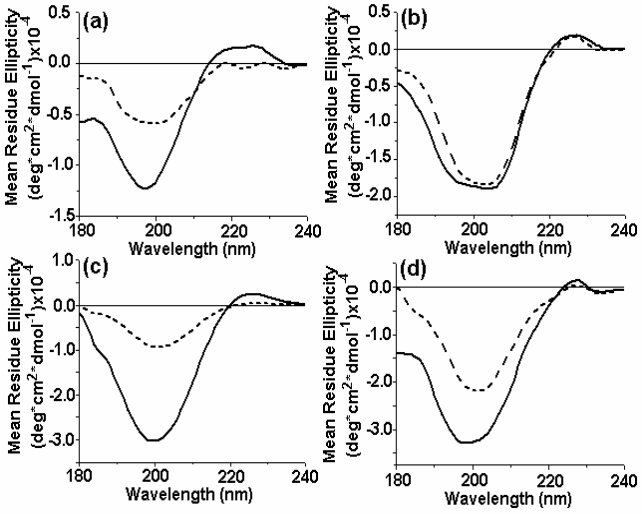

Figure 1. CD spectra were obtained at 25°C (solid line) and 60°C (segmented line) for (a) collagen (0.50 mg/ml), (b) control peptide (CP) (0.40 mg/ml), (c) $(Pro\text{-}Hyp\text{-}Gly)_{10}$ (0.20 mg/ml), and (d) (Pro-Pro-Gly)10 (0.15 mg/ml) in H_2O.

Table 1. Melting points temperatures (T_m) obtained from UV $absorbance_{225\ nm}$ measurements and RP-HPLC denaturation studies.

Compounds	T_m from RP-HPLC (°C)	T_m from UV Absorbance (°C)
Collagen	Not measured	38.0
$(Pro\text{-}Hyp\text{-}Gly)_{10}$	42.0	57.0
$(Pro\text{-}Pro\text{-}Gly)_{10}$	27.0	28.0
Control Peptide	No transition	No transition

The HPLC profiles of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ at different temperatures are given in Figure 2a to 2c. 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 stranded peptide peak was denoted as SSP.

The triple helical characteristic of the THP peaks of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was verified by denaturation studies using HPLC. From Figure 2a to 2c, it is clearly seen that the THPs disappeared completely at elevated temperature while the intensity of the SSP peak increased significantly. MALDI-TOF MS result (not shown) revealed that THP 1, THP 4, and SSP have a same constituent of molecular weight (MW) of 2691 daltons, which is for $(Pro\text{-}Hyp\text{-}Gly)_{10}$. Each of these fractions was then analyzed through HPLC. It is clearly seen from Figure 3a to 3c that the chromatogram of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was similarly reproduced in the independent HPLC analyses of THP 1, THP 4 and SSP.

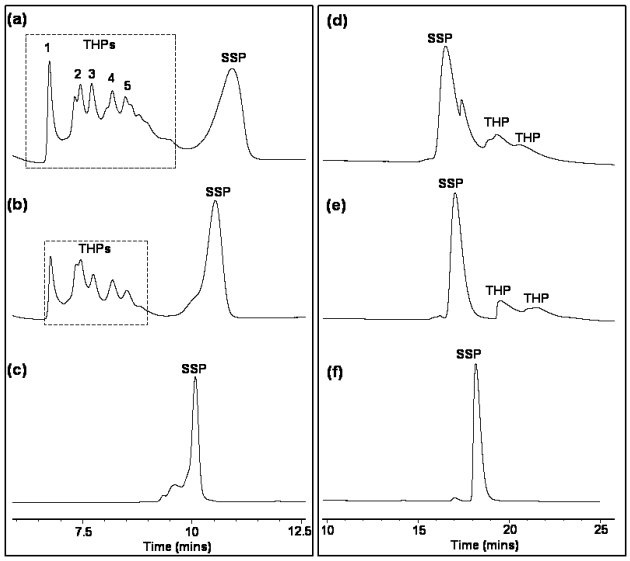

Figure 2. HPLC profiles of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ at a) 15°C, b) 30°C and c) 60°C and HPLC profiles of $(Pro\text{-}Pro\text{-}Gly)_{10}$ at d) 10°C, e) 20°C, and f) 40°C.

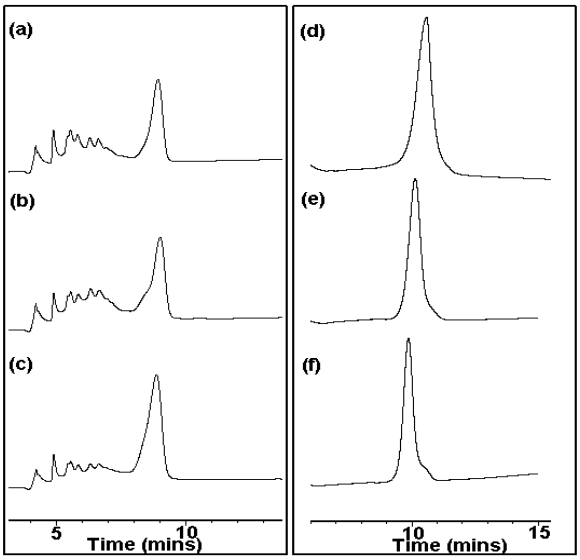

Figure 3. HPLC profiles of contents of THP 1 (a), THP 4 (b) and SSP (c) at 30°C. HPLC profiles of control peptide (CP) at different temperatures: (d) 5°C, (e) 20°C and (f) 40°C.

The HPLC profiles of $(Pro\text{-}Pro\text{-}Gly)_{10}$ were shown in Figure 2d to 2f. HPLC analyses of $(Pro\text{-}Pro\text{-}Gly)_{10}$ at temperatures lower than the T_m showed at least an additional broad peak indicative of the triple helical conformations of $(Pro\text{-}Pro\text{-}Gly)_{10}$ (denoted as THP). The triple helical characteristic of the THP peak was also examined by the denaturation experiments using HPLC. The interconversion between the THP and SSP peaks as seen in Figure 2d to 2f is due to the denaturation of the triple helical structure during the temperature titration process. The intensity of the THP peak decreases at elevated temperatures accompanied by an increase of the intensity of the SSP peak. MALDI-TOF MS result (not shown) verified that each fraction contained a similar parent ion.

The observation of multiple THP peaks is probably best explained by the misalignment within the triple helical structure during the self-assembly process. Another possible reason is the potential partial denaturation of triple helices by the combined effects of the solvent system and stationary phase. The occurrence of these multiple peaks may also be a consequence of the spontaneous assembly of deletion and desired peptide into triple helical conformation. In the MALDI-TOF MS analyses (result not shown), a very small percentage of deletion peptide

with one proline residue deleted of molecular weight 2594 daltons was detected. The *cis-trans* isomerization of prolines/hydroxyprolines is another possible factor for the occurrence for these multiple peaks. However, the contribution of the *cis-trans* isomerization could be small and limited in this case as the analyses of $(Pro\text{-}Hyp\text{-}Gly)_5$, $(Pro\text{-}Pro\text{-}Gly)_{10}$ and CP at same conditions as for the analysis of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ resulted in only a single peak instead of multiple peaks (Figure 4).

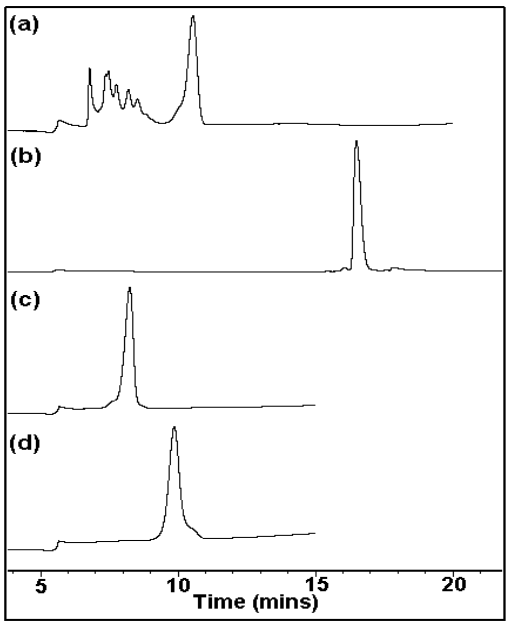

Figure 4. HPLC profiles of (a) $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (b) $(Pro\text{-}Pro\text{-}Gly)_{10}$, (c) $(Pro\text{-}Hyp\text{-}Gly)_5$, and (d) control peptide at 35°C at same flow conditions.

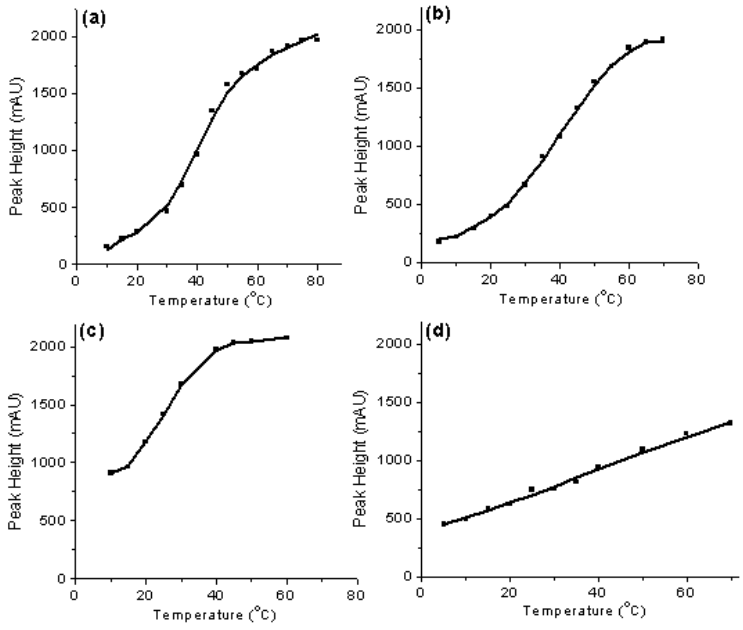

Figure 5. Thermal transition curves of (a) $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (b) THP-1 of $(Pro\text{-}Hyp\text{-}Gly)_{10}$, (c) $(Pro\text{-}Pro\text{-}Gly)_{10}$, and (d) control peptide obtained by RP-HPLC melting curve studies.

HPLC analyses of the control peptide (CP) resulted in a single peak. No changes were observed between the analysis at low and high temperatures (Figure 3d to 3f) indicating that the CP is stable at only one conformation in the temperature range of the study, which is most likely the polyproline II-like conformation. No multiple peaks were observed at both low and high temperatures again suggested 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 $(Pro\text{-}Hyp\text{-}Gly)_{10}$. This shows that the *cis-trans* isomerization is a slow process that would not be an important factor within the time-frame of the HPLC analyses.

To study the melting point temperature of the triple helix conformation, we have developed a new technique of plotting the peak intensity for the SSP peak as a function of temperature. A thermal transition curve was established for each collagen-like peptide by the new RP-HPLC denaturation studies and the results are shown in Figure 5. The results were consistent with the CD spectroscopy and UV absorbance measurements. In order to verify the triple helical characteristic of the THP peak of $(Pro\text{-}Hyp\text{-}Gly)_{10}$, the fraction of THP 1 as seen in Figure 2a was examined independently by the RP-HPLC denaturation study. Varying the temperature of THP 1 resulted in a highly cooperative transition curve similar to that of (Pro-

Hyp-Gly)$_{10}$ (see Figure 5b). All of the T_m values obtained from the RP-HPLC denaturation studies were tabulated in Table 1.

Conclusion

We established an alternative method of instrumental analysis for the conformational studies of the collagen triple helix. Characterization of the assembled, misfolded, and unassembled conformations of the collagen-like peptides is possible due to the superior separation capability of RP-HPLC. Several THP peaks were observed in addition to the single SSP peak in the RP-HPLC analyses of (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$. The multiple peaks possibly arise from the correctly folded, misfolded, and partially folded triple helical confomations. (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ exhibited a cooperative melting curve in the thermal transition study by RP-HPLC. This characterization approach is more beneficial as compared to the conventional CD and NMR spectroscopy for the separation between all of the above conformations. The ratio of the triple helix to the single stranded at different temperatures can always be examined with this technique and thus it 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. This study will aid in enabling the development of a new analytical tool for the structural and thermodynamic studies of collagen using RP-HPLC.

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