CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, 2-(N-morpholino) ethanesulfonic acid, trinitrobenzene sulfonate, beta mercapto ethanol, tannic acid, tricine, 1-anilino-8-naphthalene sulfonic acid, N-(3-[2-Furyl]acryloyl)-(L-Leu)-Gly-Pro-Ala (FA(L-L)GPA), N-(3-[2-Furyl]acryloyl)-(D-Leu)-Gly-Pro-Ala (FA(D-L)GPA), collagenase type 1A, hydroxyproline, sodium dodecyl sulfate, acrylamide, glycine, ammonium per sulfate, N,N,N',N'-tetramethylethylenediamine and coomassive brilliant blue R-250 were procured from Sigma Chemicals Co., USA. Ac-RGD-$CONH_2$, Ac-RGK-$CONH_2$,
GSOGADGPAGAOGTOGPQGIAGQRGVVGLOGQRGER,
GSOGADGP(D-A)GAOGTOGPQGIAGQRGVVGLOGQRGER peptides were custom synthesized from Tuffs University, USA. All other reagents used were of analytical grade and sourced from SRL Ltd., India.

2.2 COLLAGEN EXTRACTION

Collagen was extracted from rat tail tendon by known procedures (by acetic acid extraction and salting out with NaCl) and used throughout the studies (Chandrakasan et al 1976). The collagen content of the solution was estimated by the standard procedure using hydroxylproline estimation (Woessner 1961). Purity of collagen and structural conformation were

determined by SDS-PAGE and circular dichroism spectroscopy, respectively (Laemmli 1973).

2.3 ROLE OF CHARGED FUNCTIONAL GROUP IN COLLAGEN-COLLAGENASE BINDING

To identify the role of charged functional group in collagen collagenase binding, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was selected as a crosslinker.

2.3.1 Mass Spectral Analysis

Two peptides with the sequence of Ac-RGD-$CONH_2$ and Ac-RGK-$CONH_2$ were chosen to elucidate the formation of isopeptide bond between the side chains of carboxyl and amino group. Peptides (1 mg/mL) were dissolved in MES buffer, and then EDC was added to these peptides and stirred for 24 h at room temperature. After 24 h, the samples were injected using water/acetonitrile/methanol mixture in ESI-Mass spectrometer (Thermofinnigan LCQ 6000 advanced max ion trap). SIM mode was selected to highlight the molecular ion peak to confirm the formation of isopeptide bond. Separate mass spectra were taken for the individual peptides.

2.3.2 Electrophoretic Analysis

Collagen at constant concentration (1 μM) with varying EDC concentration (0-1000 μM) at 20 ºC and pH 4.5 was taken for SDS-PAGE. The control collagen and EDC cross-linked collagen samples were mixed with sample buffer at 1:4 ratio and denatured by heating for 5 min at 85–95 °C and loaded onto the wells of SDS polyacrylamide gel and the instrument was operated at 100 V. The gel was stained using coomassive brilliant blue for few hours and then destained to observe the bands.

2.3.3 Amino Group Assay

The free amino groups content of native and cross-linked samples were determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay (Habeeb 1966). Collagen samples were incubated for 30 min in 1 ml of 4 wt % solution of $NaHCO_3$. To this mixture, 1 ml of a freshly prepared solution of TNBS (0.5 wt %) in 4 wt % $NaHCO_3$ was added. The resulting mixture was left for 2 h at 40 °C. After the addition of HCl (3 mL, 6 M), the temperature was raised to 60 °C. Degradation of collagen was achieved within 90 min. The absorbance at 346 nm was measured using a UV-Vis spectrophotometer. A blank was prepared by applying the same procedure, except that HCl was added before the addition of TNBS.

2.3.4 Viscosity Measurements

Viscosity experiments were carried out on an Ubbelodhe viscometer, immersed in a thermostated water bath maintained at 25±0.1 °C. The concentration of collagen was 1 μM. The EDC concentration was varied (100-1000 μM).

2.3.5 Fluorescence Spectral Studies

Fluorescence spectral experiment was carried out at an excitation wavelength of 273 nm maintaining constant collagen concentration (1 μM) and varying the EDC concentration (0-1000 μM) at 20 ºC. pH was maintained at 4.5 using MES buffer. These spectral measurements were performed in Cary Eclipse fluorescence spectrophotometer.

2.3.6 CD Spectroscopy

Dichroic spectroscopy measurements were conducted on a JASCO J-815 CD spectrophotometer equipped with a peltier temperature control-

423S/15 (JASCO Inc.). The protein was used at a concentration of 1 μM, in 10 mM MES buffer, pH 4.5 with concentrations of EDC from 100-2130 μM. For CD data collection, 350 μL of protein was used in a 0.1 cm path length quartz cuvette. Wavelength was scanned between 190 to 250 nm, and spectra were recorded at 20 °C using 1.0 nm bandwidth, 0.1 nm step size, and an averaged time of 1 s. The raw data in millidegree unit was converted to molar ellipticity [θ].

2.3.7 Water Uptake Studies

The collagen was used at a concentration of 1 μM, in 10 mM MES buffer, pH 4 with the varied concentrations of EDC from 100-3000 μM. Samples were neutralized to pH 7.4 using 0.1 M NaOH and 10 mM Tris buffer. The neutralized collagen solution (10 mL) was transferred to a 15 mL centrifuge tube, vortexed for 30 s and incubated at 37 °C for 2 h. After incubation, the tubes were centrifuged at 8000Xg for 10 minute. The supernatant was discarded and the gel samples were weighed.

2.3.8 Turbidity Measurements

Turbidity curves were obtained by monitoring the optical density of samples placed in 1 mm cell at 313 nm as a function of time using a Perkin Elmer Lambda 35 spectrophotometer. The temperature was maintained at 37 ^{o}C by peltier temperature controlled system attached with spectrophotometer. All turbidity measurements were repeated a minimum of three times.

2.3.9 Thermal Analysis

The thermal stability of collagen and EDC-treated collagen were measured using differential scanning calorimetry (Q200 DSC with refrigerated cooling system 90, TA instruments, Waters). The collagen was

used at a concentration of 1 μM, in 10 mM acetate buffer, pH 4 with the concentrations of EDC from 100-3000 μM. Samples were neutralized to pH 7.4 using 0.1 M NaOH and 10 mM tris buffer. The neutralized collagen solution (10mL) was transferred to a 15 mL centrifuge tube, vortexed for 30 s and incubated at 37 °C for 2 h. After incubation the tubes were centrifuged at 8000X g for 10 minute. The supernatant was discarded and the gel was removed from the tube and blotted using a tissue. Gel samples were weighed (10 mg) and sealed in a Tzero pan with hermatic lid. The contents were heated from 30 to 90 ºC at a heating rate of 2 ºC/min.

2.3.10 Proteolysis Assay

The collagen was used at a concentration of 1 μM, in 10 mM MES buffer, pH 4 with the varied concentrations of EDC from 100-3000 μM. Samples were neutralized and centrifuged at 8000Xg for 10 minute. The supernatant was discarded and the gel samples were washed with water. The gel samples were incubated with 50 units of *Clostridium histolyticum* collagenase for 5 h at pH 7 and 37 °C. After incubation, ninhydrin mixture was added and the tubes were kept for 20 minute in boiling water for color development. The mixture was cooled, diluted with 50% n-propanol and the absorbance was read at 600 nm (Mandl et al 1953). The amount of leucine equivalent liberated from the sample was calculated using standard leucine plot. The difference in leucine equivalent was calculated as % inhibition.

2.4 EFFECT OF ISOPEPTIDE BOND ON COLLAGEN MIMICS- MOLECULAR DYNAMICS APPROACH

Three model systems were considered for understanding the role of isopeptide bond in the structure and stability of collagen like peptides. The sequences of the systems were given in **Figure 2.1**. Model-1 consists of sequence without isopeptide bonds. Model-2 and Model-3 contain isopeptide

bond between Lys-Glu and Lys-Asp respectively, which has been highlighted. Gencollagen package was used to build the three dimensional structure of collagen like peptides. Molecular dynamics (MD) simulations of all the systems were carried out using GROMACS 4.5.3 package (http://www.gromacs.org/) (Berendsen et al 1995; Lindahl et al 2001; Hess et al 2008). The details of box dimension, number of counter ions and water molecules of all the model systems are given in **Table 2.1**. Each of the systems was solvated with TIP3P water (Jorgensen et al 1983). Calculations were carried out employing AMBER FF99SB force field (Hornak et al 2006) and parameters for Hyp were taken from Park et al (2005). All the three systems were independently subjected to 500 steps of energy minimization employing the steepest descent algorithm. This step was followed by solvent equilibration and equilibration of the entire system for 250 pico second (ps) and 1 nano second (ns). The production run was carried out for all the systems for 10 nano seconds (ns) using 2 femto second (fs) time step for the integration of the equation of motion in the NPT ensemble at 27 ºC and at 1 atmospheric pressure. The velocity rescaling (V-rescale) thermostat and Parrinello-Rahman Barostat were used to control the temperature and pressure, respectively (Parrinello et al 1981; Nos´e et al 1983; Bussi et al 2007).

Table 2.1 The box dimension, number of counter ions and number of water molecules used in the MD simulation

Model System	Box dimension	Counter ion	No. of counter ion	No. of water molecules
Model-1	6x6x 15	Cl-	3	31625
Model-2	6x6x 15	Cl-	3	16802
Model-3	6x6x 15	Cl^-	3	16810

Model 1						Model 2						Model 3					
TH 1			TH 2			TH 1			TH 2			TH 1			TH 2		
D	G	K	Q	G	R	D	G	K	Q	G	R	D	G	K	Q	G	R
R	D	G	A	Q	G	R	D	G	A	Q	G	R	D	G	A	Q	G
G	R	E	G	A	E	G	R	E	G	A	E	G	R	E	G	A	E
D	G	K	V	G	P	D	G	K	V	G	P	D	G	K	V	G	P
A	D	G	M	V	G	A	D	G	M	V	G	A	D	G	M	V	G
G	A	E	G	M	N	G	A	E	G	M	N	G	A	E	G	M	N
P	G	P	F	G	I	P	G	P	F	G	I	P	G	P	F	G	I
K	P	G	P	F	G	K	P	G	P	F	G	K	P	G	P	F	G
G	K	L	G	P	F	G	K	L	G	P	F	G	K	L	G	P	F
A	G	R	P	G	P	A	G	R	P	G	P	A	G	R	P	G	P
D	A	G	K	P	G	D	A	G	K	P	G	D	A	G	K	P	G
G	D	E	G	K	P	G	D	E	G	K	P	G	D	E	G	K	P
S	G	I	A	G	K	S	G	I	A	G	K	S	G	I	A	G	K
P	S	G	A	A	G	P	S	G	A	A	G	P	S	G	A	A	G
G	P	N	G	A	P	G	P	N	G	A	P	G	P	N	G	A	P
K	G	P	E	G	T	K	G	P	E	G	T	K	G	P	E	G	T
D	K	G	P	E	G	D	K	G	P	E	G	D	K	G	P	E	G
G	D	R	G	P	D	G	D	R	G	P	D	G	D	R	G	P	D
V	G	D	K	G	P	V	G	D	K	G	P	V	G	D	K	G	P
R	V	G	A	K	G	R	V	G	A	K	G	R	V	G	A	K	G
G	R	A	G	A	K	G	R	A	G	A	K	G	R	A	G	A	K
L	G	R	E	G	N	L	G	R	E	G	N	L	G	R	E	G	N
T	L	G	R	E	G	T	L	G	R	E	G	T	L	G	R	E	G
G	T	A	G	R	D	G	T	A	G	R	D	G	T	A	G	R	D
P	G	P	V	G	K	P	G	P	V	G	K	P	G	P	V	G	K
I	P	G	P	V	G	I	P	G	P	V	G	I	P	G	P	V	G
G	I	A	G	P	H	G	I	A	G	P	H	G	I	A	G	P	H
P	G	V	P	G	A	P	G	V	P	G	A	P	G	V	P	G	A
P	P	G	P	P	G	P	P	G	P	P	G	P	P	G	P	P	G
G	P	A	G	P	L	G	P	A	G	P	L	G	P	A	G	P	L
P	G	P	A	G	A	P	G	P	A	G	A	P	G	P	A	G	A
A	P	G	V	A	G	A	P	G	V	A	G	A	P	G	V	A	G
G	A	P	G	V	A	G	A	P	G	V	A	G	A	P	G	V	A
A	G	A	P	G	R	A	G	A	P	G	R	A	G	A	P	G	R
P	A	G	A	P	G	P	A	G	A	P	G	P	A	G	A	P	G
G	P	A	G	A	A	G	P	A	G	A	A	G	P	A	G	A	A

Figure 2.1 Amino acid sequences of models and the residues involved in isopeptide bond formation are highlighted in yellow color

Bond length involving hydrogen atoms were constrained employing LINCS algorithm (Hess et al 1997). The particle-mesh Ewald (PME) was used to calculate the electrostatic interaction (Darden et al 1995). The cutoff distances for the long-range electrostatic and van der Waals energy terms were set as 10 Å. The MD simulation coordinates of all the systems were saved at 1 ps interval for further analyses. Post processing and analyses were carried out using GROMACS analysis tools. VMD and PyMol packages were used for visualization (Humphrey et al 1996; Huang et al 1998).

2.4.1 Analysis

2.4.1.1 Root-mean-square deviation (RMSD)

The RMSD of each residue of all the peptides was calculated with respect to the initial conformation as a function of time using **eq. 2.1**:

$$\mathrm{RMSD(t)} = \left[\frac{1}{N}\sum_{i=1}^{N}\|r_i(t) - r_i(0)\|^2\right]^{1/2} \qquad (2.1)$$

Here r_i is the distance between atoms at time t and N is the total number of backbone atoms. r_i is compared with the distance between the same atoms at time zero.

2.4.1.2 Principal component analysis (PCA)

PCA was carried out to examine the distribution of clusters in each model. PCA analysis was performed using g_covar and g_anaeig program implemented in GROMACS suite of packages (Berendsen et al 1995; Golbik et al 2000; DeLano 2008). Both rotational and translational motions were removed by fitting an alpha carbon atom of collagen structure of each frame to the starting conformation. Eigenvalues and eigenvectors were calculated by

diagonalizing the covariance matrix. The eigenvectors give the directions of motion, and the eigenvalues represent the amount of variation along the eigenvectors. The first two principle components (PC1 and PC2) account for a maximum variation. Projection of the trajectories onto individual eigenvectors provides information about the time dependence of protein conformation along these eigenvectors. The clusters are projected on to these first PC1 and PC2 to further understand how each population of clusters distributed in each models**.**

2.4.1.3 H-bond analysis

The variations between the intermolecular H-bonds present in the three α chain of collagen with time were obtained from the trajectory using the geometrical criteria as given in **eq 2.2**:

$$H_i = \begin{cases} 1, ((d(H_i \ldots O_i) \leq 3.5\dot{A}) \\ (140^\circ \leq \text{angle}(O_i \ldots H_i - N_i \leq 180^\circ)) \\ 0, \text{otherwise} \end{cases} \quad (2.2)$$

Here H_i is the possible i^{th} backbone intermolecular H-bond in the triple helix. The occupancy of the H-bond was determined according to the expression in **eq. 2.3**:

$$\text{Occupancy of H} - \text{bond } H_i \text{ in } \% = \left[\frac{\text{Total no. of } H_i = 1 \text{ state}}{\text{Total no. of conformation}}\right] 100 \quad (2.3)$$

2.5 ROLE OF HYDROGEN BONDING AND HYDROPHOBIC INTERACTIONS USING TANNIC ACID IN COLLAGEN-COLLAGENASE BINDING

To identify the role of hydrogen bonding and hydrophobic interactions in collagen collagenase binding, tannic acid (TA) was selected as a crosslinker.

2.5.1 Fluorescence Spectral Studies

Fluorescence spectral measurement was carried out at an excitation wavelength of 280 nm for collagen concentration of 1 μM, varying the TA concentration (0-20 μM). These experiments were carried out at 25 ºC with 10 mM acetate buffer of pH 4.

2.5.2 CD Spectroscopy

The collagen was used at a concentration of 1 μM, in 10 mM acetate buffer pH 4 with concentrations of TA from 0-200 μM. For CD data collection, 400 μl of each protein was used in a 0.1 cm path length quartz cuvette. Wavelength was scanned between 190 to 250 nm, and scanned spectra were collected at 25 °C using 1.0 nm bandwidth, 0.1 nm step size, for an averaged time of 1 s. The raw data in millidegree units was converted to molar ellipticity [θ]. For temperature-induced denaturation, the ellipticity was monitored as a function of temperature from 10 to 70 ºC at wavelength maxima 220 nm for the collagen solution. Temperature was increased at a heating rate of 1 ºC/min. The molar ellipticity at 220 nm showed a good agreement with the trimer to monomer model (T→3M) (Periskov et al 2004). The T_m was determined as the temperature at which the fraction folded was equal to 0.5 in the trimer to monomer transition curve. The T_m was determined with an accuracy of ±0.5 ºC.

2.5.3 Viscosity Measurements

Viscosity experiments were carried out on an Ubbelodhe viscometer, immersed in a thermostated water bath maintained at 25±0.1 °C. The concentration of collagen was maintained at 1 μM, where as TA concentration was varied (20-200 μM).

2.5.4 Binding of TA with collagen and Hydration Behaviour Studies

The bound and unbound study of TA with collagen was carried out by measuring the absorbance at 274 nm. The collagen was used at a concentration of 1 μM, in 10 mM acetate buffer pH 4 with concentrations of TA from 100-3000 μM. The samples (10 mL) were transferred to a 15 mL centrifuge tube after neutralization to pH 7.4 using 0.1 M NaOH and 10 mM Tris buffer and vortexed for 30 s followed by incubation at 37 °C for 2 h. After incubation the tubes were centrifuged at 8000X g for 10 min. The supernatant was subjected to absorbance measurement at 274 nm and the precipitated gel samples were weighed. Unbound TA concentration was calculated from the absorbance of supernatant solution using standard graph, which was plotted for TA with varying concentration. Difference between total and unbound concentration provides the bound concentration of TA. The gels were weighed again after drying the samples in a hot air oven for 24 h. The difference in weight was used to determine the moisture content.

2.5.5 Thermal Denaturation Analysis

The thermal stability of collagen and TA-treated collagen was measured using differential scanning calorimetry. The collagen concentration was maintained at 1 μM, in 10 mM acetate buffer, pH 4 with the varied concentrations of TA from 100-3000 μM. Samples were neutralized to pH 7.4 using 0.1 M NaOH and 10 mM Tris buffer. The neutralized collagen solution

(10mL) was transferred to a 15 mL centrifuge tube, vortexed for 30 s and the tubes were incubated at 37 °C for 2 h using a thermostat. After incubation the tubes were centrifuged at 8000X g for 10 minute. The supernatant was discarded and the gel was removed from the tube and blotted using a tissue to remove excess adhered water and sealed in a Tzero pan with Tzero hermatic lid. The contents were heated for 30 to 90 ºC at a heating rate of 2 ºC/min. All the experiments were done in triplicates.

2.5.6 Proteolytic Assay

The collagen was used at a concentration of 1 μM, in 10 mM acetate buffer, pH 4 with the varied concentrations of TA from 250-3000 μM. Samples were neutralized and centrifuged at 8000Xg for 10 minute. The supernatant was discarded and the gel samples were washed with water. The gel samples were incubated with 50 units of *Clostridium histolyticum* collagenase for 5 h at pH 7 and 37 °C. After incubation, ninhydrin mixture was added and the tubes were kept for 20 min in boiling water and examined for colour change. Upon change in colour, the mixture was cooled, diluted with 50% n-propanol and absorbance was read at 600 nm (Mandl et al 1953). The amount of leucine equivalent liberated from the sample was calculated using standard leucine plot. The difference in leucine equivalent was calculated as % inhibition.

2.5.7 Collagen Zymography

TA treated collagen was analyzed for collagenase resistance activity using collagen zymography. Zymogram gel and loading buffer (50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue, 10 mM dithiothreitol (DTT) were prepared for the collagenase zymogram. Eight percentage running gel containing 0.5 mg/mL type I collagen or TA treated collagen was used as substrate. Gels were

overlaid with 4% stacking gel. The samples (100 units of bacterial collagenase) were prepared in a non-reducing buffer, and the gel was run at 150 V until the dye front run off the gel at 4 ºC. The gel was removed from the glass plate, and washed with 2.5% triton X-100 in a shaker for 30 min at room temperature. The gel was briefly washed with collagenase buffer (50 mM tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.2% Brij-35, pH 7.5) for 48 h at 37 ºC. After rinsing with distilled water, gel was stained by commassive brilliant blue-G250 at room temperature on a rocker, followed by destaining. After completion of staining and destaining, gel was rinsed with distilled water and photographed by scanner.

2.6 ROLE OF CHIRALITY AT THE CLEAVAGE SITE OF COLLAGENASE IN COLLAGEN MIMICS

To identify the role of amino acid chirality on collagenase cleavage site in collagen, molecular dynamics and experimental studies were carried out using synthetic peptides to mimic collagen. CLPs were utilized to analyze the effect of D-amino acid on collagen triple helical structure and stability against thermal as well as enzymatic degradation.

2.6.1 Effect of D-Amino Acid on Collagen Mimics- Molecular Dynamics Approach

Host-guest peptide approach combined with MD simulation was used to analyze the effect of D-AA on structure and stability of collagen. In this study, the X_{AA} residue of the central triplet of the $(Ace\text{-}(G\text{-}P\text{-}O)_7\text{-}Nme)_3$ has been replaced with D-Pro, D-Ala, and D-Asp, which leads to the replacement of three L-AA residues in the entire triple helix.

2.6.2 Computational Details

Because of its tendency to form the most stable triple-helical conformation and highly occurring triplet in the collagen, $((G\text{-}P\text{-}O)_7)_3$ was chosen as the host peptide. Systematic experimental and theoretical studies on collagen-like peptides demonstrate that at least five triplets are essentially required to form a stable triple helix. Therefore, triple-helical models were built with a sequence having seven triplets per chain. The details of the various sequences considered in this investigation were shown in **Figure 2.2**. The host-guest peptides with D-AA as guests were built using GENCOLLAGEN, which is a tool, used to generate the three-dimensional coordinates of the triple helix for a given sequence. MD simulations were carried out using the AMBER 9.0 package, and the ff99SB force field was used for all the naturally occurring AAs (Hornak et al 2006). Hyp residues were treated using the force field developed by Park et al (2005). Despite QM studies showing the marginal difference in the energy of L-AA and D-AA, they can be handled with the same force field because other inaccuracies in the force field energy discretion will shadow this effect. All of the simulations were carried out in an implicit solvent environment using the generalized Born model (GB). All simulations were carried out in the NVT ensemble. Temperature of the system was maintained at 27 ºC using a Nose-Hoover thermostat. The nonbonded cutoff was fixed at 15 Å. The nonbonded atom pair list was updated for every 100 fs. The equilibration of the system was monitored by averaging the fluctuation in the potential energy, kinetic energy, and temperature over time. All the systems were well equilibrated within 100 ps. After equilibration, a production run was carried out for 10 ns. The trajectories were saved for every 0.2 ps interval for further analysis. The analyses of the trajectories were made using the PTRAJ package (Parrinello et al 1981; Nos´e et al 1983; Bussi et al 2007). To find the effect of D-AAs in

the collagen-like peptides, various parameters were obtained from the MD trajectories.

Host Model GPO	**Guest Model GpO**	**Host Model GAO**	**Guest Model GaO**	**Host Model GDO**	**Guest Model GdO**
G	G	G	G	G	G
P G	P G	P G	P G	P G	P G
O P G	O P G	O P G	O P G	O P G	O P G
G O P	G O P	G O P	G O P	G O P	G O P
P G O	P G O	P G O	P G O	P G O	P G O
O P G	O P G	O P G	O P G	O P G	O P G
G O P	G O P	G O P	G O P	G O P	G O P
P G O	P G O	P G O	P G O	P G O	P G O
O P G	O P G	O P G	O P G	O P G	O P G
G O P	G O P	G O P	G O P	G O P	G O P
P G O	**P** G O	**A** G O	**A** G O	**D** G O	**D** G O
O P G	O **P** G	O **A** G	O **A** G	O **D** G	O **D** G
G O P	G O **P**	G O **A**	G O **A**	G O **D**	G O **D**
P G O	P G O	P G O	P G O	P G O	P G O
O P G	O P G	O P G	O P G	O P G	O P G
G O P	G O P	G O P	G O P	G O P	G O P
P G O	P G O	P G O	P G O	P G O	P G O
O P G	O P G	O P G	O P G	O P G	O P G
G O P	G O P	G O P	G O P	G O P	G O P
P G O	P G O	P G O	P G O	P G O	P G O
O P G	O P G	O P G	O P G	O P G	O P G
O P	O P	O P	O P	O P	O P
O	O	O	O	O	O

Figure 2.2 Amino acid sequences of model collagen-like peptides (D-amino acids are represented in bold letters as well as highlighted in yellow color)

2.6.3 Analysis

2.6.3.1 Diameter of the triple helix

The possible winding and unwinding of the collagen super helix can be quantified from the diameter of the triple helix. The diameter of the circle encompassed by the three C^{α} atoms of all three chains was calculated as given in **eq. 2.4**. The triple-helix diameter (*D*) at the i^{th} position was calculated as a function of time as

$$D_i = 2\left(\frac{(a)(b)(c)}{\sqrt{(a+b+c)(b+c-a)(a+b-c)(c+a-b)}}\right) \quad (2.4)$$

Here *a*, *b*, and *c* are the distance between each C^{α} atom of each chain. The average diameter of the triple helix was calculated using the **eq. 2.5**:

$$\text{Diameter} = \text{average}(D_1, D_2, \ldots\ldots\ldots, D_i) \quad (2.5)$$

Here D_i represents the diameter of a circle encompassed by the i^{th} set of the three C^{α} atoms of the chains. The average diameter of the triple helix was calculated over the entire trajectory.

2.6.3.2 Radius of gyration

Radius of gyration is especially useful to measure the compactness of a structure. It was calculated from the trajectory by using **eq. 2.6**:

$$R_g = \left(\frac{\sum_i \|r_i\|^2 m_i}{\sum_i m_i}\right)^{1/2} \quad (2.6)$$

Here m_i is the mass of atom i, and r_i is the position of atom i with respect to the center of mass of the molecule. The radius of gyration was computed with all the C^{α} atoms present in the triple helix.

2.6.3.3 Calculation of information entropy of imino acids ring puckering

The pseudo-rotation angle of Pro and Hyp (five-member rings) was monitored as a function of time using the method developed by Westhof and Sundaralingam (1983). The puckering values were converted to entropy using Shannon information entropy and the following equation (**eq. 2.7**):

$$H(X) = E\big(I(X)\big) = -\sum_{i=1}^{n} p(x_i) \log_2 p(x_i) \qquad (2.7)$$

Here I (X) is the information content or self-information of X (puckering of imino acid), which is itself a random variable, and p (x_i) = P (X =x_i) is the probability mass function of X.

2.6.3.4 Cluster analysis

The trajectories were subjected to cluster analysis based on the RMSD between each structure. The structures were grouped into five sets in such a way that the difference in RMSD values from one group to another is at least 1.2 Å. The recently implemented clustering algorithm in AMBER package was used.

2.6.3.5 Relative destabilization energy

To estimate the changes in the energetics due to L→D conversion, the relative destabilization energy (RDE) was computed using **eq. 2.8**:

$$RDE = E_{D\ form} - E_{L\ form} \qquad (2.8)$$

Here E_{Lform} and E_{Dform} represent the total energy of the model peptides with only L-AA and D-AA substitution. The total energy of average structures

obtained from the MD simulation for the model peptides was used for the calculation. Rest of the analysis like RMSD, PCA, and H-bond analysis were carried out and mentioned above in the analysis of effect of isopeptide bond on CLPs.

2.6.4 Effect of D-Amino Acid (D-Leu) Substitution at the Cleavage Site of Collagenase in Tetrameric Peptide FALGPA

FALGPA is the well-studied peptide sequence for the collagenolysis by bacterial collagenase. The effect of D-amino acid (D-Leu) substitution on cleavage site of collagenase was studied using the tertrameric peptide, FALGPA.

2.6.4.1 Structural analysis - CD spectroscopy

Peptide solutions of concentrations 0.5 mg/mL were equilibrated at 4 $^{\circ}$C for more than 48 h prior to analysis, in 10 mM Tris buffer of pH 7.4. For CD data collection, 350 μL of each peptide was used in a 0.1 cm path length quartz cuvette. Wavelength was scanned between 190 to 350 nm, using 1.0 nm bandwidth, 0.1 nm step size, and an averaged time of 1 s. The raw data in millidegree units was converted to molar ellipticity [θ]. Structural analysis was carried using Yang's and Reed's references.

2.6.4.2 Thermal stability - CD spectroscopy

Thermal analysis was carried out by recording CD spectra with increase in temperature. For wavelength scans, the signals were collected from 190 to 350 nm at 1 nm intervals, at 10 to 65 °C by increasing temperature by 1 °C/min. For equilibrium melting temperature (T_m) transitions, the ellipticity at 202 and 192 nm was monitored while the sample temperature was increased from 10 to 65 °C at intervals of 0.5 °C for FA(L-

L)GPA and FA(D-L)GPA, respectively. The data was collected at each point. Temperature induced secondary structural changes were determined using Yang's and Reed's references (Chang et al 1978; Reed et al 1997).

2.6.4.3 Thermal stability – Differential scanning calorimetry

The temperature dependence of partial heat capacity was monitored via DSC. The instrument is equipped with reference and sample cells in which approximate volume of samples was weighed (25 µL of 2 mg/mL) in the sample cell and sealed in a Tzero pan with Tzero hermatic lid. The contents were heated from 10 to 70 ºC at a heating rate of 1 ºC/min. DSC generates data for heat capacity (C_p) as a function of temperature. The calorimetric enthalpy ΔH_{cal} was calculated from the area under the curve of the concentration-normalized DSC endotherm.

2.6.4.4 Enzymatic analysis

Peptide solutions of concentrations 1 mg/mL in appropriate amount of tricine buffer (0.05 M tricine, 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.4) was equilibrated at 4 $^{\circ}$C for more than 48 h prior to analysis. Peptide samples of concentrations 0.5 mg/mL and *Clostridium histolyticum* collagenase (100 µL of 1 mg/mL) were added, and the final volume was adjusted to 1 mL. The course of hydrolysis of FALGPA was monitored by measuring absorbance at 345 nm at 37 °C as a function of time. Peptide without enzyme was used as control and tricine buffer was used as a blank.

2.6.5 Effect of D-Amino Acid Substitution at the Cleavage Site of Collagenase on CLPs (Imino Poor Region)

To understand the effect of D-amino acid on collagenolysis, folding and stability, the synthetic short collagen like peptide with the imino

poor sequence was used for this investigation. The peptide models were constructed from the natural sequence of the collagenase cleavage site in type I collagen (Uniprot accession number P02452 residues from 936-972 residues).

Model-A: GSO GAD GPA GAO GTO GPQ GIA GQR GVV GLO GQR GER

Model-B: GSO GAD GP(D-A) GAO GTO GPQ GIA GQR GVV GLO GQR GER

Because earlier reports reveal that, collagenases unidirectionally act on collagen (C-terminal) in the under-twisted regions.

2.6.5.1 Preparation of peptide samples

Peptide samples of 1 mg/mL were prepared in aqueous solutions. The samples were heated at 60 °C for 5 min and then immediately stored at 4 °C for a minimum time period of 72 h to allow forming the triple helical conformation.

2.6.5.2 Secondary structural analysis- CD spectroscopy

CD spectra of peptide samples were recorded at 4 °C in 10 mM Tris buffer at pH 7 using 1 mm path length quartz. Wavelength was scanned between 260 to 190 nm at 4 °C using 1.0 nm band width, 0.1 nm step size, for an average time of 1s with an average of three sans at a scan speed of 100 nm/min. Data analysis was carried with JASCO software packages attached to the spectropolarimeter. For thermal unfolding measurements, ellipticity was monitored at 220 nm as a function of temperature from 4 to 70 °C to monitor thermal transitions. Effect of enzymes on collagen like peptides was determined by collagen like peptides (0.5 mg/mL) incubated at 37 °C for 2 h

with bacterial collagenase from *Clostridium histolyticum.* The wavelength selected for scanning the samples was from 260 to 190 nm.

2.6.5.3 Dynamic light scattering (DLS) measurements

DLS measurements were performed in 90-plus nanoparticle size analyser, Brookhavan instruments, with the laser wavelength of 660 nm at a 90° scattering angle and a temperature controller. Measurements were conducted at 6 °C using 1.5 mL cuvette. Prior to sample measurement, all the samples were incubated at 4 °C for 72 h and filtered through 0.2 μm pore size filters. The hydrodynamic diameter was obtained by analysing intensity autocorrelation function with 90-plus particle size software package attached to the Brookhavan instrument**.**

2.6.5.4 Transmission electron microscopy (TEM)

TEM images of peptides were obtained using Hitachi H-7650 at an acceleration voltage of 80 kV. 3 μL of peptide sample was placed on a carbon coated copper grid and allowed to dry at room temperature which was further stained with 2 μL of 1 w/v % phosphotungstic acid solution for 30 s. The excess staining solution was blotted using filter paper and TEM grids were dried at room temperature for two hours prior to imaging. Images were obtained from TEM at room temperature.

2.6.5.5 Enzyme kinetics- Circular dichroism spectroscopy

CD spectra was recorded over the range of λ= 250 to 190 nm to analyze the effect of bacterial collagenase collected from *Clostridium histolyticum* on CLP at pH 7, 37 °C for 2 h in 10 mM Tris buffer using 1 mm path length quartz, 1.0 nm band width, 0.1 nm step size, for an average time of 1 s. The CLP concentration was maintained at 0.5 mg/mL. An average of

three scans with a scan speed of 100 nm/min was taken. Data analysis was performed with CONTIN software packages (Johnson 1999; Sreerama et al 1994, 2000).

2.6.5.6 Enzyme kinetics- Reverse phase HPLC experiments

RP-HPLC experiments were performed using an Agilent 1100 semi preparative HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with auto sampler using an Zorbax 300 extend-C18RP column of 5 micron particle size (4.6X250 mm). The RP-HPLC experiments were carried out with a linear gradient flow of 0.1% TFA in water and 0.1% TFA in acetonitrile from 10 to 50% in 35 minute for collagen like peptide elution with a flow rate of 1 mL/min (Khew et al 2007). The samples were prepared with and without collagenase at 37 °C for 2 h incubation with peptide concentration of 0.5 mg/mL in Tris buffer in the presence of calcium ions. After 2 h of incubation, the samples were subjected to RP-HPLC with the same experimental conditions at 37 °C. The injection volume for the analysis of peptides was 50 µL of 0.5 mg/mL concentration of peptides. The chromatogram was observed for 35 min to know the change of elution time with respect to D-amino acid substitution. The diode array detection was set at 214 nm.

2.6.6 Effect of D-Amino Acid Substitution in CLPs (Imino Poor Region) on Hydrophilic and Hydrophobic Surfaces

2.6.6.1 Preparation of hydrophobic surfaces

Quartz slides (Erma, FRG) were cleaned using freshly prepared chromic acid followed by repeated washing with Millipore water (Milli-Q, USA) and stored in dessicator. The surfaces were further cleaned using Plasma Cleaner (Harrick Plasma, USA) for about 10 mins, to ensure complete

surface hydrophilicity. A slide from each batch was tested by measuring contact angle of water which was about 7°. For preparation of hydrophobic surface, the quartz surfaces were made hydrophobic using the Langmuir – Blodgett technique (LB). Chloroform solution of stearic acid was spread at the air/water interface and the solvent was allowed to evaporate for a period of about 20 mins. The quartz was withdrawn from the subphase which was maintained at a constant surface pressure π= 20 mN/m whereby one layer of stearic acid LB film was transferred. The transfer ratio was 0.85.

2.6.6.2 Sample preparation

Aqueous solutions of the peptides were prepared with the concentration of 1 mg/mL. The solutions were heated at 60 °C for 5 minute then immediately stored at 4 °C for a minimum period of 72 h to allow formation of triple helical conformation. 50 μL peptide solutions (1 mg/mL) were adsorbed onto hydrophilic and hydrophobic surfaces of the quartz slides to form a thin layer of peptide on the surfaces and slides were then allowed to dry at room temperature in a vacuum dessicator.

2.6.6.3 Circular dichroism (CD) measurements

CD spectra of the sample slides were scanned between 260 to 190 nm at 25 °C using 1.0 nm band width, 0.1 nm step size, for an average time of 1s with an average of three scans at a scan speed of 100 nm/min. Data analysis was performed with CONTIN software packages.

2.6.6.4 Fourier transformed infrared spectroscopy (ATR-FTIR)

50 μL peptide solution (0.5 mg/mL) was adsorbed onto low-e glass microscope slides and kept for drying in a vacuum dessicator. The dried film was investigated by reflectance infrared spectroscopy (ABB MB 3000

instruments, Pike Technologies, USA) using on an average 80 scans at a resolution of 4 cm^{-1}.

2.6.6.5 Interferometry measurements

To evaluate change in thickness on adsorption of the peptides on hydrophilic and hydrophobic surfaces, thickness was measured using Filmetrics F20 UV thin film analyzer. All values were an average of three measurements on the surface and with a minimum goodness of fit 0.97.

2.6.6.6 Linear dichroism (LD) measurements

To better understand the orientation of the collagen like peptides on hydrophilic and hydrophobic surfaces, LD measurements were recorded using Cary Bio 050 UV-Vis spectrophotometer. A prism polarizer placed in the path of the incident light is mounted on a graduated rotating base that could be dialed to generate plane-polarized light at various angles relative to the substrate of the sample. Polarized absorption spectra had been taken at angles of 0° and 90°. Order parameters from Linear Dichroic measurement of the peptides were used to analyze the orientation of the collagen like peptides upon L→D configurational changes. Here R is the ratio between absorbance for p and s-polarized light. LD is used to calculate the order parameter which is defined as (**eq. 2.9**)

$$\text{Order Parameter} = \frac{(R-1)}{(R+2)} \qquad (2.9)$$

Here $R=A_{0°}/A_{90°}$, $A_{0°}$ and $A_{90°}$ is the absorbance of peptide at an angle of 0° and 90°, respectively.

The polarized absorption spectra were corrected for scattering by using a simple algorithm that removed scattering so that the correction

satisfied two criteria. First, the scattering correction had to satisfy the simple formula $a+b\lambda^4$, where a and b are independent variables. We made further assumption that the scattering correction is identical (isotropic) for both polarization angles. This prevents the scattering correction from having an influence on the calculation of the transition dipole. The angle of orientation was calculated using the following equation (**eq. 2.10**).

$$\text{Order Parameter} = \frac{(1 - 3\text{Cos}^2\theta)}{2} \qquad (2.10)$$

2.6.6.7 Scanning electron microscopy (SEM)

Peptide films on hydrophilic and hydrophobic surfaces were sputtered with thin layer of gold (200 Å) and analyzed using Bio-SEM Hitacho S-3400 instrument.