Chapter 2

MATERIALS AND METHODS
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2.1. MATERIALS

2.1.1. Proteins
Concanavalin A, Succinyl Con A, Lipase from *Mucor miehei* and wheat germ, Stem bromelain, papain and chymopapain were purchased from Sigma Chemical Co., St. Louis, USA.

2.1.2. Reagents used in unfolding/refolding studies
2,2,2-trifluoroethanol (TFE), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), Ultra pure urea, guanidine hydrochloride were obtained from Sigma Chemical Co., St. Louis, USA. Poly(ethylene glycol)s of molecular weight 400, 6,000, and 20,000 were purchased from Sisco Research Laboratory, Mumbai, India.

2.1.3. Miscellaneous
Dialysis tubings of 1-inch width were obtained from Sigma Chemical Co, MO, and USA. Whatmann filter papers (No. 1) were product of Whatman International Ltd, Maidstone, England. Parafilm ‘M’ was obtained from American Can Company, CT, USA. Z-L-Lys-ONp hydrochloride (thiol protease substrate), BCA (Bicinchoninic acid) kit, 1-anilinonaphthalene-8-sulfonate (ANS), N-acetyl-L-tryptophanamide, 2-mercaptopethanol, iodoacetamide and glycine were obtained from Sigma Chemical Co., St. Louis, USA. Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, hydrochloric acid, sulphuric acid, sodium acetate, cysteine hydrochloride and acetic acid were obtained from Qualigens Fine Chemicals, Mumbai, India.

All glass-distilled water was used throughout these studies. All the experiments were performed at room temperature unless otherwise stated.
2.2. METHODS

2.2.1. pH measurements
pH measurements were carried out on an Elico digital pH meter, model LI 610 using a PPC's combined electrode, type CL-51 consisting of glass and reference electrodes in a single entity. The least count of the pH meter was 0.01 pH unit. The pH meter was routinely calibrated at room temperature with either 0.05 M potassium hydrogen phthalate buffer, pH 4.0 in the acidic range or 0.01M tetraborate buffer, pH 9.2 in the alkaline range.

2.2.2. Concentration determination
Protein concentration was determined from the value of specific extinction coefficient \( \varepsilon^{1\%}_{1cm} = 12.3 \) for Con A and Succinyl Con A, 25.0 for papain, 20.1 for bromelain and 18.2 for chymopapain) by measuring the absorbance of protein solutions at 280nm on a Hitachi spectrophotometer, model U-1500 or alternately by BCA kit.

2.2.3. Circular Dichroism (CD) measurements
CD measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulphonic acid. All the CD measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab's RTE – 110 water bath with an accuracy of ± 0.1°C. Spectra were collected with scan speed of 20 nm/min and response time of 1 sec. Each spectrum was the average of 3-4 scans. Far and near UV CD spectra were obtained with 1 mm and 10 mm path length cells respectively.
The results were expressed as MRE (Mean Residue Ellipticity) in deg. cm$^2$. dmol$^{-1}$ which is defined as

$$\text{MRE} = \frac{\theta_{\text{obs}}}{(10 \times n \times l \times C_p)}$$

where $\theta_{\text{obs}}$ is the CD in milli-degree, $n$ is the number of amino acid residues, $l$ is the path length of the cell and $C_p$ is mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al. [20]:

$$\% \alpha-\text{helix} = \left(\frac{\text{MRE}_{222\text{nm}} - 2340}{30300}\right) \times 100$$

2.2.4. Fluorescence Measurements

2.2.4.1. Intrinsic fluorescence: Fluorescence measurements were performed on Shimadzu spectrofluorimeter, model RF-540 equipped with a data recorder DR-3. The fluorescence spectra were measured at $25 \pm 0.1^\circ$ C with a 1 cm pathlength cell. The excitation and emission slits were set at 5 and 10 nm respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra was recorded in the range of 300–400 nm.

2.2.4.2. Extrinsic fluorescence: A stock solution of ANS was prepared in distilled water and its concentration was determined using an extinction coefficient of $\varepsilon_M = 5000 \text{ M}^{-1}\text{cm}^{-1}$ at 350 nm. For ANS fluorescence in the ANS binding experiments, the excitation was set at 380 nm and the emission spectra were taken in the range of 400-600 nm.

2.2.5. Acrylamide Quenching Experiments

Aliquots of 5 M acrylamide stock solution were added to the protein solution (0.1 mg/ml) to achieve the desired range of quencher concentration (0.1-0.5 M). To excite the tryptophan residues only, excitation wavelength was set at 295 nm and the emission spectra were recorded in the range of 300–400 nm. Quenching
experiment on NAT A, tryptophan analogue, was also done. Its concentration was determined using an extinction coefficient of $\varepsilon_M = 5690 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm. The data was analyzed according to Stern-Volmer equation [21]:

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

Where $F_0$ and $F$ are the fluorescence intensities at 340 nm in the absence and presence of quencher, respectively; $K_{sv}$ is the Stern-Volmer constant and $[Q]$ is the molar concentration of the quencher.

### 2.2.6. Unfolding studies on lipases

#### 2.2.6.1. pH-induced unfolding of lipase

*Mucor miehei* lipase experiments were carried out in 10 mM of the following buffers: pH 1-0-2-8 Glycine HCl buffer, pH 3-0-5-0 Sodium acetate buffer, pH 6-0-7-0 Sodium phosphate buffer. 6 M GdnHCl solution was prepared in 10 mM sodium phosphate buffer, pH 7-0.

Experiments on wheat germ lipase were carried out in 20 mM of the following buffers: pH 0-6-1-6 KCl-HCl buffer, pH 1-8-3-0 Glycine HCl buffer, pH 3-5-5-0 Sodium acetate buffer, pH 6-0-8-0 Sodium phosphate buffer, pH 9-0-12-0 Glycine NaOH buffer.

Lipase was incubated for 12 hours at desired pH before spectroscopic measurements were recorded.

#### 2.2.6.2. Lipase activity

Enzyme assays were done at room temperature in 50 mM tris-HCl buffer (pH 7-0) using p-nitrophenyl butyrate as the substrate. A fresh stock of substrate (50 mM) was prepared in acetonitrile and was used at concentration of 1 mM in 1 ml of reaction mixture. Upon hydrolysis by lipase, p-nitrophenyl butyrate is converted to yellow colored compound that can be read at 405 nm in UV-visible spectrophotometer.
Protein was incubated at respective pH overnight before activity measurement. The concentration was kept at 0.6 mg/ml in assay mixture. Background hydrolyses of the substrate i.e. in the absence of enzyme, were measured and used for correcting the hydrolysis rates obtained with enzymes. Absorbance at 405 nm was measured over a period of 2 minutes and enzyme activity was reported as difference in the observed absorbance value (in arbitrary units).

2.2.6.3. Guanidine hydrochloride induced denaturation
Equilibrium denaturation of wheat germ lipase in presence of GdnHCl (in 20 mM Sodium-phosphate buffer of pH 7.0 and KCl-HCl buffer of pH 0.8) was studied by far UV-CD and intrinsic fluorescence. Stock solution of 8 M GdnHCl was prepared in respective buffer. Increasing amounts of stock GdnHCl solution was added to fixed concentration of protein (0.2 mg/ml for far UV-CD and 0.1 mg/ml for intrinsic fluorescence measurements) so as to get the desired concentrations of GdnHCl in the range 0-6 M. The protein in different concentrations of GdnHCl was allowed to equilibrate for 24 hours. To check the reversibility of denaturation, protein was incubated in GdnHCl for 12 hours, followed by dilution with respective buffer upto desired concentration of GdnHCl. After incubation of 12 hours, samples were read by far UV-CD and fluorescence.

Data analysis: Data were expressed in terms of fraction unfolded ($F_u$) calculated from equation:

$$F_u = [(Y - Y_n) / (Y_d - Y_n)]$$

Where $Y$ is observed variable parameter and $Y_n$ and $Y_d$ are the values of variables characteristic of folded and unfolded conformations.

2.2.6.4. Thermal denaturation transition
For thermal-transition studies, a water-jacketed 1mm pathlength cell was used for far-UV CD attached to the RTE-110 waterbath interfaced with a microcomputer. A protein concentration of 0.2 mg/ml was used. The protein solution was
incubated for 5 minutes at desired temperature (in the range 25-90 °C) before CD measurements. The reversibility of thermal scans was monitored by the return of CD spectrum after cooling from 90°C to 25°C. The data was plotted in terms of Fraction denatured (F_d) as described above.

2.2.7. Stability studies on Thiol Proteases

2.2.7.1. Assay for Protease Activity
Enzyme assays were done at room temperature in 10mM Sodium acetate buffer (pH 4·5), containing 1 mM EDTA and 5 mM Cysteine-HCl, using Z-L-Lys-ONp hydrochloride as the substrate. A freshly made stock solution (5 mM) of the substrate in DMSO was added to 3 ml buffer so that final concentration of the substrate was 80 μM in the reaction mix. Protein concentration of 30 μg/ml was used for the activity assay. Protein concentration was determined spectrophotometrically using E^\text{1%}_{\text{em}} of 25·0 for papain, 20·1 for bromelain and 18·2 for chymopapain.
Upon hydrolysis by proteases, Z-L-Lys-ONp hydrochloride is converted to yellow colored compound that can be read at 326 nm in UV-visible spectrophotometer. Background hydrolyses of the substrate i.e. in the absence of enzymes, were measured and used for correcting the hydrolysis rates obtained with enzymes. Enzyme solutions (enzyme + 10% w/v PEG- 20000, 6000 and 400) were incubated at different temperatures for 5 minutes before activity measurements were done. Protease activity was measured as rate of change in absorbance of the reaction mix at 326 nm.

2.2.7.2. Incubation of papain in PEGs
To study the effect of PEGs on the conformation of papain at pH 4·5, different volumes of concentrated PEGs (% w/v) were added to the protein solution taken in
different volumes of the buffer (in 10 mM Sodium acetate buffer, pH 4-5), so as to get the desired concentration of the PEGs. The final volume in each tube was 1-0 ml. All the spectroscopic measurements were made after half an hour of incubation at room temperature.

2.2.7.3. Thermal Denaturation studies
For thermal-transition studies, a water-jacketed 1mm pathlength cell was used for far-UV CD attached to the RTE-110 waterbath interfaced with a microcomputer. Protein concentration of 0·5 mg/ml was used. The protein solution was incubated for 3 minutes at desired temperature before CD measurements. The data was plotted in terms of Fraction denatured ($F_d$) as described above.

2.2.8. Unfolding studies on Succinyl Con A

2.2.8.1. TFE and HFIP induced denaturation of Con A and Succinyl Con A
To study the effect of fluoroalcohols on the concentration of Succinyl Con A in 10 mM sodium-acetate buffer at pH 5·0, different volumes of concentrated alcohols (% v/v) were added to the protein solution taken in different volumes of the buffer so as to get the desired concentration of the alcohols. The final volume in each tube was 1-0 ml. All the spectroscopic measurements were made after 12 hours of incubation at room temperature.

2.2.8.2. Aggregation studies
To measure the level of aggregation in protein samples (at different fluoroalcohol concentration) turbidity at 350 nm was monitored, either by spectrophotometer or by fluorimeter by exciting the samples at 350 nm. No absorbing species are present at this wavelength, so that scattering from the aggregating protein particles attenuates the light beam. To measure the dependence of temperature-induced
aggregation of Con A and Succinyl Con A on protein concentration and pH, the protein was heated for 5 min in a water bath at various temperatures (25-96°C) and Optical Density (OD) at 350 nm was monitored.

2.2.8.3. Urea and GdnHCl induced unfolding and refolding of Succinyl Con A
To study the effect of denaturants on Succinyl Con A (in 10 mM sodium acetate buffer, pH 5.0), different volumes of stock GdnHCl solution (8 M) or Urea (10 M) were added to the protein solution taken in different volumes of the buffer so as to get the desired concentration of the denaturants. The final volume in each tube was 1.0 ml. All the spectroscopic measurements (CD and fluorescence) were made after 24 hours of incubation at room temperature. Data was plotted in terms of fraction native ($F_n$), related to $F_d$ by the following relation:

$$F_n = 1 - F_d$$

For refolding studies, protein was incubated with increasing concentration of GdnHCl and urea for 12 hours and was then diluted with pH 5.0 buffer upto required denaturant concentration. CD and fluorescence measurements were performed on the samples after 12 hours of incubation.