Results

&

Discussion
Chapter 1

Purification and characterization of goat pancreatic thiol proteinase inhibitor
3.1 RESULTS

3.1.1 PURIFICATION OF THE INHIBITOR

In the present work, pancreatic thiol proteinase inhibitor (PTPI) has been purified from goat pancreas by the method of Priyadarshini and Bano [2009]. As detailed in the methods section, the procedure involved four steps after homogenisation, alkaline treatment (pH 11.0), acetone fractionation, ammonium sulphate precipitation (20-80%) and gel filtration chromatography on Sephacryl S 100 HR column. The progress of typical purification is summarized in Table 6. The initial homogenate contained free cathepsins, so presumably the inhibitors were entirely complexed. The alkali treatment destroyed the lysosomal cysteine proteinases liberating the inhibitors in assayable form. A large amount of inactive proteins was precipitated during the readjustment to pH 7.5, and could be removed by centrifugation. Fractionation of the soluble material with acetone decreased the total amount of protein providing a fold purification of 18.84 and a percent yield of 58.8.

3.1.2 GEL FILTRATION

The protein precipitate obtained after ammonium sulphate fractionation was dissolved in minimum amount of 50 mM sodium phosphate buffer (pH 7.5) and was dialyzed against several changes of the same buffer (also containing 0.15 M NaCl). The dialyzed protein was filtered on Whatman paper and chromatographed on Sephacryl S-100HR column (60x1.7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. A single peak giving significant papain inhibition was obtained [Fig. 12]. The fractions corresponding to the peak were pooled and were used for further analyses. The procedure provided a fold purification of 498.84 (~500) and percent yield of 20.4.

3.1.3 HOMOGENEITY OF THE PURIFIED INHIBITOR

As observed in Fig.12, the inhibitor eluted as a single symmetric peak with constant specific activity suggesting a homogenous preparation. In addition, the preparation did not inhibit bovine trypsin, chymotrypsin or pepsin. Physical evidence for homogeneity was further provided by gel electrophoresis under non-denaturing conditions. The electrophoretic pattern of PTPI is shown in Fig. 13, lane e. The inhibitor moved as a single band.
<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total Activity (Units)</th>
<th>Specific Activity (Units/mg protein)</th>
<th>Fold Purification</th>
<th>Percent Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>200</td>
<td>8000</td>
<td>106</td>
<td>0.013</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Alkaline treatment</td>
<td>100</td>
<td>2899</td>
<td>76</td>
<td>0.026</td>
<td>2</td>
<td>71.6</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>13</td>
<td>254.05</td>
<td>62.39</td>
<td>0.245</td>
<td></td>
<td>18.84</td>
</tr>
<tr>
<td>Ammonium sulphate cut (20-80%)</td>
<td>10</td>
<td>147.7</td>
<td>54.8</td>
<td>0.371</td>
<td></td>
<td>28.53</td>
</tr>
<tr>
<td>Sepharcl S-100 HR</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.6</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.4</td>
</tr>
</tbody>
</table>

*Protein concentration was determined by the method of Lowry et al. [1951].

*One unit of enzyme inhibitory activity is defined as the amount of inhibitor bringing about 0.001 change in O.D. / ml min.
Fig. 12  Gel filtration chromatography on Sephacryl S-100 HR

The precipitate obtained from 20 to 80% ammonium sulphate saturation (after acetone fractionation) was dissolved and dialyzed against several changes of 50 mM sodium phosphate buffer, pH 7.5 containing 0.15 M NaCl. The sample was applied on Sephacryl S-100 HR column (60 x 1.7 cm) and fractions were eluted with the same buffer at a flow rate of 15 ml h⁻¹. Fractions of 5 ml were collected and monitored by inhibition of caseinolytic activity of papain. Fractions 7, 8, 9 were pooled for further studies.
Fig. 13  Gel electrophoresis of PTPI during various stages of purification

Electrophoresis was performed on 7.5% acrylamide gel as described in methods section. Lane a contained 60 μg pancreas homogenate, lane b contained 60 μg homogenate after alkaline treatment, lane c is 60 μg fraction obtained after acetone treatment, lane d is 60 μg dialyzed fraction after ammonium sulphate fractionation, lane e is 60 μg pancreatic thiol proteinase inhibitor after Sephacryl S-100 gel filtration.
3.1.4 REDUCING AND NON-REDUCING PAGE

Purified PTPI was analyzed by SDS-PAGE under non-reducing (in the absence of β-mercaptoethanol) or reducing conditions (in the presence of β-mercaptoethanol). PTPI migrated as two bands with different mobilities, suggesting a double subunit structure with subunits held together by non-covalent forces [Fig. 14].

3.1.5 PROPERTIES OF THE PURIFIED PANCREATIC THIOL PROTEINASE INHIBITOR

Molecular weight determination

The molecular weight of pancreatic thiol proteinase inhibitor was determined under native as well as denaturing conditions. The molecular weight of native PTPI was determined using gel filtration chromatography on Sephacryl S 100 HR. The marker proteins—Trypsin (23 kDa), Pepsin (35 kDa), Ovalbumin (43 kDa) and BSA (66 kDa) were chromatographed on the Sephacryl S 100 HR column (60 x 1.7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5 and their elution volume was determined. Analysis of the data indicated linear relationship between Ve/Vo and log M by the method of Andrews [1964] [Fig. 15], where Ve is the elution volume of the protein and Vo is the void volume of the column. The Ve/Vo of the native cystatin corresponds to molecular weight of 43650 (~44000) [Fig. 15].

The molecular weight of PTPI under denaturing conditions was calculated from its mobility in SDS-PAGE [Fig. 16a] by the procedure of Weber and Osborn [1969]. The mobilities of marker proteins were plotted against the logarithm of their molecular weights [Fig. 16b]. The least square analysis of the data indicated a linear relationship between log M and relative mobility (Rm). The molecular weight obtained was 43940 (~44000) (positions of two subunits corresponded to 23980 and 19960, without reduction). In the presence of βME the molecular weight was found to be 47194 (positions of two subunits corresponded to 25860 and 21334) [Fig. 16b].

Stokes radius

Stokes radius of a protein correlates well with its elution behaviour from gel filtration column. The stokes radius of PTPI was determined by its elution volume from a calibrated Sephacryl S 100 HR column (60 x 1.7 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5 using marker proteins. The column was
Fig. 14  **SDS Polyacrylamide gel electrophoresis of purified PTPI**

Electrophoresis was performed on 12.5% gels as described in methods section. SDS PAGE was performed under non reducing and reducing conditions, lane a: non reducing condition (in the absence of β-mercaptoethanol), lane b: reducing conditions (in the presence of β-mercaptoethanol). Lanes a and b each contained 40μg of the inhibitor, respectively.

Fig. 15  **Molecular weight estimation of purified PTPI using Sephacryl S-100 HR gel filtration chromatography**

Purified PTPI was applied on a column of Sephacryl S-100 HR (60 x 1.7 cm) and eluted with 50 mM sodium phosphate buffer, pH 7.5 at a flow rate of 15 ml h⁻¹. The molecular weight markers used were, A trypsin (23 kDa); B pepsin (35 kDa); C ovalbumin (45 kDa); D bovine serum albumin (66 kDa). Arrow shows the position of PTPI elution.
**Fig. 16**  
**Molecular weight determination of PTPI by SDS-PAGE electrophoresis**

**(A)**  
Electrophoresis was performed on 12.5% polyacrylamide gel. The middle gel lane contained the molecular mass standards: A, phosphorylase b (97.4 kDa); B, bovine serum albumin (68 kDa); C, ovalbumin (45 kDa); D, carbonic anhydrase (29.1 kDa); E, soyabean trypsin inhibitor (20 kDa); F, lysozyme (14.3 kDa). Lane a contained 40 μg PTPI without β-mercaptoethanol and lane b contained 40 μg of β-mercaptoethanol treated purified inhibitor.

**(B)**  
Plot of log M vs relative mobility (Rm) using least square analysis. The line indicates the positions of Subunit I and Subunit II of PTPI.
2.4- 2 - 1.6- H 1.2 - 0.8 - 0.4 - 0

Subunit I

0 0.2 0.4 0.6 0.8 1

Relative mobility (Rm)

(B)

Log M

0 0.4 0.8 1.2 1.6 2 2.4

Relative mobility (Rm)

Subunit I
Subunit II
calibrated by determining the elution volume of several globular proteins with known stokes radii, such as Trypsin (20.2 Å), BSA (35.6 Å), ovalbumin (27.3 Å). The data was analyzed according to the equation,

\[ K_{av} = \frac{V_e - V_o}{V_t - V_o} \]

Where, \( V_e = \) elution volume, \( V_o = \) void volume and \( V_t = \) total volume. \( K_{av} \) is the partition coefficient. The linear plot between known stokes radius and \([-\log K_{av}]^{1/2}\) of the marker proteins was used for the calculation of PTPI's stokes radius [Laurent and Killander, 1964]. As depicted in Fig. 17, the value was found to be 27.3 Å for the purified inhibitor.

**Diffusion coefficient**

Diffusion coefficient (D) of PTPI was found to be \(7.87 \times 10^{-7}\) cm\(^2\) s\(^{-1}\), computed from the value of its stokes radius by using the equation,

\[ D = \frac{kT}{6\pi\eta r} \]

Where \( k = 1.38 \times 10^{-16}\) erg/deg is Boltzmann's constant, \( T \) is the absolute temperature and \( \eta \) is the coefficient of viscosity of the medium (0.01 g/cm-sec for water and dilute aqueous salt solutions at 20°C). The sedimentation coefficient is given by the formula

\[ S = \frac{M (1-\nu_2 \rho)}{N_o f} = \frac{M (1-\nu_2 \rho)}{N_o 6\pi \eta r} \]

Where \( M \) is the mass of the protein molecule in Da, \( N_o \) is the Avogadro’s number, \( 6.023 \times 10^{23} \), \( \nu_2 \) is the partial specific volume of the protein (0.73 g/cm\(^3\)), and \( \rho \) is the density of the solvent (1.0 g/cm\(^3\) for water). The ratio of \( S_{max}/S \) can be used to interpret the shape of the protein. In the hydrodynamic parameter \( S_{max}/S \), \( S_{max} \) is the maximum possible sedimentation coefficient for a protein of the given mass, corresponding to a sphere of the minimum diameter, to contain mass of protein, with no water of hydration. The ratio of \( S_{max}/S \) is the same as \( f/f_{min} \), where \( f \) is the actual frictional coefficient of the hydrated protein and \( f_{min} \) is the frictional coefficient of unhydrated minimal sphere [Tanford, 1961]. The sedimentation coefficient of PTPI was found to be 3.83 s. The \( S_{max}/S \) ratio of PTPI is 1.2.
Marker proteins and the purified cystatin were subjected to gel filtration on Sephacryl S-100 HR (60 x 1.7 cm). The $K_{av}$ values were computed from the elution volume of marker proteins. Stokes radii of the marker proteins were: A trypsin (20.2 Å); B ovalbumin (27.3 Å); C bovine serum albumin (35.6 Å). Arrow shows the stokes radius of purified inhibitor. The experimental conditions were same as in Fig. 1.
Carbohydrate content

Type 1 and type 2 cystatins, generally, lack carbohydrate content. Dissimilar to this PTPI was found to possess 2.32% carbohydrate content.

Sulphydryl group content

The sulphhydryl groups in PTPI were titrated against DTNB. Colourless solution obtained indicated that no free sulphhydryl groups are present in the purified inhibitor.

Effect of pH on activity of PTPI

Effect of pH on the thiol proteinase inhibitory activity of pancreatic thiol proteinase inhibitor was examined at various pH values. Fig. 18 shows that the inhibitor is stable in the pH range 3.0-10.0 and has maximum activity at pH 7.5.

Effect of temperature on activity of PTPI

Stability of PTPI was investigated as a function of temperature between 30 and 90°C in 50 mM sodium phosphate buffer pH 7.5, by means of inhibitory activity assay. PTPI remained maximally active within temperature range of 30-70°C [Fig. 19].

Temperature stability of PTPI

Goat PTPI was exposed to 90°C for varying time intervals, rapidly cooled and inhibitory activity determined by method of Kunitz [1947]. As illustrated in Fig. 20, PTPI retained approximately 40% of its activity till 120 min. The inhibitor was thus stable for 120 min at 90°C.

3.1.6 IMMUNOLOGICAL PROPERTIES

Antibody titre

The PTPI caused good immune response and the resulting antiserum had a titre of 25,118.86 as determined by direct binding ELISA in rabbit serum [Fig. 21].

Cross-reactivity

PTPI was immunogenic and induced antibody formation in rabbits. The antiserum raised against purified inhibitor showed cross reactivity with the inhibitor (indicated by single precipitin line on immunodiffusion plate) exhibiting immunogenic purity
Fig. 18  Effect of pH on activity of PTPI

50 μg of the inhibitor was incubated in 50 mM sodium acetate buffer, pH 3.0-6.0, sodium phosphate buffer, pH 7.0-8.0, tris-HCl buffer, pH 9.0, for 30 min at 37°C. After the incubation the pH of the mixture was neutralized and then 50 μg of activated papain was added and the mixture was further incubated for 60 min at 37°C. The following procedure was same as described in methods section for assaying the inhibitor using casein as substrate.

Fig. 19  Effect of temperature on PTPI

50 μg of the inhibitor was incubated in 50 mM sodium phosphate buffer, pH 7.5, at various temperatures for 30 min and then rapidly cooled. 50 μg of activated papain was added and kept for 60 min at 37°C. The remaining procedure for determining thiol proteinase inhibitory activity was same as described in methods section using casein as substrate.
Fig. 20 Thermal stability of PTPI

50 μg of the inhibitor was incubated in 50 mM sodium phosphate buffer, pH 7.5, at 90°C for different time intervals, rapidly cooled. 50 μg of activated papain was added and incubated for 60 min at 37°C. Rest of the procedure was same as described in methods.

Fig. 21 Direct binding ELISA

Serially diluted antiserum and pre-immune serum were incubated with 0.5 μg/100 μl antigen. The procedure has been described in methods. The curve with hollow circles is for post-immunized sera, whereas the curve with solid circles is for pre-immunized sera.
and homogeneity of the inhibitor preparation [Fig. 22]. It exhibited no immunogenic identity with goat lung and brain cystatins isolated in our laboratory.

3.1.7 KINETIC PROPERTIES OF PTPI

Stoichiometry of Inhibition

The inhibition of proteinases was studied by varying their molar concentration at a fixed molar concentration of PTPI. The remaining activity of proteinase showed that as its concentration is increased from 0.01-0.06 μM it is progressively inhibited by 0.06 μM PTPI giving a stoichiometric ratio of 1:1, thus one molecule of PTPI inhibits one molecule of active proteinase. Same result was obtained for ficin and bromelain.

Inhibition of different proteinases

The inhibitory activity of PTPI towards thiol proteinases, papain, ficin and bromelain and serine proteinases, trypsin and chymotrypsin was examined using casein as substrate. PTPI inactivated papain and ficin very efficiently and bromelain to a slightly lesser extent. The order of inhibition was papain > ficin > bromelain. However it failed to inhibit bovine trypsin and chymotrypsin [Fig. 23].

Ki determination

Dissociation equilibrium constants (measured as Ki), for the binding of PTPI to plant cysteine proteinases papain, ficin and bromelain, were determined by monitoring the loss of enzyme activity and after lowering the respective inhibitor and proteinase concentration, which favour the dissociation of the complex. Ki values were determined using the steady state equation derived by Krupka and Laidler [1959],

\[
\frac{[I]_0}{1 - (V_t/V_o)} = \frac{1 + [S]_e}{K_m} \left( \frac{V_t}{V_o} \right) + [E]_0
\]

The increasing values of Ki (app) with an increase in the substrate concentration suggested a competitive mechanism of inhibition. The true Ki values were obtained from the replot of Ki (app) versus substrate concentration. The Ki values obtained for papain, ficin and bromelain are 5.88, 9.02 and 22.28 nM, respectively, implying the highest affinity of inhibitor for papain [Fig. 24-26: inset].
Fig. 22  **Ouchterlony immunodiffusion**

Anti-PTPI antiserum was raised in rabbits. For the immunodiffusion study, the antiserum was allowed to react with inhibitor (60 µg) on agarose plates as described in methods section. The central well contained the antiserum, whereas the surrounding three well contained purified PTPI.

Fig. 23  **Inhibitory activity of PTPI with different proteinases**

50 µg of thiol proteinases papain, ficin, bromelain and serine proteinases, trypsin and chymotrypsin were incubated with varying concentrations of PTPI (0-30 µg) for 30 min. The inhibitory activity of PTPI towards these proteinases was measured by using 2% casein as substrate.
Fig. 24  **Determination of inhibition constant with (Ki) papain**  

Papain was used at final concentration of 0.06 µM with increasing amounts of PTPI (0.06-0.24 µM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2 Km and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate give the true Ki.

Fig. 25  **Determination of inhibition constant (Ki) with ficin**  

Ficin was used at final concentration of 0.06 µM with increasing amounts of PTPI (0.06-0.24 µM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2 Km and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate give the true Ki.
Fig. 26  **Determination of inhibition constant (Ki) with bromelain**

Bromelain was used at final concentration of 0.06 μM with increasing amounts of PTPI (0.06-0.24 μM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2 Km and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate give the true Ki.
IC50 value

IC50 value is the concentration of the inhibitor at which 50% of the enzyme is inhibited. The IC50 values obtained with various thiol proteinases are summarized in table 7. The values obtained for the three proteinases, papain, ficin and bromelain, are 0.08, 0.078 and 0.154 μM, respectively, again suggesting greater affinity of the inhibitor for papaya proteinase.

Dissociation rate constant (K_{-1})

The conditions for the dissociation were taken such that the enzyme-inhibitor complex obeys first order kinetics during the initial part of the reaction. In this case the integrated form of the equation is given by,

\[\ln \left( \frac{[EI]}{[EI]_0} \right) = K_{-1} t\]

\[\log \left( \frac{[EI]}{[EI]_0} \right) = K_{-1} t / 2.303\]

Figures 27, 28 and 29 show the respective plots for papain, ficin and bromelain. The K_{-1} values obtained for papain, ficin and bromelain are 8.76 x 10^{-5}, 3.35 x 10^{-4} and 1.32 x 10^{-4} s^{-1}, respectively.

Association rate constant (K_{+1})

Association rate constants calculated from measured dissociation rate and dissociation equilibrium constants by the relation,

\[K_{+1} = \frac{K_{-1}}{K_i}\]

and hence the affinity of the inhibitor for proteinases is in the following order: papain (1.49 x 10^{4} M^{-1} s^{-1}) > ficin (1.39 x 10^{4} M^{-1} s^{-1}) > bromelain (5.94 x 10^{3} M^{-1} s^{-1}).

Half life of the complex

The half life values of enzyme-inhibitor complexes were calculated using K_{-1} values by the equation,

\[t_{1/2} = \frac{0.693}{K_{-1}}\]

The calculated half-life values of papain-PTPI complex was 7.91 x 10^{3} s, for
### TABLE 7: KINETIC CONSTANTS OBTAINED ON INTERACTION OF PTPI WITH PROTEINASES – PAPAIN, FICIN AND BROMELAIN

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Papain</th>
<th>Ficin</th>
<th>Bromelain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki (nM)</strong></td>
<td>5.88±0.06</td>
<td>9.02±0.08</td>
<td>22.28±0.11</td>
</tr>
<tr>
<td><strong>K⁺� (M⁻¹ s⁻¹)</strong></td>
<td>1.49±0.03x10⁴</td>
<td>1.39±0.02x10⁴</td>
<td>5.94±0.02x10³</td>
</tr>
<tr>
<td><strong>K⁻� (s⁻¹)</strong></td>
<td>8.76±0.01x10⁻⁵</td>
<td>3.35±0.02x10⁻⁴</td>
<td>1.32±0.02x10⁻⁴</td>
</tr>
<tr>
<td><strong>Half life value (s)</strong></td>
<td>7.91x10³</td>
<td>5.51x10³</td>
<td>5.23x10³</td>
</tr>
<tr>
<td><strong>IC50 (µM)</strong></td>
<td>0.08</td>
<td>0.078</td>
<td>0.154</td>
</tr>
</tbody>
</table>

Results represent the mean ± SEM calculated from three independent experiments.
Fig. 27  Determination of dissociation rate constant ($K_{-1}$) with papain
Papain-PTPI complex (1μM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.

Fig. 28  Determination of dissociation rate constant ($K_{-1}$) with ficin
Ficin-PTPI complex (1μM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.
Fig. 29  Determination of dissociation rate constant ($K_d$) with bromelain

Bromelain-PTPI complex (1μM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.
ficin-PTPI complex was $5.51 \times 10^3$ s and $5.23 \times 10^3$ s for bromelain-PTPI complex.

3.1.8 N-TERMINAL ANALYSIS

The N-terminal 24 amino acid residues of the heavier subunit (23.98 kDa) of PTPI were sequenced by the automated Edman degradation method. Table 8 shows the amino-terminal sequence of goat pancreatic thiol proteinase inhibitor and its sequence homology with other known mammalian cystatins. As in other cystatins, PTPI was found to possess a conserved glycine residue at 11th position. Maximum sequence homology was observed with bovine skin cystatin C as compared to other cystatins like bovine parotid cystatin C, bovine colostrum cystatin C, human cystatin C, human stefin A & B, human placental cystatin and cystatin E.

HYDROPATHY PLOT

Using the sequence obtained from N-terminal analysis of PTPI, a hydropathy plot was made using the respective hydropathy indices [Kyte and Doolittle, 1982]. Among 24 residues sequenced, the stretch of 3–7, 7–11 and 15–19 residues has maximum hydropathy index suggesting that these residues might be present inside the hydrophobic core of the protein [Fig. 30].

3.1.9 SPECTRAL ANALYSES

Absorption spectrum

PTPI gave typical protein absorption with a maximum at 278 nm and a minimum at 250 nm. The ratio of the absorbance at 280/260 nm is 1.3 [Layne, 1957]. The millimolar absorption coefficient at 280 nm of PTPI is found to be 22.1 mM$^{-1}$cm$^{-1}$ by taking molecular weight value of 44,000. The interaction of inhibitor and papain at its stoichiometric ratio was studied at pH 7.5. Absorption difference spectrum of papain-PTPI complex showed peaks in the region of 245-255 nm, at 280 nm, negative peaks at around 290 nm and a shoulder at 260 nm [Fig. 31].

Fluorescence emission spectrum

After excitation at 280 nm, where phenol and indole groups absorb, the PTPI exhibits an emission spectrum with a maximum ($\lambda_{max}$) at $335 \pm 1$ nm which points to the non-polar tryptophyl side chains in the protein [Burstein et al., 1973].
TABLE 8: N-TERMINAL AMINO ACID SEQUENCE OF PTPI: A COMPARISON

<table>
<thead>
<tr>
<th></th>
<th>Goat PTPI</th>
<th>Human placental cystatin (8.33%)</th>
<th>Human cystatin E (8.33%)</th>
<th>Bovine parotid cystatin C (12.5%)</th>
<th>Human cystatin C (12.5%)</th>
<th>Bovine skin cystatin- C (20%)</th>
<th>Bovine colostrum cystatin C (11.8%)</th>
<th>Human stefin B (5.8%)</th>
<th>Human stefin A (5.8%)</th>
<th>Human α2 kininogen (14.3%)</th>
<th>Bovine α2 kininogen (7.1%)</th>
<th>Sheep plasma LMW K1 (10%)</th>
</tr>
</thead>
</table>

The highly purified cystatin was transferred to PVDF membrane by Western blotting before doing amino acid analysis. Percent homology with other known cystatin N-terminal sequences is indicated in parenthesis.
Fig. 30  Hydropathy plot of the N-terminal residues of heavy subunit of PTPI

The hydropathy calculation was made according to the method of Kyte and Doolittle [1982] using already known hydropathy index of the 24 amino acid residues. A window size of 4 was selected for plot formation. The upper part of the graph shows the hydrophobic, and lower part hydrophilic regions.
The binding of PTPI to papain was accompanied by appreciable changes in fluorescence emission [Fig. 32]. There was a shift of fluorescence maximum to longer wavelength (from 335 to 345 nm) with considerable enhancement (+21.62%) of fluorescence intensity at \( \lambda_{\text{max}} \).

**Circular dichroism spectra of native PTPI and its complex with papain**

The far-UV CD spectra of PTPI revealed \( \alpha \)-helical structure of 17.18% [Fig. 33] [calculated using equation described by Chen et al., 1972]. Complexation of PTPI with papain resulted in complete loss of negative peaks and a strong positive peak is observed [Fig. 34]. Complex formation with papain abolished the native secondary structure and resulted in shifting of absorption maximum.
Fig. 31 UV-Absorption difference spectra measured for PTPI-papain complex

PTPI (2.66 μM) was incubated with activated papain for 30 min and an absorbance difference spectrum was calculated between 240 nm to 320 nm. PTPI and papain were in a molar ratio of 1:1.

Fig. 32 Fluorescence spectra of PTPI alone and PTPI in complex with papain

Fluorescence spectra of the inhibitor alone PTPI, papain and papain-PTPI complex was measured at excitation wavelength ($\lambda_{ex}$) of 280 nm and emission wavelength ($\lambda_{em}$) of 300-400 nm. The concentration of PTPI was 2 μM. The fluorescence of complex of PTPI with papain was measured at a molar ratio of 1:1. The slit width was 5 nm for excitation and 10 nm for emission beams.
Fig. 33  **Far UV-CD spectra of native PTPI**

The concentration of PTPI was 1.7 μM. Cells of 1 mm path length were used. The buffer used was 50 mM sodium phosphate buffer, pH 7.5. Cells of 1 mm path length were used. The unit on the ordinate is mean residue ellipticity.

Fig. 34  **Far UV-CD Spectra of PTPI in complex with papain**

Far UV-CD spectra of native PTPI alone and papain-PTPI complex. The concentration of PTPI was 1.73 μM and PTPI and papain molar ratio was 1:1. The complex was obtained after incubation at 37°C for 30 minutes in 50 mM sodium phosphate buffer, pH 7.5. Cells of 1 mm path length were used. The unit on the ordinate is mean residue ellipticity.
Discussion
Chapter 1
DISCUSSION

Most of the evidences indicate that the proteolytic activity of both endogenous and exogenous cysteine proteinases is primarily regulated by a group of proteinase inhibitors belonging to the cystatin superfamily. These cysteine proteinase inhibitors (cystatins) are ubiquitous in organisms, ranging from bacteria to mammals. Cystatins are classified into three distinct families based on their sequence homology, presence of disulphide bonds and molecular mass [Abrahamson et al., 2003]. These inhibitors are of physiological importance because inhibition is achieved at physiological concentration of the inhibitor in a sufficiently short time with negligible dissociation of the complex [Beith, 1980]. TPIs (cystatins) are known to play important function in various pathophysiologic conditions [Turk et al., 2008; Shah and Bano, 2009] implying that cystatin functioning is pivotal for proper health maintenance.

Cystatins have been purified and characterized extensively from various mammalian sources like goat lung [Khan and Bano, 2009a], brain [Sumbul and Bano, 2006], human placenta [Rashid, et al. 2006a], goat kidney [Zehra et al., 2005], goat sheep plasma [Baba et al., 2005], human liver [Green et al., 1984] and human spleen [Jarvinen and Rinnie, 1982]. The presence of a thiol proteinase inhibitor in pancreas has been shown earlier [Ni et al., 1997] however; its isolation and physico-chemical characterization remained unattempted. Role of cathepsins in mediating exocrine and endocrine functions of pancreas has already been established (e.g. cathepsin B-conversion of trypsinogen to trypsin [Otto & Riesenkonig, 1975; Teich et al., 2002; Thrower et al., 2006], involvement of cathepsin B and H in the formation of insulin from proinsulin [Ansorge et al., 1977]). Recent literature implicates the role of calpains in insulin secretion and action [Sreenan et al., 2001; Kalbe et al., 2005]. Altered cysteine proteinase activity in pancreas is often correlated to deleterious consequences like onset or enhanced severity of pancreatitis [van Acker et al., 2002; Shikimi et al., 1987], diabetes [Weber et al., 2002; Sreenan et al., 2001] and tumors [Lorenzo et al., 2000]. However information about the regulation of cysteine proteinase activity by their proteinaceous inhibitors in pancreas is scarce.

In the present work, a thiol proteinase inhibitor (PTPI) was purified from goat pancreas by the method of Priyadarshini and Bano [2009]. The four step procedure involved alkaline treatment, acetone fractionation, ammonium sulphate fractionation and gel filtration chromatography. The procedure used provided a percent yield of 99
20.4 and fold purification of 500 [Table 6]. Purification of TPIs from other sources has been reported using affinity chromatography, chromatofocusing, gel filtration and ion exchange chromatography [Anastasi et al., 1983; Evans and Barrett, 1987; Khan and Bano, 2009a]. The purified inhibitor was found to be homogenous on the basis of charge and molecular weight as shown by native PAGE [Fig. 13]. In SDS-PAGE, both under reducing as well as non reducing conditions, PTPI gave two bands suggestive of presence two subunits [Fig. 14] held together by non covalent bonds.

The molecular weight of the isolated inhibitor ascertained from gel filtration chromatography on Sephacryl S 100 HR column [Fig. 15] was 43650 (~44000). Quite similar result was obtained in denaturing PAGE [Fig. 16b]. A slightly higher molecular weight obtained under reducing conditions (47194 compared to 43940 under non reducing conditions) is however, anomalous. The estimation of molecular weight on dodecyl sulphate gels is based on the hypothesis that all proteins bind fairly equal amount of detergents and that the complexes formed by protein and detergent adopt the same shape and conformation such that the electrophoretic mobility is function of the molecular weight and pore size of the gel. If a protein does not show such behaviour the molecular weight determination by this method becomes erroneous. Known examples of such proteins include polypeptide with unusual charge [Tung and Knight, 1971; Panyim and Chalkev, 1971], conformation [Bjork et al., 1972] or with unreduced disulphide bonds [Trayer et al., 1971] and glycoproteins [Mitchell et al., 1973]. The ambiguity of this method in case of PTPI could be that it deviates from the general behaviour of proteins in dodecyl sulphate solution. The SDS-PAGE was repeated thrice and same result was obtained.

Cystatins type 1 and type 2 have been classified on the basis of molecular weight and presence of disulphide bonds [Abrahamson et al., 2003]. Cystatins from tissues are usually small single subunit proteins with low molecular masses. Molecular masses of 11.4 kDa and 12 kDa have been reported for cysteine proteinase inhibitors isolated from human spleen [Jarvinen and Rinnie, 1982], 14 kDa-14.3 kDa for cystatins from bovine muscle [Zabari et al., 1993]. The molecular mass of PTPI is higher than those of stefins and cystatins (~11 kDa and 13 kDa, respectively) but lower than those of kininogens (~50 kDa-100 kDa) [Turk and Bode, 1991]. Low molecular mass of tissue cystatins is now contrasted with reports on high molecular mass. Ylonen et al. [1999] isolated and purified high molecular mass thiol proteinase inhibitors (of 43 kDa and ~52 kDa) from the skin of Atlantic salmon. Cystatins isolated from goat kidney were
reported to have a molecular mass of 67 kDa [Zehra et al., 2005]. A high molecular mass cystatin (of 70.8 kDa) has also been isolated from goat brain [Sumbul and Bano, 2006]. Recently, the work from our lab, has reported the purification of high molecular mass cystatins from goat lung [Khan and Bano, 2009a].

The purified inhibitor was characterized for its various hydrodynamic properties. Stokes radius of PTPI as deduced from its gel filtration behaviour was 27.3 Å. Diffusion coefficient of PTPI was found to be $7.87 \times 10^{-7}$ cm$^2$ s$^{-1}$. The sedimentation coefficient of PTPI was calculated to be 3.83 s. The values of stokes radius and $S_{max}/S$ ratio can be used to predict the shape of the protein molecule [Schurmann et al., 2001]. Globular proteins typically have $S_{max}/S$ ratio of 1.2-1.3 (for example catalase and serum albumin have $S_{max}/S$ of 1.20 and 1.29), and the ratio increases to 1.6-2.0 or more for elongated proteins [Erickson, 1982]. For PTPI $S_{max}/S$ ratio was calculated to be 1.2. The values of stokes radius and $S_{max}/S$ ratio for PTPI are in close agreement with those of ovalbumin, suggesting that PTPI is a globular in shape.

Generally, cystatins type 1 and type 2 isolated from tissues lack carbohydrates [Bode et al., 1988] whereas the presence of carbohydrates is a distinguishing property of type 3 cystatins, the kininogens [Ohkubo et al., 1984]. However, presence of carbohydrate has been reported in some tissue thiol proteinase inhibitors like cystatins E, F, M and those isolated from goat kidney and brain [Ni et al., 1997; 1998; Sotiropoulou et al., 1997; Zehra et al., 2005; Sumbul and Bano, 2006]. PTPI contains 2.32% carbohydrates and has no thiol group. Analysis of the influence of pH on activity of PTPI reveals that the inhibitor remains fairly active in the pH range of 3.0-10.0 [Fig. 18]. PTPI also exhibited stability in a wide temperature range of 30-70°C [Fig. 19] and remained active upto 120 min when heated to 90°C [Fig. 20]. High stability of the purified inhibitor in broad temperature and pH ranges is consistently found in other cystatins like goat lung cystatins [Khan and Bano, 2009a], goat brain cystatins [Sumbul and Bano, 2006], human placental cystatin [Rashid et al., 2006a], goat kidney cystatins [Zehra et al., 2005], stefin A and B [Zerovnik et al., 1997], etc.

The PTPI or pancreatic cystatin gave a good immune response with an antibody titre of 25,118.86 as determined by direct binding ELISA in rabbit serum [Fig. 21]. The antibodies raised against purified inhibitor gave a reaction of identity with the inhibitor as indicated by a single precipitin line on immunodiffusion suggesting that the isolated PTPI is pure. Experiments also showed that the antiserum had no immunogenic identity with goat brain and lung cystatins purified in our lab. This
indicates that the epitopes of goat pancreatic thiol proteinase inhibitor are different from goat brain and lung thiol proteinase inhibitors. The purified inhibitor was found to be specific for cysteine proteinases since no activity against aspartic (pepsin) and serine proteinases (trypsin and chymotrypsin) was detected. This has been reported for many other cystatins along with the thiol proteinase inhibitor isoforms purified from human spleen [Jarvinen and Rinnie, 1982], and other goat cystatins [Khan and Bano, 2009a; Sumbul and Bano, 2006; Zehra et al., 2005]. The stoichiometry of binding of purified cystatin to papain, ficin and bromelain was 1:1. This value shows that PTPI is a tight binding inhibitor of these proteinases and essentially all enzyme molecules are able to bind to the inhibitor. Anastasi et al. [1983] have also reported equimolar complexes of cystatin with cysteine proteinase. Abrahamson et al. [1987b] also reported the rapid formation of 1:1 complex between cystatin C and papain. There are other reports also demonstrating similar binding of thiol proteinase inhibitors with papain like that of the recombinant human cystatin C [Bjork et al., 1994], recombinant bovine cystatin C [Olsson et al., 1999], recombinant stefin A [Nicklin and Barrett, 1984]. Members of cystatin superfamily also show different binding stoichiometries with papain. The high molecular weight kininogens from human and sheep plasma as well as low molecular weight kininogens from the latter show 1:2 stoichiometry of interaction with papain [Baba et al., 2005; Turk et al., 1996b].

The IC50 values of the PTPI obtained for papain, ficin and bromelain were 0.08, 0.078 and 0.154 μM, respectively [Table 7]. Lower IC50 value suggests a greater affinity of the inhibitor towards the enzyme. The values obtained for PTPI indicate its greater affinity towards papain than for ficin and bromelain. Katunuma and Kominami [1985] have found IC50 value for the inhibitor isolated from rat liver as 0.16 μg for papain, 0.46 μg for ficin, 4.2 μg and 0.14 μg for cathepsin B and H, respectively.

Accurate Ki values were determined by working at lower enzyme concentrations and using equations derived by Krupka and Laidler [1959] and Henderson [1972]. Ki values were calculated from the slope of the curve obtained for the inhibition of caseinolytic activity of papain, ficin and bromelain. PTPI is a strong inhibitor of thiol proteinases as indicated by their Ki values. The data shows that PTPI inhibited papain, ficin and bromelain with Ki values of 5.88, 9.02 and 22.28 nM, respectively, under conditions of routine assay system [Table 7, Fig. 24-26]. Thus, of the enzymes studied,
PTPI binds most tightly to papain. These values are in good comparison with other thiol proteinase inhibitors. Sumbul and Bano [2006] obtained Ki values of $1.87 \times 10^{-8}$ M and $3.125 \times 10^{-8}$ M, for inhibition of papain by goat brain cystatins. Quite similar values were obtained for goat kidney cystatins [Zehra et al., 2005]. Human placental cystatin also gave a Ki value of $5.5 \times 10^{-8}$ M for papain as reported by Rashid et al. [2006a]. Ki values of nanomolar range have been documented for cathepsin B, H and L with cystatin A [Barrett et al., 1984], cystatin C [Machleidt et al., 1986], cystatin D [Balbin et al., 1994]. Studies of the kinetics of binding of chicken cystatin to several cysteine proteinases have shown that these reactions are best described by the simple reversible bimolecular mechanism:

\[
P + I \overset{k_{+1}}{\underset{k_{-1}}{\rightleftharpoons}} PI
\]

Where P is the proteinase, I represents inhibitor and PI their complex [Björk and Ylinenjärvi, 1990; Henderson, 1972]. The same conclusion is strongly indicated by studies with PTPI in this work. The linear increase in the observed pseudo-first order rate constant for cysteine proteinases studied with PTPI concentration is consistent with a simple reversible bimolecular reaction mechanism [Nicklin and Barrett, 1984]. Moreover, the increasing value of Ki with an increase in substrate concentration suggests the inhibition to be competitive as reported earlier by Li et al., [2000] and by Nicklin and Barrett [1984] for inhibition of human cathepsin B by chicken cystatin. Latter obtained Ki (app) values of 1.85 and 3.68 nM with the substrate concentration of 0.05 and 0.39 mM, respectively. Possibly, all cystatins of family II, and perhaps also those of other families, interact with the target enzymes in same general manner.

Association constant obtained for papain-PTPI interaction was $1.49 \times 10^4$ M$^{-1}$s$^{-1}$. In general, proteinases having low Ki also have high $K_{+1}$ and low $K_{-1}$ values suggesting the stability of the enzyme inhibitor complex and rapidity of its formation. Our data is in accordance with this, thereby suggesting that the interaction of PTPI with papain is rapid and stable. The association rate constants obtained for ficin and bromelain were $1.39 \times 10^4$ M$^{-1}$s$^{-1}$ and $5.94 \times 10^3$ M$^{-1}$s$^{-1}$, respectively. Thus, reiterating the order of affinity as, papain > ficin > bromelain. The Ki and rate constants for association of the inhibitor with papain and ficin are also comparable with the values measured for chicken cystatin [Björk et al., 1989], human cystatin C and bovine cystatin C [Björk et al., 1994] and cystatin D [Balbin et al., 1994] for their interactions with various cysteine proteinases (papain, ficin and cathepsins B, H and L).
The dissociation constant \((K_{i})\) values for the enzyme-inhibitor complex was determined by displacement procedure, in which the inhibitor released from the complex was trapped by excess substrate (casein) with increase in time. The amount of enzyme released from the complex was monitored by continuous measurement of enzyme activity. The respective \(K_{i}\) values obtained for papain, ficin and bromelain are \(8.76 \times 10^{-5}\), \(3.35 \times 10^{-4}\) and \(1.32 \times 10^{-4}\) s\(^{-1}\), respectively [Fig. 27-29, Table 7]. The values of \(K_{i}\) obtained in the present work are comparable to \(K_{i}\) values obtained for chicken cystatin, \(5 \times 10^{-5}\) s\(^{-1}\) with papain [Nicklin and Barrett, 1984]. The published data on rate constants for other goat cystatins [Khan and Bano, 2009a; Sumbul and Bano, 2006; Zehra et al., 2005] is in similitude with our results. This resemblance in inhibitory mechanism of cystatins isolated from goat earlier reflects the species similarities. The above data gives comprehensive information about the kinetics of inhibition of purified thiol proteinase inhibitor with papain, ficin and bromelain and the overall comparison showed that PTPI inhibits papain more effectively compared to other two proteinases.

The N-terminal 24 amino acid residues of the heavier subunit (23.98 kDa) were sequenced, and some interesting results were obtained. As in other cystatins [Table 8], PTPI possesses a conserved glycine residue at 11\(^{th}\) position rather than the conserved position 9 in various species [Brzin et al., 1984]. Maximum sequence homology was observed with bovine skin cystatin C (20%) [Cimerman et al., 1996]. Fair sequence homology of PTPI was also observed with bovine parotid cystatin C (12.5%), bovine colostrum cystatin C (11.8%), and human cystatin C (12.5%) [Cimerman et al., 1996], sheep plasma LMW K1 [Baba et al., 2005], placental cystatin [Rashid et al., 2006a] and cystatin E [Ni et al., 1997]. Using sequence of these N-terminal amino acid residues, a hydropathy plot was made using the respective hydropathy indices [Kyte and Doolittle, 1982]. Among 24 residues sequenced, the stretch of 3-7, 7-11 and 15-19 residues has maximum hydropathy index suggesting that these residues might be present inside the hydrophobic core of the protein [Fig. 30].

The absence of disulphide bonds is a unique character of type 1 cystatin family members. Molecular masses of type 1 and 2 cystatin families range in 11 kDa to 13 kDa [Turk and Bode, 1991]. Presence of carbohydrates is however limited only to kininogens, with few exceptions found for members of type 2 cystatin family. PTPI is devoid of any disulphide linkage, has high molecular mass (44 kDa) and bears sequence similarities to both stefins and cystatins [Table 8]. Studies on kinetics of
inhibition as well as on biophysical interaction of PTPI with thiol proteinases shows that it bears resemblances with other members of type 2 cystatin family. Based on this the purified inhibitor can be regarded as a variant of type 1 and type 2 cystatin families. This is not unusual as there is growing list of proteins possessing some structural and functional motifs of cystatin superfamily, but bearing substantial differences causing them to be classified as variants of cystatin superfamily.

The interaction between papain and PTPI was studied using various spectroscopic techniques. The complex spectroscopic changes (observed in UV-difference spectrum of PTPI-papain complex) [Fig. 31] accompanying the binding of PTPI with papain indicate that the environment of several aromatic residues in the proteins has been perturbed upon interaction. Peak at 280 nm is indicative of changes around tyrosine residues [Donovan, 1969; 1973a; 1973b]. The shoulder around 260 nm may be partly due to phenylalanine and may also contain contribution from aromatic residues [Donovan, 1973a]. The changes at 290 nm suggest that aromatic amino acid residues are involved in binding with papain, like tryptophan. Such results are consistent with earlier reported results for interaction of low and high molecular weight kininogens, human placental cystatin, goat brain cystatin and rat cystatin with papain [Baba et al., 2005; Rashid et al., 2004; Sumbul & Bano, 2006; Takeda et al., 1983].

Fluorescence studies showed that complexation of PTPI with papain resulted in changes in intensity and shape of the emission spectrum. The maxima of cystatin shifted from 335 nm to 345 nm for the PTPI-papain complex which was accompanied by an increase in the fluorescence intensity. The largest increase of intensity was observed between 330-345 nm [Fig. 32] indicating that these changes arise predominantly from perturbations around tryptophan residues, either by exposure of tryptophan residues to the solvent or may originate from local interactions affecting chromophoric groups of the two proteins.

A CD spectrum in the far UV region depicts the contributions of the secondary structure of the protein [Jirgensons, 1970]. The α-helical structure of the protein in the far UV region is characterized by negative peaks at 208-210 nm, at 222 nm and a positive peak between 190-192 nm [Jirgensons, 1970; Chen et al., 1972]. PTPI has an α-helical content of 17.18%. The α helical content was calculated from the ellipticity values at 222 nm using equation given by Chen et al. [1972]. The CD spectra of PTPI (α helical content 17.18%) resembled that of cystatin A (with a low α helical content ~15%) [Stubbs et al., 1990; Pol et al., 1995]. This type of structure has also been
reported for chicken cystatin which has an α helical content of about 20% [Schwabe et al., 1984].

The goat pancreatic cystatin loses its native structure on formation of complex with papain [Fig. 34]. Far UV-CD spectra of the inhibitor-papain complex showed intense positive peak and complete loss of any negative peaks, characteristic of random coil structure [Ramasarma et al., 1994]. However, earlier studies for the interaction of chicken cystatin, human cystatin C and cystatin A with several proteinases reported no appreciable conformational adaptation of either protein [Pol et al., 1995; Lindahl et al., 1992; Takeda et al., 1983]. The positive ellipticity of PTPI-papain complex observed in the present case is in similitude with reports of Rashid et al., [2004] and Baba et al., [2005]. This feature of PTPI-papain interaction is comparable to that of serine proteinase inhibitors with target enzymes involving conformational change [Luthy et al., 1973; Quast et al., 1974; Olson and Shore, 1982].

The results indicate that the UV absorption, fluorescence emission and far UV CD changes are more due to conformational changes in proteins rather than any local interaction affecting the chromophoric groups of the two constituent proteins of the complex. The positive ellipticity observed for PI (proteinase-inhibitor) complex in far UV CD region further confirms that cystatin and papain both lose their native structures on formation of complex. The conformation of this complex resembles neither of the constituent proteins, rather indicates attainment of random coil structure due to this interaction. The kinetic studies also suggest formation of tight complex on interaction of inhibitor with papain.

PTPI is a good partner of other reported goat tissue cystatins in terms of its physical properties. Also, the pH and heat stability, interaction with papain, affinities towards other proteinases and N-terminal sequence analysis of the purified PTPI are quite similar to other cystatins, but differences in terms of its molecular mass, subunit structure, sulphydryl groups, and carbohydrate content from other tissue cystatins imply different routes of biosynthesis, different in vivo distribution and suggest a variety of physiological functions. It is interesting to speculate on the physiological role of this endogenous proteinase inhibitor. It seems likely that this endogenous inhibitor would at least serve a protective function against inappropriate proteolysis both with in the cell and outside the cell.
Chapter 2

Denaturing action of guanidine hydrochloride and urea towards pancreatic thiol proteinase inhibitor
3.2 RESULTS

The possibility of measuring the conformational stability of proteins with precision is important to resolve the protein folding problem, to define the structural characteristics of the proteins for studying their structure and function through mutational analysis and to applied research.

With this view, a systematic investigation on the effect of increasing concentration of guanidine hydrochloride (GdnHCl) and urea on the functional and structural parameters of pancreatic thiol proteinase inhibitor (PTPI) was performed using the following methods:

(1) Enzyme catalytic activity, to indicate the disruption of active site regions.
(2) Red shift of wavelength of the maximum fluorescence emission ($\lambda_{\text{max}}$), to monitor global structural changes induced by the denaturant.
(3) Ellipticity at 222 nm in the CD spectrum, to detect the secondary structural changes induced by GdnHCl and urea.
(4) ANS- binding to detect the appearance of hydrophobic patches in the enzyme molecule during unfolding.

Time dependent changes in the structural parameters and enzymatic activity of PTPI at increasing GdnHCl and urea concentrations were monitored to standardize the incubation time required for achieving equilibrium under these conditions. Under all of the conditions studied, the changes occurred within maximum of 2 h with no further alteration up to 12 h (data not shown). These observations demonstrated that an incubation time of 2 h is sufficient for achieving equilibrium under any condition of denaturant studied.

3.2.1 CHANGES IN MOLECULAR PROPERTIES OF PTPI ASSOCIATED WITH GdnHCl INDUCED UNFOLDING

Effect of GdnHCl on papain inhibitory activity of PTPI

Enzyme activity can be regarded as the most sensitive probe for studying protein unfolding and refolding as it reflects subtle readjustments at the active site, allowing very small conformational variations of an enzyme structure to be detected. Fig. 35 shows the effect of increasing concentration of GdnHCl on the papain inhibitory activity of PTPI. The inhibitor was incubated with increasing GdnHCl concentration
Fig. 35  Effect of increasing GdnHCl concentration on the activity of PTPI

Native PTPI (1 μM) was incubated with increasing concentration of GdnHCl (0-6 M) for 2 h at room temperature. PTPI was assayed for loss of antiproteinase activity by caseinolytic assay of Kunitz [1947]. Values are Mean ± SEM of four independent determinations.
in 50 mM sodium phosphate buffer, pH 7.5 and its thiol proteinase inhibitory activity was monitored by the method of Kunitz [1947]. The activity of native PTPI was taken as 100. A sigmoidal dependence of enzymatic activity on GdnHCl concentration was observed. No significant effect of the denaturant on enzymatic activity of native PTPI was observed upto 1 M GdnHCl. Approximately, 20% loss of the papain inhibitory activity of PTPI occurred at 2 M GdnHCl. At 4 M GdnHCl concentration only 35% of the native enzyme activity was left. PTPI was found to be almost completely inactivated beyond 4 M GdnHCl.

**Effect on spectroscopic properties of PTPI in the presence of GdnHCl**

Optical spectroscopic studies on PTPI in the presence of increasing GdnHCl concentrations were performed to study the effect of denaturant on its structural properties.

**Fluorescence spectra of PTPI in the presence of GdnHCl**

The spectral parameters of fluorescence emission spectra such as position, shape and intensity are dependent on the electronic and dynamic properties of the chromophore environment; hence steady-state fluorescence has been extensively used to obtain information on the structural and dynamic properties of protein [Prajapati et al., 1998]. Fluorescence measurements were performed by using a 280 nm excitation wavelength in order to detect the contribution of both tryptophan and tyrosine residues. This choice is advantageous in having fluorescence signals that reflect the global conformational changes of the tertiary structure, rather than local modifications.

The modification of the microenvironment of aromatic residues of PTPI due to denaturant has been monitored by studying changes in the intensity and wavelength of emission maxima ($\lambda_{\text{max}}$) as a function of denaturant concentration. Fig. 36 illustrates changes in fluorescence emission intensity and $\lambda_{\text{max}}$ (inset) of PTPI with increasing GdnHCl concentration (0-6 M). The wavelength of maximum emission ($\lambda_{\text{max}}$) is a robust signal for measuring the unfolding of proteins. The fluorescence emission spectrum of native PTPI shows a maximum of 335 nm that shifts to 350 nm in 6 M GdnHCl [Fig. 36 inset]. Prominent but a gradual red shift was observable only at concentrations beyond 3 M GdnHCl. There was little or no effect on the fluorescence intensity till 1 M GdnHCl. 2-4 M GdnHCl caused an increase in the emission intensity. Further, increment of denaturant concentration to 5 and 6 M, quenched the
Fig. 36  Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of GdnHCl

The concentration of PTPI was 1 µM. PTPI was preincubated for 2 h at 25°C in 50 mM sodium phosphate buffer (pH 7.5) containing the increasing concentration of GdnHCl (0-6 M). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slitwidth of 5 nm. The inset shows changes in wavelength of maximum emission with increasing concentration of GdnHCl.
fluorescence intensity below native. This effect has also been documented for GdnHCl mediated alteration in fluorescence emission of other multimeric proteins like alcohol dehydrogenase [Sacchetta et al., 2001], glutamate dehydrogenase [Ruiz et al., 2003], β-nerve growth factor [Timm & Neet, 1992]. It is well known that fluorescence spectra of proteins with maxima around 335 nm are characteristic of tryptophan residues well buried in the hydrophobic core, whereas fluorescence spectra with maximum around 350 nm are characteristic of tryptophan residues exposed to the aqueous solvent [Lakowicz, 1983]. Thus, treatment of PTPI with higher concentrations of GdnHCl results in exposure of the buried tryptophan moieties present in native inhibitor to the solvent. Also, decrease in emission intensity at high denaturant concentration may result from quenching of the tryptophan fluorescence by aqueous solvent. Such a situation can happen only when the denaturant induces unfolding of PTPI.

Secondary structure of PTPI in GdnHCl solutions

Far UV-CD studies on GdnHCl induced unfolding of PTPI were carried out to study the effect of GdnHCl on the secondary structure of the inhibitor. The CD observed below 230 nm is due to the peptide amide chromophore [Johnson, 1985] and can be used to estimate the content of secondary structure. In the far UV region, the CD spectrum of native PTPI shows the presence of fair amount of α-helical conformation [Priyadarshini and Bano, 2009]. It was found to possess 17.2% of α-helical content determined by the method of Chen et al. [1972]. Fig. 37 summarizes the effect of GdnHCl on secondary structure of PTPI. Upto 1 M GdnHCl, in similitude to activity and fluorescence results, minute effects are seen on mean residue ellipticity (MRE) at 222 nm. Beyond 4 M GdnHCl a complete loss of signal was observed. These observations suggest that treatment of PTPI with higher GdnHCl concentration results in complete unfolding of the inhibitor.

Midpoint of denaturation transition (Cm) in the presence of GdnHCl

The midpoint of chemical denaturation transition of proteins, abbreviated as Cm, can be defined as the concentration of the denaturant, at which 50% of the transition is complete, that is where \( f_d = f_n \), fraction of denatured protein molecules is equal to fraction of native species.

Transition curves were constructed from various parameters determined in GdnHCl
Fig. 37  Secondary structure analysis of PTPI in the presence of GdnHCl

The figure shows changes in far UV CD spectra of PTPI denatured in increasing concentrations of GdnHCl (0-6 M). The conditions were same as for figure 3 except that the concentration of PTPI was 1.73 μM. The inset shows changes in MRE at 222 nm with increasing concentration of GdnHCl.
denaturation of PTPI. The results are depicted in Fig. 38. Cm determined from the equilibrium transition curve derived from activity data [Fig. 38a] was 3.2 M GdnHCl. Cm is a function of protein concentration in dimer coupled systems [Timm & Neet, 1992]. To analyze if the dissociation of dimeric PTPI and inactivation are coupled denaturation curves were constructed over a range in PTPI concentration (0.1, 1 and 10 μM). A concentration dependent shift in the midpoint of transition was observed, consistent with two-state model of denaturation [Fig. 38a]. Fig. 38b shows the normalized transition curve of changes in λ_{max} of PTPI as a function of GdnHCl concentration. The midpoint of transition (Cm) was again determined to be 3.2 M. Cm deduced from the normalized transition curve of MRE_{222 nm} [Fig. 38c] was also 3.2 M. Changes in the molecular properties of PTPI such as enzymatic activity, MRE_{222 nm}, λ_{max} with increasing GdnHCl concentration reveal a monophasic sigmoidal dependence, with coincidental profiles [Fig. 38d], characteristic of cooperative unfolding, suggesting a two state model for denaturation of the inhibitor.

### 3.2.2 CHANGES IN MOLECULAR PROPERTIES OF PTPI ASSOCIATED WITH UREA INDUCED UNFOLDING

Although urea and GdnHCl are believed to have similar modes of action [Nandi & Robinson, 1984], GdnHCl is a monovalent salt that has both ionic and chaotropic effects [Mayr and Schmid, 1993; Makhatadze et al., 1998; Myers et al., 1995], whereas urea has only chaotropic effects. Thus, urea is an ideal control agent for distinguishing between the ionic and chaotropic effects of GdnHCl.

Urea induced changes in structural and functional properties of PTPI were studied by changes in papain inhibitory activity, fluorescence emission intensity and λ_{max} of emission and MRE_{222 nm} with increasing urea concentration.

**Effect of urea on papain inhibitory activity of PTPI**

PTPI was incubated with increasing concentration of urea in 50 mM sodium phosphate buffer (pH 7.5). Its thiol proteinase inhibitory activity was determined at each concentration of the denaturant using casein as substrate, by the method of Kunitz [1947]. Activity of native PTPI was taken to be 100. Fig. 39 shows that urea concentration lower than 1 M did not substantially affect the activity. 20% of activity was lost at 2 M urea concentration. At 4 M urea concentration 40% loss of
Changes in functional and structural properties of PTPI in the presence of GdnHCl

The figure shows normalized transition curves for GdnHCl-induced unfolding of PTPI as obtained from:

(a) enzymatic activity
(b) wavelength of maximum emission
(c) MRE at 222 nm

(d) shows the coincidence of transition curves, (a), (b) and (c).

Panel (a) also shows the protein concentration dependence of PTPI denaturation. Inhibitory activity of PTPI was monitored at different concentrations, 0.1 μM -○-, 1 μM -□-, 10 μM -△- of the native protein each incubated with increasing concentrations of GdnHCl. Fraction of unfolded protein was calculated at each concentration as explained in methods section.
Fig. 39  **Effect of increasing urea concentration on activity of PTPI**

Native PTPI (1 μM) was incubated with increasing concentration of urea (0-9 M) for 2 h at room temperature. PTPI was assayed for loss of antiprotease activity by caseinolytic assay of Kunitz [1947]. Values are Mean ± SEM of four independent determinations.
activity was obtained and only 18% of native PTPI papain inhibitory activity was observed at 6 M urea. Beyond this concentration the inhibitor was barely active.

**Effect of urea on spectroscopic properties of PTPI**

**Fluorescence spectra of PTPI in the presence of urea**

Intrinsic fluorescence emission spectra of native and unfolded proteins in the presence of increasing urea concentration are shown in Fig. 40. An increase in fluorescence emission of native PTPI without any shift in emission $\lambda_{\text{max}}$ was observed till 2 M urea. Fluorescence emission enhancement was again noticed at 6 M urea, preceded by a plateau (at 4 and 5 M urea). A sharp increase in emission intensity was observed beyond 8 M urea with a marked red shift of $\lambda_{\text{max}}$ to 350 nm which indicates that treatment of PTPI with high concentrations of urea leads to unfolding of the inhibitor. The maximum value of $\lambda_{\text{max}}$ obtained for both denaturants was same.

**Secondary structure of PTPI in urea solutions**

Far UV-CD spectroscopy was used to monitor changes in secondary structure of PTPI upon urea induced unfolding. Incubation of PTPI in urea solutions results both in changes the shape of CD spectra and in urea-concentration dependent loss in ellipticity [Fig. 41]. Upon denaturation the negative CD below 230 nm diminishes significantly, consistent with the loss of ordered secondary structure that should accompany protein unfolding. At urea concentrations above 5 M, there is complete loss of negative peaks. A sigmoidal dependence of decrease in MRE$_{222}$ nm with increasing urea concentrations was observed with no change in the value observed for the native inhibitor upto 1 M urea.

**Midpoint of denaturation transition (Cm) in presence of urea**

As in GdnHCl-denaturation, Cm (the concentration at which 50% of the protein molecules exist in the unfolded state) was determined for urea denaturation also from the data derived on various parameters studied. Fig. 42a depicts the normalized transition curve for the loss of enzymatic activity in response to urea. The Cm value deduced from the curve was 3.6 M. No dependence of Cm on protein concentration was detected. It may be that inactivation and dimer dissociation in case of urea denaturation of PTPI are not coupled or as has been explained in the case of
Fig. 40  Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of urea

The concentration of PTPI was 1 μM. PTPI was preincubated for 2 h at 25°C in 50 mM sodium phosphate buffer (pH 7.5) containing the increasing concentration of urea (0-9 M). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slit width of 5 nm. The inset shows changes in wavelength of maximum emission with increasing concentration of urea.
Fig. 41  Secondary structure analysis of PTPI in the presence of urea

The figure shows changes in far UV CD spectra of PTPI denatured in increasing concentrations of urea (0-9 M). The conditions were same as for figure 3 except that the concentration of PTPI was 1.73 μM. The inset shows changes in MRE at 222 nm with increasing concentration of urea.
Fig. 42  Changes in functional and structural properties of PTPI in presence of urea

The figure shows normalized transition curves for urea induced unfolding of PTPI as obtained from
(a) enzymatic activity
(b) wavelength of maximum emission
(c) MRE at 222 nm
(d) shows the coincidence of transition curves, (a), (b) and (c).
Fraction of unfolded protein was calculated at each concentration as explained in methods section.
denaturation of the dimeric myosin rod, the equilibrium constants are likely to be very sensitive to denaturant concentration such that the effect of protein concentration is overshadowed [Nozais and Bechet, 1993]. The midpoint of $\lambda_{\text{max}}$ transition curve was determined to be 4 M [Fig. 42b]. The Cm value deduced from MRE$_{222}$ nm transition curve was 3.6 M urea [Fig. 42c].

The urea denaturation profiles of PTPI as studied by monitoring the changes in inhibitory activity, fluorescence emission maximum and MRE$_{222}$ nm at increasing urea concentrations are non-coincident [Fig. 42d]. The inactivation of the inhibitor and loss of secondary structure can be seen as concomitant events. However, the modification (or substantial loss) in tertiary structure seems to occur at higher urea concentration since the midpoint of $\lambda_{\text{max}}$ transition curve is located around 4 M [Fig. 42c].

### 3.2.3 ANS FLUORESCENCE

ANS has been widely used as a sensitive reporter of apolar regions in proteins and as a probe for protein non-native, partially unfolded conformations [Semisotnov et al., 1991; Stryer, 1965; Ptitsyn, 1995]. Such intermediates are characterized by the presence of solvent exposed hydrophobic clusters. The binding of ANS to apolar region of proteins results in a significant enhancement of ANS fluorescence intensity and in a pronounced blue-shift of $\lambda_{\text{max}}$ [Semisotnov et al., 1991]. For ANS in the presence of native PTPI, significant fluorescence intensity with emission $\lambda_{\text{max}}$ at 505 nm was observed indicating the hydrophobic interaction between ANS and native PTPI that may be present due to the presence of some exposed hydrophobic patches in native PTPI. Reports with native proteins interacting considerably with ANS are present. Bovine liver catalase binds to ANS in native state [Prakash et al., 2002] so does cytoplasmic creatine kinase [Couthon et al., 1995].

To check if any non-native intermediate is present in unfolding of PTPI by GdnHCl and urea, ANS binding to denatured PTPI at increasing concentrations of the denaturants was analyzed. Denatured protein samples were incubated with 50 molar excess of ANS and fluorescence was monitored. In GdnHCl denaturation there was absence of any notable binding of ANS to GdnHCl-treated PTPI. ANS emission intensity slightly decreased during urea denaturation indicating disruption of native PTPI structure. Also no appreciable changes were detected in ANS emission $\lambda_{\text{max}}$ in both the cases, suggestive of no increase in apolar surface exposure during the process.
[Fig. 43]. Similar results have been reported for creatine kinase. ANS binding fluorescence data for creatine kinase denaturation in 6 M urea and 3 M GdnHCl showed no hydrophobic surface exposure [Huang et al., 2001].

3.2.4 RENATURATION OF PTPI AFTER GdnHCl- AND UREA-INDUCED DENATURATION

For studying the renaturation of GdnHCl- and urea- treated PTPI, refolding studies were performed. PTPI was incubated with increasing concentrations of GdnHCl (0-6 M) or urea (0-9 M) for 2 h. For performing refolding studies, these samples were then diluted to 50-fold in 50 mM sodium phosphate buffer (pH 7.5). The extent of renaturation was monitored by the recovery of inhibitory activity and fluorescence properties; at regular intervals till 24 h. Table 9 depicts the properties of refolded PTPI. Regain of inhibitory activity of PTPI was taken to be the main criterion for refolding as the fully active enzyme under these conditions is the manifestation of active site being present in proper (native) conformation. The inhibitor did not regain its papain inhibitory activity on urea denaturation. In case of GdnHCl induced denaturation only 10% of the native activity was observed even at low denaturant concentrations. Also, as seen in table 9 at all time intervals, both in urea and GdnHCl mediated denaturation (at all denaturant concentrations) the inhibitor refolded to a species having significantly different spectroscopic properties, $\lambda_{\text{max}}$ of which was within the range of 325-330 nm, instead of 335 nm (for native PTPI). Upon incubation with ANS, the refolded species obtained both in case of urea and GdnHCl, did not alter the emission spectra of the dye, in intensity or in $\lambda_{\text{max}}$. 
Fig. 43  ANS fluorescence of PTPI at various concentrations of GdnHCl and urea

The figure shows dependence of fluorescence emission of ANS bound to PTPI on the denaturant concentration. No significant change in the fluorescence intensity (at 505 nm) is observed at any concentration of GdnHCl. In presence of urea, fluorescence intensity continuously decreases.
TABLE 9: FLUORESCENCE EMISSION MAXIMA AND PAPAIN INHIBITORY ACTIVITY DURING REFOLDING OF PTPI AT VARIOUS TIME INTERVALS AFTER 50 FOLD DILUTION OF DENATURED SAMPLES

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Papain inhibitory activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GdnHCl</td>
<td></td>
<td>10</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Fluorescence emission maximum (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
<td>4 h</td>
<td>8 h</td>
<td>24 h</td>
</tr>
<tr>
<td>GdnHCl</td>
<td></td>
<td>325</td>
<td>330</td>
<td>330</td>
<td>320</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td>325</td>
<td>332</td>
<td>328</td>
<td>327</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity of the refolded PTPI was determined by the method of Kunitz [1947]. The activity is expressed as percent of native PTPI activity which was taken to be 100%. ND: None Detected.
Discussion

Chapter 2
The altered levels (or activities) of cysteine proteinases (cathepsins) has been established as a cause for rheumatoid arthritis, osteoarthritis and osteoporosis [Vasiljeva et al., 2007], neurological disorders [Nakanishi, 2003], pancreatitis [van Acker et al., 2002], cancer [Gocheva & Joyce, 2007], etc. This fact prompted an increase in the interest in the enzymes that regulate CP activity, in vivo. Such proteinaceous inhibitors of CPs, ubiquitously distributed in organisms as well as tissues, primarily belong to the cystatin superfamily. Modified expression or function of the members of cystatin superfamily marks predisposition to diverse pathological states [Turk et al., 2008]. Studies that delve into the structural and functional properties of these inhibitors in response to various externally imposed conditions thus intend to provide a good approximation of the conformational stabilities of these inhibitors.

An interplay of various physicochemical forces- like hydrophobic interactions, ionic interactions, disulfide bonds and other local as well as non-local interactions maintain the three dimensional structure of proteins. The conformational stability of the native protein is a function of external variables, such as, temperature, pH, ionic strength and solvent composition and can be measured by equilibrium unfolding studies using urea and guanidine hydrochloride (GdnHCl) [Pace, 1986]. CD measurements in the far-UV region, detecting changes in the secondary structure and steady-state fluorescence measurements, detecting changes in the tertiary structure, are complementary tools to investigate the conformational stability of globular proteins.

Equilibrium denaturation studies usually focus primarily on monomeric globular proteins. However, multidomain and oligomeric proteins remain relatively little explored [Jeanicke, 1991].

A number of investigations on unfolding have already been conducted for various members of cystatin superfamily [Khan & Bano, 2009; Rashid et al., 2005; Jankowska et al., 2004; Zerovnik et al., 1992; etc.]. On these lines, equilibrium denaturation of the purified cystatin, PTPI, was undertaken.

The unfolding of PTPI in GdnHCl and urea suggests different unfolding pathways. The two possible pathways in urea and GdnHCl are represented schematically in Fig. 44. In the figure dotted arrow in case of GdnHCl denaturation represents partial reversibility of the process, whereas in case of urea denaturation it represents the
uncertainty in presence and placement of intermediate states. Analysis of PTPI denaturation by fluorescence emission $\lambda_{\text{max}}$, MRE$_{222}$ nm, and enzymatic activity measurements reveals a coincident transition, with $C_m$ value of 3.2 M for GdnHCl denaturation. These results are consistent with two-state transition involving a folded dimer and unfolded monomer.

Equilibrium denaturation studies of the small dimeric globular proteins, fl gene V protein [Liang & Terwilliger, 1991], P22 Arc repressor [Bowie and Sauer, 1989a], mouse-β-nerve growth factor [Timm & Neet, 1992], revealed that stable intermediates are not detected at equilibrium. The experimental data were consistent with a two-state model involving only the native dimer and denatured monomer. However, recent reports catalogue the presence of intermediates in denaturant induced unfolding of multimeric or large proteins [Garrido et al., 2005; Akhtar et al., 2002; Guo et al., 2004b; etc]. Two-state denaturation of dimeric proteins by GdnHCl has also been reported earlier [Timm & Neet, 1992].

Inactivation and loss of secondary structure appear to be concomitant events in urea denaturation [Fig. 42]. The midpoint of transition curves for ellipticity and inactivation by urea was 3.6 M. The midpoint value of the $\lambda_{\text{max}}$ transition curve is shifted to a slightly higher concentration of urea (4 M). The non-coincidence of transition curves, as measured by probes that are sensitive to different levels of protein structure are consistent with a mechanism involving intermediate states [Kim & Baldwin, 1982]. The non-coincidence of the $\lambda_{\text{max}}$ transition curve with those monitoring the changes in enzymic activity and ellipticity suggests the existence of intermediate states during urea denaturation. It may illustrate the unfolding of portions enclosing tryptophan residues. However, the intermediates are not clearly observed. As for example, no compact intermediate state possessing hydrophobic areas have been detected through ANS fluorescence. Intermediates in urea-induced unfolding of oligomeric enzymes have been documented for cytoplasmatic creatine kinase [Couthon
et al., 1995], bovine liver catalase [Prakash et al., 2002], glucose dehydrogenase [Mendoza-Hernandez et al., 2000]. Creatine kinase denaturation by urea revealed presence of intermediate states adjudged by non-coincidence of the ellipticity curve with the curves presenting changes in enzymic activity, tertiary structure and molecular dimensions. However, intermediates were clearly observed in case of GdnHCl mediated creatine kinase unfolding [Couthon et al., 1995]. Presence of intermediate states in urea denaturation has also been documented for glucose dehydrogenase monitored easily by CD and fluorescence spectroscopy. Contrary to this, glucose oxidase was found to denture following a two-state model for urea [Akhtar et al., 2002].

Although monomeric, but high molecular mass goat lung cystatin are also known to denature in the presence of urea by following a multistep process [Khan and Bano, 2009b], suggesting the presence of aggregated/non-native intermediates in urea unfolding of high molecular mass proteins.

Refolding and reactivation of the denatured PTPI (both for urea- and GdnHCl-denaturation) was incomplete. The partially refolded PTPI gave maximum emission in range of 325-330 nm. Although close to the native enzyme and suggestive of a compact structure, its specific tertiary structure is quite different from the native PTPI, as no ANS binding was observed. This points to a disrupted native from. Activity gain was only 10% in case of GdnHCl, while in case of urea it remained undetected. This agrees with the generally recognised opinion that conformational integrity is important for maintaining the enzyme activity and the slight changes at the active site can lead to complete enzyme inactivation. In case of urea, it may point to large kinetic barrier for the unfolded monomer to reassociate into an active dimer. Or under in vivo conditions assisted folding might be taking place. Conditions for 100% recovery of active enzyme thus require optimization, in case of GdnHCl.

The data derived from these studies indicate that unfolding pathways in GdnHCl and urea are different. This is not surprising since the two denaturants interact with proteins in a different way. Apart from their hydrogen bond breaking effect, urea also acts on hydrophobic bonds [Kamoun, 1988] and ions arising from GdnHCl are known to modulate electrostatic interactions [Hagihara et al., 1994]. Urea is generally known to be about half as effective in protein unfolding and dissociation as GdnHCl [Pace, 1986]. This is suggestive of a ‘2-fold rule’ [Mayers et al., 1995; Smith & Scholtz, 1996]. Multimeric proteins have higher ratio of \( \frac{C_m(\text{urea})}{C_m(\text{GdnHCl})} \) [Akhtar et
al., 2002], which suggests that multimeric proteins are more susceptible to GdnHCl denaturation. Although, for proteins with non-two state transitions (as seen for urea induced denaturation in the present case) the Cm value may not be an accurate measure of the stability to the chaotrope, it does provide some indications for the differences in the interactions of the denaturant with different classes of proteins. The results of the present study show that PTPI is an exception to the ‘2-fold rule’ of urea and GdnHCl denaturation. Fig. 38 and Fig. 42 present the results of denaturation of PTPI as monitored by the change in enzyme activity, MRE$_{222}$ nm, and $\lambda_{\text{max}}$ of fluorescence emission. The midpoint of transition for urea unfolding was 3.6 M (4 M from the $\lambda_{\text{max}}$ curve), and that for GdnHCl was 3.2 M. Violation of ‘2-fold rule’ for urea and GdnHCl denaturation has also been reported for the dimeric enzyme glucose oxidase [Akhtar et al., 2002]. This further points to significant contributions of both electrostatic and hydrophobic interactions in maintaining the active site and/or dimeric nature of the PTPI.

The results obtained in this study give an idea about the stability of PTPI and can be extrapolated to physiological conditions. Studying the behaviour and stability of proteins under denaturing conditions allows one to understand and quantify the forces that contribute to the conformational stability of protein in their aqueous environment [Pace, 1986]. Further the data on the stability of proteins can be used in algorithms to predict the structure or docking of proteins and to design new proteins with better activities and stabilising changes.
Chapter 3

In vitro fibrillation of pancreatic thiol proteinase inhibitor
3.3 RESULTS

Amyloid fibril formation is generally the result of the alteration of native conformation of proteins. The partially folded conformations are favoured by mutations or changes in pH and ionic strength [Pedersen et al., 2004]. Members of cystatin superfamily show predisposition for fibrillization. A number of reports are available on in vitro fibrillation of cystatins under experimentally exposed mild denaturational conditions, besides, cystatin C and stefin B fibrillation been incriminated in human cystatin C amyloid angiopathy (HCCAA) and progressive myoclonus epilepsy (EPM1), respectively [Turk et al., 2008].

The ability for rational design of conditions promoting amyloid formation with a wider range of proteins (than those so far identified in specific diseases) provides an opportunity to investigate in detail the mechanism of the underlying processes (in vivo) and hence to explore the factors that predispose individual sequences to form ordered aggregates under particular conditions. This could be an important factor in the development of strategies to combat amyloid formation/deposition.

On these footings, present study was designed to investigate the conditions under which the purified goat pancreatic thiol proteinase inhibitor fibrillates and to probe the inhibition or deaggregation of fibrils in the presence of divalent metal ions.

3.3.1 ACID DENATURATION OF PTPI

It has been well established that fibril formation requires appropriate physicochemical conditions, among which pH exerts a significant influence on the whole process of fibrillogenesis [Lai et al., 1996]. pH engendered deformation of proteins is common in biology. A number of proteins have been reported to exist as partially folded intermediates or as molten globules under acidic conditions [Rashid et al., 2006b; Kuwajima, 1989; Zerovnik et al., 1992; Ahmad and Khan, 2006; Brahma et al., 2005], a condition already emphasized as an important start point for amyloid aggregation. Thus to explore the existence of any non-native, partially unfolded intermediate under acidic conditions, PTPI was subjected to denaturation over a pH range of 1.0-7.0.

Fluorescence spectra of PTPI in the presence of varying pH

Acid induced unfolding of PTPI was followed by intrinsic fluorescence properties of the protein. PTPI incubated in buffers of different pH was excited at 280 nm to assess global conformational changes inflicted by pH. In its native state PTPI is
characterized by a peak at 335 nm (at pH 7.5) [Priyadarshini and Bano, 2009]. Till pH 5.0, no change is observed in $\lambda_{\text{max}}$ of emission [Fig. 45a]. However, a 10 nm blue shift is observed at pH 3.0. Lowering the pH to 2.0 caused a red shift of 2 nm (compared to the state at pH 3.0) further followed by a red shift of 3 nm, at pH 1.0. On the other hand, as the pH is lowered from 7.0 to 1.0, fluorescence intensity is quenched [Fig. 45a]. This decrease in fluorescence intensity and a blue shift of 10 nm at pH 3.0 is indicative of burial of aromatic residues in the hydrophobic core of the protein and formation of a more compact structure. Red shift observed at pH 2.0 and 1.0 can be ascribed to slight unfolding of the structure.

**Effect of pH on ANS spectrum of PTPI**

In order to study the exposure of hydrophobic clusters of the PTPI during acid induced unfolding, effect of pH was followed by ANS fluorescence at 505 nm after exciting the ANS-protein complex at 380 nm [Fig. 45b]. Till pH 4.0, there was no appreciable increase in ANS binding. A sharp increase is noticeable at pH 3.0, reaching maximum at pH 1.0. Further, there was a shift in $\lambda_{\text{max}}$ of ANS emission at pH 3.0, the $\lambda_{\text{max}}$ of ANS-protein complex was blue shifted to 490 nm with no further change with pH reduction. These results show that at pH 3.0, small amount of hydrophobic clusters are present which bind ANS and also suggest the presence of large number of solvent accessible non-polar clusters in protein molecule at pH 1.0.

**Perturbation of secondary structure of PTPI by pH**

The effect of acid induced unfolding on secondary structure of PTPI was monitored by ellipticity measurements at 222 nm. The results are shown in Fig. 46. The ellipticity at 222 nm decreased markedly below pH 7.0, to a minimum value at around pH 3.0. A further decrease in pH below 3.0 resulted in a second transition corresponding to the formation of secondary structure which became maximum at pH 1.0. At pH 3.0, ~40% of secondary structure is lost. At pH 1.0, ~34% of secondary structure is regained and it is marked by the presence of 82% secondary structure. Thus it may be concluded that PTPI at pH 3.0 exists as a partially unfolded intermediate which has ~60% of residual secondary structure, but almost completely altered tertiary structure. PTPI at pH 1.0 exhibits more of molten globule like features with native like secondary structure contents, enhanced ability to bind ANS, however with tertiary contacts quite similar to native PTPI [Fig. 45a].
Fig. 45  pH dependence of intrinsic and ANS fluorescence properties of PTPI

(a) Figure shows variation in intrinsic fluorescence intensity at 335 nm and $\lambda_{\text{max}}$ of emission of PTPI with pH. PTPI (1 $\mu$M) was incubated in 50 mM solutions of glycine/HCl (pH 2.0), sodium acetate (pH 3.0-5.0), sodium phosphate (pH 6.0-7.0) at room temperature for 4 h. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm.

(b) Figure shows variation in ANS fluorescence intensity at 505 nm and $\lambda_{\text{max}}$ of emission of ANS-PTPI complex with pH. The samples were prepared as described above. The ANS to protein molar ratio was 1:50. ANS fluorescence was measured at an excitation wavelength of 380 nm in the emission range of 400-600 nm with a slit width of 5 nm.
Fig. 46  pH dependence of mean residue ellipticity (MRE) of PTPI at 222 nm

For far UV CD, 1.73 μM of PTPI was incubated in 50 mM solutions of glycine/HCl (pH 2.0), sodium acetate (pH 3.0-5.0), sodium phosphate (pH 6.0-7.0) at room temperature for 4 h. Cells of 1 mm pathlength were used.
3.3.2 EFFECT OF 2, 2, 2-TRIFLUOROETHANOL (TFE) ON ACIDIC pH INDUCED STATE OF PTPI

TFE has been used widely with other proteins to induce amyloid fibril formation [Zerovnik et al., 2007]. TFE has a double edged effect on proteins and peptides, on one hand, it can destroy the tertiary structure of proteins on the other hand, it can increase the \( \alpha \)-helical structure of proteins and peptides via strengthening of the hydrogen bonds [Luo and Baldwin, 1998; 1997]. Effect of TFE on pH 3.0 state of PTPI was studied by means of intrinsic and extrinsic fluorescence and far UV CD.

**Effect of TFE on the secondary structure of PTPI at pH 3.0**

Fig. 47a shows changes in ellipticity at 222 nm of pH 3.0 state as a function of TFE concentration. As shown earlier approximately 40% loss in secondary structure at pH 3.0 occurred as compared to the native state at pH 7.5. Increase in concentration of TFE leads to increase in MRE \( \text{222 nm} \) value. It is interesting to note that at 10% TFE the induced secondary structure reached almost the value for the native state. At higher TFE concentration denatured states with very high \( \alpha \)-helical content were formed.

Native PTPI was also titrated with various concentrations of TFE. At all TFE concentrations accumulation of secondary structure was noticed with native PTPI transforming to an all-helical state beyond 10% TFE (data not shown).

**Effect of TFE on intrinsic fluorescence of PTPI at pH 3.0**

Since the pH 3.0 state of PTPI in presence of 10% (v/v) TFE shows native like secondary structure content it was of interest to study properties of pH 3.0 state in presence of TFE. Fig. 47b shows intrinsic emission spectra of PTPI at pH 7.5, pH 3.0 and pH 3.0 state in the presence of 10% (v/v) TFE. The emission spectrum of PTPI shows \( \lambda_{\text{max}} \) at 335 nm under native conditions. At pH 3.0, there was \( \sim 31\% \) loss in fluorescence intensity and a change in \( \lambda_{\text{max}} \) to 325 nm suggesting internalization of tryptophan residues to a more hydrophobic environment [Haq et al., 2002]. At 10% (v/v) TFE concentration, there is \( \sim 17\% \) increase in fluorescence intensity with further blue shift in \( \lambda_{\text{max}} \) to 323 nm, suggesting further internalization of tryptophan residues in the hydrophobic environment and creation of a more compact structure.
Fig. 47 **Effect of TFE on PTPI at pH 3.0**

(a) Figure shows the effect of increasing concentration of TFE (0-20%) on pH 3.0 state of PTPI as monitored by changes in MRE value at 222 nm. Cells of 1 mm pathlength were used.

(b) Intrinsic emission spectra of PTPI at pH 7.5, at pH 3.0 alone and in presence of 10% (v/v) TFE. The concentration of PTPI used was 1 μM. The excitation wavelength was 280 nm and emission was recorded in the range of 300-400 nm with a slit width of 5 nm.
(a) 

![Graph showing the relationship between % (v/v) TFE and $\text{MRE}_{222\text{nm}}$ (deg. cm$^2$ dmol$^{-1}$)].

(b) 

![Graph showing fluorescence intensity vs. wavelength for different conditions: Native PTPI at pH 7.5, pH 3.0 state + TFE (10%), and pH 3.0 state.]}
Effect of TFE on ANS spectrum of PTPI at pH 3.0

Fluorescent hydrophobic probe ANS has higher affinity for the partially unfolded intermediates and molten globules than for the proteins in the native or fully unfolded state. Fig. 48a shows that ANS binds maximally to pH 3.0 state of PTPI in presence of 10% (v/v) TFE compared to minimal ANS binding to native PTPI. The emission maximum blue shifts to 485 nm in the presence of TFE as compared to those of pH 3.0 state (490 nm) and at pH 7.5 (505 nm), further suggesting that the protein is adopting an altered structure. Titration of the pH 3.0 state with increasing concentration of TFE as studied by ANS binding at 490 nm [Fig. 48b] indicates slight disruption of compact structure at 10% (v/v) TFE. However at higher TFE concentrations [15 and 20% (v/v)] the pH 3.0 state gets altered as there is decrease in exposed hydrophobic clusters available for ANS binding.

Similar studies were also conducted for acid induced pH 2.0 state and pH 1.0 state of PTPI. Unlike pH 3.0 state, no stabilized intermediates were observed. However, 10% (v/v) TFE was found to be predenaturational concentration in both the cases.

3.3.3 OPTIMAL pH AND TFE CONCENTRATION TO INDUCE PTPI AMYLOID FIBRIL GROWTH

Above results show that on decreasing the pH beyond 4.0 there is decrease in the dichroicity of PTPI at 222 nm and a blue shift of the \( \lambda_{\text{max}} \) of fluorescence emission. With further decrease in pH to 2.0 and 1.0 there are further alterations in conformation of the protein. As reported for many other proteins acidic conditions prevent complete unfolding of the protein molecule [Rashid et al., 2006b; Gupta et al., 2003; Artigues et al., 1994], and result in the formation of compact, non-native intermediates or molten globules. Acidic conditions render PTPI to adopt a partially unfolded conformation at pH 3.0 referred here as pH 3.0 state having substantial amount of secondary structure but devoid of native like tertiary state. At pH 1.0 more of a molten globule like intermediate is formed characterized by its compactness (compared to native state), persistence of secondary structure without well defined tertiary packing and a strong affinity for ANS as a consequence of the exposure of hydrophobic areas. TFE is known to induce \( \alpha \)-helical states and to stabilize partially unfolded intermediates in proteins. PTPI at pH 3.0 gains native like secondary structure at 10% (v/v) TFE concentration and exhibits substantially exposed
Fig. 48  **Fluorescence spectra of ANS bound to PTPI under different conditions**

(a) Figure shows fluorescence emission spectra of ANS bound to PTPI at pH 7.5, at pH 3.0 alone and in presence of 10% (v/v) TFE.

(b) Figure shows the effect of increasing concentration of TFE on pH 3.0 state as monitored by changes in ANS binding at 490 nm. The ANS to protein molar ratio was 1:50. ANS fluorescence was measured at an excitation wavelength of 380 nm in the emission range of 400-600 nm with a slit width of 5 nm.
(a) 

![Graph showing fluorescence intensity vs. wavelength (nm) with curves for pH 3.0 state, pH 3.0 state + TFE (10%), and Native PTP1 at pH 7.5.](a)

(b) 

![Graph showing ANS fluorescence intensity vs. % (v/v) TFE with data points at various TFE concentrations.](b)
hydrophobic patches (revealed by ANS binding). However, there is absence of native
like tertiary fold. Beyond this concentration of TFE, denaturing states with very high
α helical content were formed.

Based on these findings PTPI for amyloid growth was incubated in acidic medium, as
it is known that TFE accelerates amyloid growth [Zerovnik et al., 2002a], 10% (v/v)
TFE was added. Amyloid formation was followed by two standard procedures,
thioflavin T (ThT) assay and transmission electron microscopy (TEM) [Nilsson,
2004], in the following six samples at regular time intervals,
PTPI at pH 1.0, 2.0 and at pH 3.0 and at these three pH in presence of 10% (v/v) TFE.

3.3.4 AMYLOID TYPE AGGREGATION/FIBRILLATION OF PTPI
MONITORED BY ThT FLUORESCENCE

The enhancement in the fluorescence intensity of ThT upon binding to ordered protein
aggregates is a rapid and sensitive method to show presence of fibrils. Free ThT has
emission maximum at 450 nm. However, upon binding to fibrils the emission λmax
changes to 485 nm [Levine, 1999]. 50 μM PTPI was incubated at pH 2.0 and at pH
3.0, in presence of 0.15 M NaCl and 0.05% sodium azide. Changes in fluorescence
emission of ThT were monitored at regular time intervals for both the samples.
Results are depicted in Fig. 49a and 50a. The pH 2.0 sample, showed only ~5 fold
enhancement in ThT fluorescence even after 55 days of incubation [Fig. 49a],
suggesting a very lengthy lag phase. At pH 3.0 however, ~14 fold increment was
observed within 5 days of incubation [Fig. 50a]. This indicates that at pH 3.0 the
amyloid growth is rapid. PTPI samples incubated at neutral pH served as controls and
showed no ThT binding. Results obtained for PTPI incubated at pH 1.0 were quite
similar to those obtained for pH 3.0 sample. These results thus reveal that amyloid
aggregates from PTPI form under acidic conditions and more readily at pH 3.0 and
1.0 than at pH 2.0.

Influence of TFE on amyloid aggregation of PTPI

To study the effect of TFE on the formation of aggregates of PTPI, 50 μM protein
was incubated at pH 3.0 and at pH 2.0, with 0.15 M NaCl, 0.05 % sodium azide and
10% (v/v) TFE. Fig. 49b and 50b show the time course of the change of ThT
fluorescence as the protein aggregates form at pH 2.0 and pH 3.0 in presence of 10%
Typical fibrillation processes involve a lag phase followed by a relatively rapid elongation phase, which stabilizes at a plateau level when all the protein molecules have been incorporated into fibrils [Harper and Lansbury, 1997]. Whereas at pH 2.0 aggregates develop after a long lag phase and exhibit relatively less ThT fluorescence enhancement [observed at 55th day of incubation, Fig. 49a], in the presence of TFE the maximum ThT fluorescence is reached at 30th day of incubation with several fold increase in the intensity [Fig. 49b]. Similarly for the sample incubated at pH 3.0 in presence of TFE, amyloid aggregation started without a distinct lag phase with a profound fluorescent intensity enhancement [Fig. 50b]. The absence of lag phase suggests that amyloid aggregation of PTPI at pH 3.0 in presence of TFE, proceeds without a nucleation phase or the nucleus might have formed very rapidly (in the dead time of mixing). For PTPI incubated at pH 1.0, a rapid fibrillation was observed without any lag phase in presence of TFE. But ThT fluorescence intensity enhancement was lower to that obtained for pH 3.0.

3.3.5 TRANSMISSION ELECTRON MICROSCOPY IMAGES

Fibril formation was also followed by TEM. Fig. 51 shows TEM image of native dehydrated PTPI. No distinct fibril morphology is seen. Fig. 52 shows the TEM image of PTPI at pH 1.0 obtained after 15 days of incubation. Peculiar foliage like pattern is observed. Fig. 53 shows the TEM image obtained after one month of incubation of sample under similar conditions but at pH 2.0. It shows fibrils. Fig. 54 shows TEM image of PTPI at pH 3.0 in presence of 10% (v/v) TFE obtained after 15 days of incubation. Sharp fibril is observed.

3.3.6 EFFECT OF DIVALENT METAL IONS, Cu^{2+} AND Zn^{2+} ON PTPI FIBRILLATION

To date there is no potential cure to amyloidosis. Different effects of metal ions on amyloid aggregation have been documented. Fig. 55 shows the effect of Cu^{2+} and Zn^{2+} on ThT fluorescence of preformed fibrils. PTPI fibrils grown at pH 3.0 in the presence of 10% (v/v) TFE were used in the experiment. Just before the ThT assay metal solutions were added to the sample. Controls of each concentration of Cu^{2+} and Zn^{2+} were run to nullify the possibility of interaction with the dye. A concentration dependent decline in ThT fluorescence intensity at 485 nm was observed, about ~35%
Fig. 49  PTPI fibrillation at pH 2.0

(a)  Fluorescence emission spectra of Thioflavin T (ThT) dye in the presence of PTPI at pH 2.0

Figure shows increase of ThT fluorescence on binding to the fibrils of PTPI obtained after 55 days of incubation. 50 µM protein was incubated at pH 2.0 at room temperature.

(b)  Time course of fibril growth of PTPI followed by ThT fluorescence at 485 nm at pH 2.0 and in presence of 10% (v/v) TFE

50 µM PTPI was incubated at pH 2.0 alone and in presence of 10% (v/v) TFE. Excitation was at 440 nm and spectra were recorded from 455 to 600 nm with a slit width of 5 nm. For ThT assay 5 µM of incubated protein was used with a protein to ThT molar ratio of 1:6. The data were obtained after subtraction of the signal of the buffer containing ThT.
Fig. 50  PTPI fibrillation at pH 3.0

(a)  Fluorescence emission spectra of Thioflavin T (ThT) dye in the presence of PTPI at pH 3.0
Figure shows increase of ThT fluorescence on binding to the fibrils of PTPI obtained after 5 days of incubation. 50 μM protein was incubated at pH 3.0 at room temperature.

(b)  Time course of fibril growth of PTPI followed by ThT fluorescence at 485 nm at pH 3.0 and in presence of 10% (v/v) TFE
50 μM PTPI was incubated at pH 3.0 alone and in presence of 10% (v/v) TFE. Excitation was at 440 nm and spectra were recorded from 455 to 600 nm with a slit width of 5 nm. For ThT assay 5 μM of incubated protein was used with a protein to ThT molar ratio of 1:6. The data were obtained after subtraction of the signal of the buffer containing ThT.
**Fig. 51** Electron micrograph of PTPI incubated under native conditions

Figure shows image obtained for native dehydrated PTPI. 50 μM of PTPI in 50 mM sodium phosphate buffer (pH 7.5) was used.

**Fig. 52** Electron micrograph showing amyloid aggregation of PTPI incubated at pH 1.0

Figure shows image obtained for PTPI incubated at pH 1.0 in presence of 10% (v/v) TFE. Sample used was obtained after 15 days of incubation. Experimental details are provided in methods section.
Fig. 53  Electron micrographs showing amyloid aggregation of PTPI incubated at pH 2.0

Figure shows image obtained for PTPI (at different magnifications as shown by the size of bar lines) incubated at pH 2.0 (50 mM glycine/HCl) in presence of 10% (v/v) TFE. Sample used was obtained after 30 days of incubation. Experimental details are provided in methods section.
Fig. 54  Electron micrographs showing amyloid aggregation of PTPI incubated at pH 3.0

Figure shows image obtained for PTPI (at different magnifications as shown by the size of bar lines) incubated at pH 3.0 (50 mM sodium acetate buffer) in presence of 10% (v/v) TFE. Sample used was obtained after 15 days of incubation. Experimental details are provided in methods section.
decrease in intensity was observed at 10 μM Cu$^{2+}$ ion concentration, which increased to ~42% at 20 μM culminating into 98% decline in ThT fluorescence at 50 μM Cu$^{2+}$ ion concentration, beyond this concentration fluorescence intensity was too diminished for any discernible peak to be observed at 485 nm [Fig. 55a]. Similar effects were observed for Zn$^{2+}$. Minute decline (1-2%) in ThT fluorescence was observed till 6 μM Zn$^{2+}$ ion concentration. At 10 μM Zn$^{2+}$ ion concentration almost 97% of the intensity was quenched. Beyond this concentration no perceivable effect was seen [Fig. 55b].

When Cu$^{2+}$ and Zn$^{2+}$ were added at 50 μM and 10 μM concentration respectively, to 50 μM PTPI (pH 3.0, 10% (v/v) TFE) at the start of incubation, no ThT binding was observed, indicative of absence of any amyloid type aggregation in the presence of metal ions, suggesting Cu$^{2+}$ and Zn$^{2+}$ help in preventing amyloid formation.
Fig. 55  Inhibition of fibrillation of PTPI by Cu\(^{2+}\) and Zn\(^{2+}\) as probed by ThT fluorescence

(a) Figure shows inhibition of fibrillation as a function of Cu\(^{2+}\) concentration

(b) Figure shows inhibition of fibrillation as a function of Zn\(^{2+}\) concentration
For both the experiments 50 \(\mu\)M PTPI was incubated at pH 3.0 in presence of 10\% (v/v) TFE. Metal solutions were added prior to ThT assay. Excitation was at 440 nm and spectra were recorded from 455 to 600 nm with a slit width of 5 nm. For ThT assay 5 \(\mu\)M of incubated protein was used with a protein to ThT molar ratio of 1:6. The data were obtained after subtraction of the signal of the buffer containing ThT.
Discussion

Chapter 3
DISCUSSION

Cathepsin knockouts have demonstrated that cathepsins have specific and individual functions, which are very important for the normal functioning of the organism [Turk et al., 2001]. Among others they degrade the extracellular matrix, of importance in tumor progression and invasion [Gocheva and Joyce, 2007]. Accidentally escaped cathepsins from lysosomes are trapped by cystatins, their endogenous protein inhibitors. Cystatins comprise large family of thiol proteinase inhibitors. Members of cystatin superfamily have propensity to form fibrillar aggregates [Skerget et al., 2009]. Lately, various non-pathogenic proteins have been shown to form amyloid fibrils in vitro, such as acylphosphatase [Chiti et al., 1999], cold shock protein [Wilkins et al., 2000], hen lysozyme [Krebs et al., 2000], SH3 domain [Zurdo et al., 2001], cytochrome C [Pertinhez et al., 2001] and myoglobin [Fandrich et al., 2001], etc.

The aim of the present study was to define the requisite conditions for the in vitro fibrillation of pancreatic thiol proteinase inhibitor and thus test the generic ability of proteins to aggregate to β-sheet rich fibrillar structures. The ‘generic amyloid’ hypothesis essentially states that the ability of proteins to form ordered fibrillar cross β-structures is inextricably linked to the nature of the protein backbone [Chiti et al., 1999; Dobson, 1999] and is independent of the structure of the native state. Thus, while some proteins obviously fibrillate more readily than others, it should be possible to find conditions that are sufficiently destabilizing for the native state to allow the protein to explore alternative conformations and lock on to the stable β-sheet aggregated state.

Exploring conditions for PTPI fibrillation

It is generally believed that globular proteins need to unfold, at least partially, to aggregate into amyloid or amyloid like fibrils [Chiti et al., 1999; Krebs et al., 2000; Fandrich et al., 2001; Rabzelj et al., 2005; Holm et al., 2007; Zerovnik et al., 2007]. Such a “conformational change hypothesis” is widely supported by a large body of experimental data. Proteins normally adopting a compact and well defined three-dimensional fold have a higher propensity to aggregate under conditions that promote their partial unfolding such as high temperature, high pressure, low pH or moderate conditions of organic solvents [Konno, 2001; Rabzelj et al., 2005; Soldi et al., 2005]. As noted for various proteins, amyloid formation by PTPI in vitro was achieved by
destabilizing the native state of the protein under conditions in which non-covalent interactions still remain favourable. PTPI when subjected to acid denaturation yields a 'partially unfolded intermediate', the pH 3.0 state and a molten globule like intermediate at pH 1.0 [Fig. 45 and 46]. From these results it can be inferred that the structure remaining at these low pH environments may not be residual native like structure that the acid unfolding has failed to disrupt, but rather newly formed organisations, and consequently with little resemblance to native structure [Buck et al., 1993].

PTPI at pH 3.0, 2.0 and 1.0 possess significant non-native secondary structure, which is likely to transform to amyloid aggregates and this tendency was judged by ThT fluorescence assay. PTPI incubated under these three acidic conditions tested positive for amyloid by ThT fluorescence assay. However, there were differences in ThT binding [Fig. 49a and Fig. 50a]. Distinct fibrillar states under different solution conditions have also been reported for stefin B [Zerovnik et al., 2007] where different type of fibrils originate from pH 4.8-native like and pH 3.3-molten globule like intermediate. Fibril growth has also been documented under (mild) acidic conditions for insulin [Ahmad et al., 2005], endostatin [He et al., 2006], acylphosphatase [Chiti et al., 1999], stefin B [Rabzelj et al., 2005] and stefin A and B [Jenko et al., 2004; Zerovnik et al., 2002b]. Rapid fibrillation was observed at pH 3.0 and 1.0 then at pH 2.0; suggesting that partially folded and native like molten globule states have a higher propensity to form aggregates [Fig. 49a and Fig. 50a]. This is supported by reports on fibrillation of bovine serum albumin [Holm et al., 2007].

It is a well known fact that 2, 2, 2-trifluoroethanol (TFE), a hydrogen bond promoting solvent has an accelerating effect on amyloid fibrillation. To find out the most appropriate concentration of TFE, required for PTPI fibril growth, influence of increasing concentration of TFE on pH 3.0 state of PTPI was studied. It was observed that at 10% (v/v) TFE, pH 3.0 state of PTPI gained sufficient amount of non-native secondary structure with increased amount of exposed non polar clusters whereas at higher concentrations of TFE, \( \alpha \)-helical content was profoundly enhanced with decreased hydrophobic effect. Binary mixtures of water with alcohols like methanol, ethanol, or TFE have been shown to cause conformational transition of proteins into new stable conformational states with high \( \alpha \)-helical content and disrupted tertiary and quaternary structure resembling that of molten globule intermediate [Polverino de Laureto et al., 2002]. Alcohols weaken non-local hydrophobic interactions while
enhancing local polar interactions (i.e. hydrogen bond) of proteins [Rashid et al., 2006b]. Alcohols induce significantly higher α-helical structures in partially or completely unfolded proteins as compared to those in folded proteins [Bhakuni, 1998]. Among various alcohols, TFE is often preferred because of its remarkable potential in stabilizing the α-helical structure [Gupta et al., 2003]. 10% (v/v) TFE was found to stabilize pH 3.0 state of PTPI drawing credence from previous reports. In this study, amyloid fibril growth was accelerated by addition of 10% (v/v) TFE to samples incubated at pH 2.0 as well as at pH 3.0 [Fig. 49b and 50b]. The lag observed initially for fibrillation at pH 2.0 was considerably reduced by addition of TFE and at pH 3.0 fibrillation initiated without any lag phase in presence of TFE. Quite similar results were obtained for sample incubated at pH 1.0. This suggests that pH 1.0 and pH 3.0 states of PTPI (in presence of TFE) have a higher tendency to aggregate and fibrillate. Enhancement of the rate of fibrillation by TFE has also been noted before. Stefin A and stefin B showed accelerated fibril growth in the presence of predenaturational TFE concentrations [Jenko et al., 2004; Rabzelj et al., 2005; Zerovnik et al., 2007]. This effect was also observed for fibrillation of acylphosphatase. Chiti et al. [1999] found that fibrillation of acylphosphatase proceeds at moderate concentration of trifluoroethanol. Chiti et al. [2001] reiterated similar effects of TFE, with HypF-N terminal domain. TFE was also found to promote fibrillation in case of endostatin [He et al., 2006].

Based on above discussion, possible mechanism of PTPI aggregation could be presented as,

\[
\begin{align*}
N \xrightarrow{\text{Acidic pH}} & \quad \text{PFI} \\
& \quad \xrightarrow{\text{TFE (moderate concentration)}} \\
& \quad N_l \rightarrow \text{Agg}
\end{align*}
\]

Fig. 56: Scheme for mechanism of PTPI aggregation, where N stands for native state, PFI for acid induced partially folded intermediate; N_l for TFE induced native like intermediate and Agg for amyloid aggregate.

The proposed mechanism shows that the protein may form fibrils from a partially folded intermediate stabilized by organic solvent TFE to a more native like structure, N_l. Thus it can be propounded that partially folded predenaturational intermediates
appear to be critical species in the fibrillation process. A probable explanation was offered by He et al. [2006] for the observation that destabilization of native fold and presence of non-native α-helix promotes fibrillation. They suggested that hydrophobic surface exposure and stability are two important factors controlling protein conformation. Unstable proteins with exposed hydrophobic surfaces result in molecular adhesion [Wodak and Janin, 2003]. In present case also PTPI at acidic pH [1.0, 2.0 and 3.0] was found to possess exposed non-polar clusters revealed by enhanced ANS binding [Fig. 45b]. In an environment that favors the conformational stability, α-helix would prevent the polypeptide chain from converting into fibril structure [Fandrich and Dobson, 2002]. Contrarily, under other conditions where mutagenesis or organic solvents reduce the stability of the α-helical structure formed in proteins, the polypeptide is therefore ready to form fibrils. As for PTPI the α-helical structure revealed by far UV CD in Fig. 47a is not in the fully structured native conformation but is induced by TFE. This non-native α-helical structure of PTPI being unstable can easily transform to amyloid like aggregates [as shown by ThT fluorescence and TEM [Fig. 50b and 54]. Thus, unstable α-helix induced in proteins can facilitate fibril formation [He et al., 2006].

The fibrils obtained for PTPI incubated at pH 3.0 [Fig. 54, TEM image] were similar to classical straight needle-form structures characteristic of bona fide fibrils. Those obtained for PTPI incubated at pH 2.0, also exhibited fibrillar aggregates. However, peculiar foliage like pattern was obtained for PTPI incubated at pH 1.0. Distinct fibrillar patterns have also been shown for bovine serum albumin [Holm et al., 2007], endostatin [He et al., 2006], insulin [Grudzielanek et al., 2006] and β2 microglobulin [Hong et al., 2002]. This further suggests that PTPI is able to form different type of fibrils. It has already been noted that fibril morphologies differ depending upon solvent conditions [Zerovnik et al., 2007], which in the present case refer to differing pH.

**Strategies against fibrillation of PTPI**

A range of human disorders is associated with the extracellular deposition of insoluble protein aggregates of regular arrays of β sheet rich filaments or fibres of indefinite length, often coiled together in higher order structures [Frokjaer and Otzen, 2005; Dobson, 2003], example, Alzheimer’s disease, prions disease, Huntington’s and Parkinson’s disease, type 2 diabetes etc. [Sambashivan and Eisenberg, 2006; Merlini
and Bellotti, 2003; Engel et al., 2008]. Because of the connection of amyloid fibrils to conformational diseases which are hitherto incurable, various compounds (natural and synthetic) are under experimentation for their efficacy in destabilizing and disaggregating amyloid fibrils or inhibiting the basic conformational change of the protein responsible for fibrillation. A number of compounds like curcumin, antibiotics, anticancer agents, nicotine etc. have been demonstrated to inhibit formation of fibrillar assemblies or are under trials [Zerovnik, 2002; Findies, 2000; Riviere et al., 2008].

Recent reports have shown that Cu$^{2+}$ and Zn$^{2+}$ retard the amyloid growth [Zerovnik et al., 2006; Raman et al., 2005]. These divalent metal ions were also used for inhibiting PTPI fibrillation and for disintegrating the formed fibrils. 50 μM Cu$^{2+}$ and 10 μM Zn$^{2+}$, almost completely abolished ThT fluorescence enhancement. The same concentration when added to the PTPI sample prior to fibril induction, fibrillation seemed to be arrested. In this case, Cu$^{2+}$ and Zn$^{2+}$ may stabilize the protein, preventing the aggregation. In the former case, Cu$^{2+}$ and Zn$^{2+}$ salts might exert GdnHCl like action, disintegrating the ordered structure of fibrils.

Conclusively, these results sustain the generic hypothesis of amyloid fibrillation and outline the conditions necessary for PTPI fibrillization. The work also foreshadows the use of redox active metals like copper as therapeutic agents against amyloid formation. Lastly, it connects to the susceptibility of cystatin superfamily members for amyloid aggregation.
Chapter 4

Modification of pancreatic thiol proteinase inhibitor by reactive species:

1. Photosensitized riboflavin
2. Hydrogen peroxide
3. Hypochlorous acid
4. Nitric oxide
3.4 RESULTS

Pancreatic tissue exhibits particular sensitivity to oxidative stress, contributing to impaired functioning characteristic of diabetes, pancreatitis and fibrosis [Lenzen, 2008a]. Proteins offer the most potential targets of radical species and are usually rendered with compromised functions.

The purified protein, pancreatic thiol proteinase inhibitor (PTPI) was presumed to be equally sensitive (may be with somewhat enhanced susceptibility to reactive species, because of its host tissue). The experiments were thus focused to examine the effects of various reactive species on PTPI and also to investigate the potential of bioflavonoids quercetin (QE), caffeic acid (CA) and curcumin (Cur) to protect its damage against deleterious effects of the radicals by analyzing, papain inhibitory activity, along with intrinsic fluorescence and polyacrylamide gel electrophoresis behaviour of treated PTPI.

3.4.1A INTERACTION OF PTPI WITH PHOTOSENSITIZED RIBOFLAVIN

Riboflavin upon irradiation with fluorescent light generates reactive oxygen species like superoxide anion (O$_2^-$), singlet (1O$_2$) and triplet oxygen (3O$_2$), flavin radicals and substantial amounts of hydrogen peroxide [Husain et al., 2006].

Functional inactivation of PTPI by riboflavin

Effect of riboflavin on PTPI function was assessed by monitoring the changes in its antiproteolytic activity by caseinolytic assay of papain [Kunitz, 1947]. 1 μM PTPI was photo illuminated with increasing concentrations of riboflavin (5-50 μM) or with 40 μM riboflavin for various time intervals. The results obtained are summarized in Table 10. As shown in Table 10 (A) exposure of PTPI to increasing concentration of riboflavin, resulted in rapid decline of antiproteolytic activity (83% loss) towards papain, with half of the inactivation (56%) taking place at a concentration of riboflavin as low as 10 μM. Similarly, increase in length of exposure (0-60 min) of PTPI with riboflavin caused loss of inhibitory activity towards papain (Table 10 B), with more than 50% inhibition taking place after 30 min of incubation. To detect the ROS type involved in PTPI inactivation various free radical scavengers were used, Fig. 57 (A) illustrates the results obtained.
<table>
<thead>
<tr>
<th>TABLE 10: LOSS OF ANTIPROTEOLYTIC ACTIVITY OF PTPI ON TREATMENT WITH PHOTOILLUMINATED RIBOFLAVIN AS A FUNCTION OF (A) CONCENTRATION OF RIBOFLAVIN AND (B) TIME OF INCUBATION</th>
</tr>
</thead>
</table>

(A)  
<table>
<thead>
<tr>
<th>Riboflavin (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>61.08±2.2*</td>
<td>44.37±1.8*</td>
<td>33.51±1.6*</td>
<td>28.23±1.2*</td>
<td>20.48±1.3*</td>
<td>17.38±0.58*</td>
</tr>
</tbody>
</table>

(B)  
<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity*</td>
<td>100</td>
<td>85.76±3.8*</td>
<td>65.28±2.5*</td>
<td>27.16±2.3*</td>
<td>25.10±2.0*</td>
<td>22.12±1.3*</td>
<td>20.12±0.58*</td>
</tr>
</tbody>
</table>

PTPI (1µM) was incubated with increasing concentrations of riboflavin in light for 1 h or with 40 µM riboflavin for increasing intervals of time.  
*PTPI was assayed for loss in antiproteolytic activity by caseinolytic method of Kunitz [1947]. The activity of native PTPI is taken to be 100. Results are Mean±SEM for three or more separate experiments.  
* Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA. Values in parentheses represent percent change from control.
Fig. 57 Effect of scavengers on riboflavin mediated inactivation of PTPI

(A) 1 μM PTPI was incubated with riboflavin (40 μM) alone or in presence of sodium azide/potassium iodide/thiourea/mannitol/sodium benzoate/uric acid/glucose (final concentration, 25 mM) or ascorbic acid (final concentration, 100 mM) for 30 min at room temperature under fluorescent light and assayed for antiproteolytic activity. None of the scavengers used had any effect on PTPI assay. Data are expressed as Mean ± SEM for four experiments. * p < 0.05 compared to PTPI+riboflavin alone.

(B) Native PTPI (1 μM) was incubated with riboflavin (40 μM) alone or in presence of curcumin (Cur , final concentration, 100 μM) or caffeic acid (CA )/quercetin (QE ) (final concentration, 350 μM). Rest of the experimental conditions were same as above. Data are expressed as Mean ±SEM for four experiments. * p < 0.05 compared to PTPI+riboflavin alone.
Maximum suppression of PTPI inactivation was caused by potassium iodide closely followed by sodium azide. Sodium azide is a scavenger of singlet oxygen and potassium iodide scavenges flavin triplet state. Sodium benzoate, mannitol and thiourea eliminate hydroxyl radicals [Khan and Khan, 2004; Martinez-Cayuela, 1995]. These hydroxyl radical scavengers also offered some protection explicating moderate role of these radicals in riboflavin mediated PTPI inactivation. Biological antioxidants such as uric acid, glucose and ascorbic acid were analysed for suppression of PTPI inactivation by riboflavin. Ascorbic acid was found to be most effective.

**Structural modification of PTPI by riboflavin**

The effects of various concentrations of photoilluminated riboflavin were also determined on the structure of PTPI. The samples incubated at increasing riboflavin concentrations (5-50 μM) were subjected to PAGE [Fig. 58]. At 5μM riboflavin concentration, a lighter intensity band was observed. At concentrations above that, till 30 μM, diffused bands were observed. At higher concentration, 40 and 50 μM, aggregation was observed with concomitant loss of native bands.

No aggregated products were observed in treated PTPI in presence of potassium iodide, sodium azide and ascorbic acid [Fig. 59]. Again propounding, flavin triplet and singlet state as main culprits for photodynamic modifications PTPI.

Treatment of PTPI with photo illuminated riboflavin drastically altered its intrinsic fluorescence properties. The fluorescence emission spectra of native PTPI gave an emission maximum at 335 nm when excited at 280 nm [Fig. 60] [Priyadarshini and Bano, 2009]. 1 μM PTPI photoilluminated with increasing concentration of riboflavin 5-50 μM was subjected to fluorescence spectroscopy to assess the effect on global conformation of protein, the samples were excited at 280 nm and emission range was 300-400 nm. Riboflavin treatment upto 10 μM did not induce any change in emission λ_max however ~ 44% decline in fluorescence intensity was precipitated. Beyond this concentration a blue shift of 5 nm (λ_max shifted to 330 nm compared to 335 nm for native) was observed with a profound decline (~75%) in fluorescence intensity [Fig. 60]. Effect of 40 μM riboflavin on intrinsic fluorescence of PTPI (1 μM) was also studied for various time periods (0-60 min). A time dependent gradual loss in fluorescence intensity with a similar blue shift was also observed after 20 min of incubation. To ascertain the effect of various scavengers in subjugating the detrimental consequences of the photodynamic action of riboflavin on structure of
Fig. 58  PAGE of native and riboflavin treated PTPI

Native PTPI (1 μM) was incubated with increasing riboflavin concentrations (5-50 μM) for 30 min under fluorescent light and subjected to non denaturing PAGE. Gels were silver stained.

Fig. 59  PAGE of PTPI exposed to riboflavin in presence of various scavengers

Native PTPI (1 μM) was incubated with 40 μM riboflavin in presence of various scavengers, sodium azide/potassium iodide (final concentration, 25 mM)/ascorbic acid (final concentration, 100 mM) or in presence of curcumin (Cur, final concentration, 100 μM) or caffeic acid (CA)/quercetin (QE) (final concentration, 350 μM) and subjected to non denaturing PAGE. The experimental conditions were same as defined for Fig. 58. Gels were silver stained.
<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribofavin (μM)</td>
<td>-</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribofavin (40 μM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scavenger</td>
<td>-</td>
<td>-</td>
<td>KI</td>
<td>Sod. azide</td>
<td>Ascorbic acid</td>
<td>CA</td>
<td>QE</td>
</tr>
</tbody>
</table>
Fig. 60  Intrinsic fluorescence analysis of PTPI treated with different concentrations of riboflavin

1 μM PTPI was incubated with increasing concentrations of riboflavin (5-50 μM) under fluorescent light for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm. Trace 1 is native PTPI, or PTPI + riboflavin 5 μM (trace 2), 10 μM (trace 3), 20 μM (trace 4), 30 μM (trace 5), 40 μM (trace 6), 50 μM (trace 7).
TABLE 11: RETENTION OF TRYPTOPHAN FLUORESCENCE IN THE PRESENCE OF DIFFERENT SCAVENGERS AND ANTIOXIDANTS

<table>
<thead>
<tr>
<th>Condition</th>
<th>% retention of tryptophan fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM PTPI + 40 μM Riboflavin</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>27.29</td>
</tr>
<tr>
<td>+ Sod. Benzoate</td>
<td>37.15</td>
</tr>
<tr>
<td>+ Mannitol</td>
<td>25.00</td>
</tr>
<tr>
<td>+ KI</td>
<td>77.17</td>
</tr>
<tr>
<td>+ Sod. Azide</td>
<td>77.60</td>
</tr>
<tr>
<td>+ Glucose</td>
<td>66.68</td>
</tr>
<tr>
<td>+ Ascorbic acid</td>
<td>76.35</td>
</tr>
<tr>
<td>+ Cur</td>
<td>59.15</td>
</tr>
<tr>
<td>+ CA</td>
<td>75.16</td>
</tr>
<tr>
<td>+ QE</td>
<td>66.70</td>
</tr>
</tbody>
</table>

Results are % retention of tryptophan fluorescence emission intensity (which is dependent upon but not synonymous with the number of tryptophan residues) measured at 280 nm excitation and 300-400 nm emission with a slit width of 5 nm. Experiment details are as described in methods section.
PTPI, fluorescence spectra of treated PTPI in presence of scavengers was obtained and percent retention of tryptophan fluorescence was determined. The results are summarized in Table 11. Treated PTPI retained only 27% of native tryptophan fluorescence. Reaffirming the involvement of flavin triplet state and singlet oxygen in photodynamic modification of PTPI by riboflavin, maximum retention of tryptophan fluorescence (~78%) was seen in presence of potassium iodide and sodium azide. Mannitol and sodium benzoate exhibited <50% retention, implicating only faint involvement of hydroxyl radicals. Among biological antioxidants, ascorbic acid again showed maximum protection.

3.4.1B PROTECTIVE EFFECT OF BIOFLAVONOIDS

Curcumin (Cur), Caffeic acid (CA) and Quercetin (QE) against photodynamic modifications of PTPI

In quest of finding natural alternatives to scavengers, Cur, CA and QE were examined for their potency to revert/prevent any modification induced in PTPI by photosensitized riboflavin. Preliminary experiments were conducted to determine the most effective concentration of above three compounds. 350 μM CA and QE prevented loss of antiproteolytic activity of PTPI [Fig. 57B]. Aggregation of PTPI was also restrained by QE and CA [Fig. 59]. In presence of 350 μM CA, ~75% of tryptophan fluorescence was retained [Table 11]. QE afforded retention of tryptophan fluorescence to an extent similar to glucose. Cur (100 μM) however exerted only moderate preventive influence in all the features studied.

3.4.2A INTERACTION OF PTPI WITH HYDROGEN PEROXIDE (H₂O₂)

Hydroperoxides such as H₂O₂ and lipid hydroperoxides have been implicated as mediators of cellular injuries in a variety of clinical conditions including pancreatitis, cancer, etc. [Pryor et al., 2006]. Thus, deleterious effects of H₂O₂ and protective effects of polyphenols on PTPI were assessed.

Functional modification of PTPI by H₂O₂

H₂O₂ caused only modest inactivation of PTPI. Even at high concentrations of H₂O₂ (500 mM) only 50% of antiproteolytic activity of PTPI was compromised
[Table 12A]. To study the impact of H$_2$O$_2$ on activity of PTPI as a function of time, 1 µM PTPI was incubated with 250 mM H$_2$O$_2$ in dark for varying time periods (0-60 min) and activity of PTPI was determined [Kunitz, 1947]. A slow and gradual decline in activity was observed culminating to only a 50% loss [Table 12B]. The effect of various scavengers on H$_2$O$_2$ induced PTPI inactivation was studied. Sodium benzoate and thiourea did not show any protection. Mannitol offered 27% enhancement of the papain inhibitory activity of treated PTPI. Since mannitol, sodium benzoate and thiourea are specific hydroxyl scavengers, this suggests only partial involvement of these radicals in H$_2$O$_2$ mediated PTPI damage [Fig. 61]. Among biological antioxidants 25 mM glucose offered better protection than 100 mM ascorbic acid.

**Structural modification of PTPI by H$_2$O$_2$**

1 µM PTPI was exposed to (1-500 mM) H$_2$O$_2$ in dark for 30 min and the samples were analyzed by polyacrylamide gel electrophoresis. There was only a slight decline in band intensity as compared to the untreated PTPI, which was maximized at 500 mM H$_2$O$_2$. In parallel to modest inactivation of the inhibitor, the gross conformational status of PTPI remained largely unaffected even when exposed to high H$_2$O$_2$ concentration [Fig. 62]. In the presence of the scavenger mannitol and physiological antioxidant, glucose, the band intensity was retrieved to that of native PTPI [Fig. 63].

Gel results are presented for most conspicuous effects only.

The intrinsic fluorescence spectra of samples treated with various concentrations of H$_2$O$_2$ are depicted in Fig. 64. Complex changes were observed in contrast to the results of inactivation and PAGE. PTPI under native conditions gives fluorescence emission spectrum with maximum at 335 nm. Even at 1 mM H$_2$O$_2$, the emission $\lambda_{max}$ suffered a red shift of 5 nm, with pronounced enhancement in intensity. No changes were noticeable till 50 mM H$_2$O$_2$ (spectra not shown). At higher concentrations fluorescence intensity decreased, reaching to that of native at 250 mM H$_2$O$_2$, while red shift to 340 nm was maintained. At 500 mM H$_2$O$_2$, $\lambda_{max}$ suffered another red shift of 5 nm, reaching a value of 350 nm. When studied as a function of time, within 20 min, 250 mM H$_2$O$_2$ produced a red shift of 5 nm in $\lambda_{max}$. Within 60 min $\lambda_{max}$ shifted to 350 nm from 335 nm for the native. Conclusively, H$_2$O$_2$ causes unfolding of PTPI.

Keeping in view the complex alterations in fluorescence intensity of PTPI in presence of H$_2$O$_2$, regain of native $\lambda_{max}$ of emission was chosen as a criterion to judge the effects of various scavengers on PTPI (1 µM) in presence of 250 mM H$_2$O$_2$. 
TABLE 12: LOSS OF ANTIPROTEOLYTIC ACTIVITY OF PTPI ON TREATMENT WITH H₂O₂ AS A FUNCTION OF
(A) CONCENTRATION OF H₂O₂ AND
(B) TIME OF INCUBATION

(A)

<table>
<thead>
<tr>
<th>H₂O₂ (mM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity(^a)</td>
<td>100</td>
<td>97.00±3.8 ((-3.0))</td>
<td>96.00±3.9 ((-4.0))</td>
<td>83.00±3.9 ((-17.0))</td>
<td>74.70±3.4 ((-25.3))</td>
<td>66.40±2.1* ((-33.6))</td>
<td>41.5±1.8* ((-58.5))</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity(^a)</td>
<td>100</td>
<td>92.12±3.8 ((-8.0))</td>
<td>87.18±3.5 ((-13))</td>
<td>68.10±2.3* ((-32))</td>
<td>59.25±2.8* ((-41))</td>
<td>50.18±2.3* ((-50))</td>
<td>43.53±1.9* ((-56))</td>
</tr>
</tbody>
</table>

PTPI (1μM) was incubated with increasing concentrations of H₂O₂ in dark for 1 h or with 250 mM H₂O₂ for increasing intervals of time.
\(^a\)PTPI was assayed for loss in antiproteolytic activity by caseinolytic method of Kunitz [1947]. The activity of native PTPI is taken to be 100. Results are Mean±SEM for three or more separate experiments.
* Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.
Values in parentheses represent percent change from control.
Fig. 61  Effect of scavengers on H₂O₂ mediated inactivation of PTPI

Native PTPI (1 µM) was incubated with H₂O₂ (250 mM) alone or in presence of mannitol/glucose (final concentration, 25 mM)/ascorbic acid (final concentration, 100 mM) or curcumin (Cur 100 µM)/ caffeic acid (CA 60 µM )/quercetin (QE 50 µM ) for 30 min in a final reaction volume of 1 ml at room temperature in dark and was assayed for its antiproteolytic activity. None of the scavengers used had any effect on PTPI assay. Data are expressed as Mean ± SEM for four experiments. * p < 0.05 compared to PTPI+H₂O₂ alone.

Fig. 62  PAGE of native and H₂O₂ treated PTPI

Native PTPI (1 µM) was incubated with increasing H₂O₂ concentrations (1-500 mM) for 30 min in dark subjected to non denaturing PAGE. Gels were silver stained.
Native PTPI +H₂O₂

Lane | a | b | c | d | e | f | g
---|---|---|---|---|---|---|---
PTPI | + | + | + | + | + | + | +
H₂O₂ (mM) | - | 1 | 10 | 50 | 100 | 250 | 500

Scavengers

- Mannitol
- Ascorbic acid
- Cur
- CA
- QE
Fig. 63  PAGE of PTPI exposed to $\text{H}_2\text{O}_2$ in presence of various scavengers

Native PTPI (1 $\mu$M) was incubated with 250 mM $\text{H}_2\text{O}_2$ in presence of various scavengers, mannitol/glucose (final concentration, 25 mM) or in presence of curcumin (Cur, final concentration, 100 $\mu$M) or caffeic acid (CA)/quercetin (QE) (final concentration, 60 and 50 $\mu$M, respectively) and subjected to non denaturing PAGE. The experimental conditions were same as defined for Fig. 61. Gels were silver stained.

Fig. 64  Intrinsic fluorescence analysis of PTPI treated with different concentrations of $\text{H}_2\text{O}_2$

1 $\mu$M PTPI was incubated with increasing concentrations of $\text{H}_2\text{O}_2$ in dark for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm. Trace 1 is native PTPI. Trace 2 is PTPI treated with 1 mM $\text{H}_2\text{O}_2$. Trace 3 represents PTPI treated with 100 mM $\text{H}_2\text{O}_2$. Trace 4 and 5 are PTPI treated with 250 mM and 500 mM $\text{H}_2\text{O}_2$. 
<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H$_2$O$_2$ (mM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scavenger</td>
<td>-</td>
<td>-</td>
<td>Mannitol</td>
<td>Glucose</td>
<td>Cur</td>
<td>CA</td>
<td>QE</td>
</tr>
</tbody>
</table>

![Fluorescence Intensity vs Wavelength Graph](image)
Thiourea and sodium benzoate did not show any impact. Glucose and ascorbic acid brought back the $\lambda_{\text{max}}$ to 335 nm. Mannitol gave same effect however fluorescence intensity of the treated PTPI in its presence remained quenched below that of untreated PTPI.

3.4.2B PROTECTIVE EFFECT OF BIOFLAVONOIDS

Curcumin (Cur), caffeic acid (CA) and quercetin (QE) against $\text{H}_2\text{O}_2$ mediated modifications of PTPI

There is limited number of reports indicating direct subjugation of oxidative damage inflicted by $\text{H}_2\text{O}_2$ on proteins in vitro. Curcumin (Cur), caffeic acid (CA) and quercetin (QE) were probed for their suppressive effects against the adverse consequences of $\text{H}_2\text{O}_2$ on PTPI.

As shown in Fig. 61, among the three bioantioxidants, most substantial increment (45%) in the treated PTPI’s inhibitory activity was brought about by 100 $\mu$M Cur, followed by a 35% enhancement spawned by 60 $\mu$M CA. QE (50 $\mu$M) showed a mediocre gain of 30% in activity. However, all of the three antioxidants, generated native like band pattern of PTPI even in the presence of 250 mM $\text{H}_2\text{O}_2$ [Fig. 63]. Intrinsic fluorescence properties of treated PTPI were also brought back to native in the presence of Cur, CA and QE.

3.4.3A INTERACTION OF PTPI WITH HYPOCHLOROUS ACID (HOCl)

Myeloperoxidase released by phagocytic cells at sites of inflammation, catalyzes the formation of potent chlorinating/oxidising agent hypochlorous acid from $\text{H}_2\text{O}_2$ and chloride ions. It is a recognized DNA and protein damaging agent [Jenner et al., 2002]. Effects of HOCl on function and structure of the purified inhibitor were analyzed.

Functional modification of PTPI by HOCl

The exposure of 1 $\mu$M PTPI to varying HOCl concentrations resulted in a dramatic change in its antiproteolytic potential. At concentration as low as 0.5 $\mu$M, HOCl incited 34% loss in activity of PTPI. At equimolar concentrations with the inhibitor, 60% PTPI activity was compromised. The inhibitor was barely active at 5 $\mu$M HOCl (~90% loss in activity). Beyond which no papain inhibitory activity was detected
Since in vivo many physiological antioxidants will be present together with PTPI, the protective effect of two, namely glucose and ascorbic acid was studied [Fig. 65]. The HOCl mediated loss in activity, was diminished in the presence of ascorbic acid and glucose, with ascorbic acid exerting a better protection.

**Structural modification of PTPI by HOCl**

1 μM PTPI was incubated with increasing concentrations of HOCl. The samples were analyzed for impact on structural integrity of PTPI employing PAGE. As shown in Fig. 66, significant loss of the parent protein band was observed even at HOCl: PTPI ratio of 0.5:1. There was a progressive increase in fragmentation of the inhibitor (as assessed by the loss of parent protein band) at higher ratios with complete loss of any staining material at HOCl: PTPI ratio of 10:1. Physiological antioxidants glucose and ascorbic acid were expected to protect PTPI fragmentation. Fig. 67 illustrates the result obtained. 25 mM glucose and 100 mM ascorbic acid afforded significant protection.

The intrinsic fluorescence properties of PTPI were also adversely affected. As shown in Fig. 68 fluorescence intensity declined with increase in HOCl concentration. No fluorescence was observed beyond 5 μM HOCl. Interestingly, emission $\lambda_{\text{max}}$ remained unaffected at all concentrations of HOCl. The protective effects of glucose and ascorbic acid were observed in retaining tryptophan fluorescence (~40% and ~45%, respectively) even in the presence of 5 μM HOCl.

**3.4.3B PROTECTIVE EFFECT OF BIOFLAVONOIDS**

**Curcumin (Cur), caffeic acid (CA) and quercetin (QE) against HOCl mediated modifications of PTPI**

Flavonoids have been extensively studied for their antioxidant properties against various free radicals. However, their effect on hypochlorous acid mediated damage is less known. Thus, present work was also aimed at exploring the potential of Cur, CA and QE against hypochlorite inflicted damage. As depicted in Fig. 65, 100 μM each of CA, Cur and QE, diminished the extent of loss of PTPI activity by several times causing the papain inhibitory potential of PTPI to remain close to that of native. Similar protection was also noticed in PAGE [Fig. 67]. All three antioxidants subjugated structural damage of PTPI caused by HOCl. Tryptophan fluorescence, lost
TABLE 13: LOSS OF ANTIPROTEOLYTIC ACTIVITY OF PTPI ON TREATMENT WITH HOCl AS A FUNCTION OF CONCENTRATION OF HOCl

<table>
<thead>
<tr>
<th>HOCl (μM)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>2.5</th>
<th>3.0</th>
<th>5.0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity*</td>
<td>100</td>
<td>66.40±2.8*</td>
<td>39.84±1.1*</td>
<td>22.34±0.82*</td>
<td>17.93±0.68*</td>
<td>9.21±0.13*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-33.6)</td>
<td>(-60.16)</td>
<td>(-78.0)</td>
<td>(-82.07)</td>
<td>(-90.04)</td>
<td></td>
</tr>
</tbody>
</table>

PTPI (1μM) was incubated with increasing concentrations of HOCl for 30 min.
*PTPI was assayed for loss in antiproteolytic activity by caseinolytic method of Kunitz [1947]. The activity of native PTPI is taken to be 100. Results are Mean±SEM for three or more separate experiments.
* Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.
Values in parentheses represent percent change from control.
Fig. 65  Effect of scavengers on HOCl mediated inactivation of PTPI

Native PTPI (1 μM) was incubated with HOCl (5 μM) alone or in presence of glucose (final concentration, 25 mM)/ascorbic acid (final concentration, 100 mM) or curcumin (Cur) / caffeic acid (CA) /quercetin (QE) (final concentration, 100 μM, respectively) for 30 min in a final reaction volume of 1 ml at room temperature and was assayed for its antiproteolytic activity. None of the scavengers used had any effect on PTPI assay. Data are expressed as mean ± SEM for four experiments. * p < 0.05 compared to PTPI+HOCl alone.

Fig. 66  PAGE of native and HOCl treated PTPI

Native PTPI (1 μM) was incubated with increasing HOCl concentrations (0.5-10 μM) for 30 min followed by the addition of 100 μM GSH to quench residual oxidants and subjected to non denaturing PAGE. Gels were silver stained.
The graph shows the inhibitory activity of different treatments compared to Native PTPI. The treatments include PTPI + HOCl, + Glc., + Ascorbic acid, + Cur, + CA, and + QE. The graph indicates a significant increase in inhibitory activity with certain treatments, as marked with asterisks. The table below provides the concentrations of HOCl in each lane:

<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HOCl</td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
Fig. 67  **PAGE of PTPI exposed to HOCl in presence of various scavengers**

Native PTPI (1 μM) was incubated with 5 μM HOCl in presence of scavengers, glucose (final concentration, 25 mM) or ascorbic acid (final concentration, 100 mM) or in presence of curcumin (Cur)/caffeic acid (CA)/quercetin (QE) (final concentration, 100 μM) followed by the addition of 100 μM GSH to quench residual oxidants and subjected to non-denaturing PAGE. The experimental conditions were same as defined for Fig. 66. Gels were silver stained.

Fig. 68  **Intrinsic fluorescence analysis of PTPI treated with different concentrations of HOCl**

1 μM PTPI was incubated with increasing concentrations of HOCl for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The reaction was stopped by adding 100 μM GSH. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm. Trace 1 is native PTPI, or PTPI + HOCl 0.5 μM (trace 2), 1 μM (trace 3), 3 μM (trace 4), 5 μM (trace 5).
PTPI  HOCl (5 nM)  Scavenger

<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HOCl (5 μM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scavenger</td>
<td>-</td>
<td>-</td>
<td>Glucose</td>
<td>Ascorbic acid</td>
<td>Cur</td>
<td>CA</td>
<td>QE</td>
</tr>
</tbody>
</table>

Wavelength (nm)

Fluorescence Intensity

Wavelength (nm)
to 5 μM HOCl was ~99% retrieved [Fig. 69]. Percent retention of tryptophan fluorescence was ~71% and ~44% in presence of Cur and QE, respectively.

3.4.4A INTERACTION OF PTPI WITH NITRIC OXIDE (NO)

It is widely accepted that enhanced NO formation [and subsequent generation of complex cocktail of cytotoxic and oxidant reactive nitrogen species] contributes to oxidative and nitrosative stress in a variety of pancreatic and cardiovascular pathologies [Lenzen, 2008a; Denicola and Radi, 2005]. Thus, studies were undertaken to study the effects of NO on function and structure of purified pancreatic thiol proteinase inhibitor (PTPI). Also Cur, CA and QE were analyzed for their efficacies in ameliorating the detrimental effects of NO on PTPI.

**Generation of NO**

Sodium nitroprusside (SNP) was used as a selective NO donor. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ion which can be estimated using Griess reagent. Incubation of solutions of SNP in phosphate buffered saline at 25°C resulted in linear time-dependent nitrite production.

**Functional modification of PTPI by NO**

1 μM PTPI was incubated with increasing concentrations of SNP (0.05 to 10 mM) for 30 min and its papain inhibitory activity was determined by caseinolytic assay of papain [Kunitz, 1947]. The functional loss of PTPI inhibitory activity was proportional to SNP concentration [Table 14]. Approximately 37% loss in activity was incurred by 0.05 mM SNP. At 1 mM SNP, a significant decline (72%) of native PTPI activity was observed with complete loss at 10 mM SNP concentration. Time dependent loss in PTPI activity was also monitored. PTPI (1 μM) was incubated with 0.05 mM SNP, and activity was determined for varying time intervals (0-180 min). There was a gradual decrease in inhibitory activity till 45 min. A sharp decline (60%) was observed after 60 min of incubation, 94% at 150 min, with no activity detected after 180 min of incubation.

**Structural modification of PTPI by NO**

Effect of NO on structural integrity of PTPI was examined by PAGE. No major
1 μM PTPI was incubated with 5 μM HOCl in presence of curcumin (Cur)/caffèic acid (CA)/quercetin (QE) (final concentration, 100 μM) for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The reaction was stopped by adding 100 μM GSH. The excitation wavelength was 280 nm and emission was recorded in range of 300–400 nm with a slit width of 5 nm.
<table>
<thead>
<tr>
<th>SNP (mM)</th>
<th>0</th>
<th>0.05</th>
<th>1.0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PTPI Activity$^a$</td>
<td>100</td>
<td>62.75±2.4* (-37.30)</td>
<td>28.00±0.92* (-72.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>%PTPI Activity$^a$</td>
<td>100</td>
<td>87.27±3.8 (-13.0)</td>
<td>65.42±3.1* (-35.0)</td>
<td>61.09±2.7* (-39.0)</td>
<td>40.13±1.5* (-60.0)</td>
<td>28.61±0.72* (-71.4)</td>
<td>12.10±0.56* (-88.0)</td>
<td>6.12±0.09* (-94)</td>
<td>-</td>
</tr>
</tbody>
</table>

PTPI (1μM) was incubated with increasing concentrations of SNP for 30 min or with 0.05 mM SNP for increasing intervals of time.
$^a$PTPI was assayed for loss in antiproteolytic activity by caseinolytic method of Kunitz [1947]. The activity of native PTPI is taken to be 100.
Results are Mean±SEM for three or more separate experiments.
* Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.
Values in parentheses represent percent change from control.
modifications were observed. The parent band pattern was conserved at all concentrations except that band intensity was slightly increased [Fig. 70].

The modifications of amino acid residues of PTPI consequential to NO exposure were analyzed by intrinsic fluorescence spectroscopy by monitoring the changes in intensity and $\lambda_{\text{max}}$. The results are summarized in Fig. 71. Fluorescence intensity was considerably quenched at 0.05 mM SNP with a 5 nm red shift (from 335 nm for untreated PTPI to 340 nm). A further increase in concentration of SNP to 1 mM, quenched the intensity to 77.4% with another 5 nm red shift (to 350 nm). At 10 mM SNP, fluorescence was completely quenched. Conclusively, NO lead to quenching of fluorescence coupled to 15 nm red shift in $\lambda_{\text{max}}$.

When analyzed as a function of time, the decrease in fluorescence intensity was triggered within 15 min of incubation and culminated to ~71% decline at 180 min of incubation. The red shift of 15 nm precipitated within 60 min of incubation.

### 3.4.4B PROTECTIVE EFFECT OF BIOFLAVONOIDS

Curcumin (Cur), caffeic acid (CA) and quercetin (QE) against NO mediated modifications of PTPI

A great deal of evidence has amassed pointing to reactive nitrogen species (RNS) as the main contributors to nitrosative stress in a variety of pathologies. Therefore, targeting NO (and NO congeners) either directly by scavengers or indirectly by inhibitors of its downstream targets are exciting therapeutic strategies. In this respect, plant polyphenols offer to be attractive candidates. Present study was undertaken to examine the anti-nitrosative efficacies of CA, QE and Cur in reclamation of the NO induced modifications of PTPI.

For these studies, 1 pM PTPI was incubated with 1 mM SNP and the samples were analyzed by caseinolytic assay of papain [Kunitz, 1947] and fluorescence spectroscopy. NO mediated a decline in activity (72% loss, Table 14). 20 pM Cur prompted restoration of lost activity near to native [Fig. 72]. 80 pM each of QE and CA inhibited the loss to a similar extent. These bioflavonoids were also analyzed for their potency to restore the structural changes induced by NO on PTPI. As shown in Fig. 73 all the three natural antioxidants negated the loss in tryptophan fluorescence and retrieved the native like fluorescence pattern in treated PTPI. Maximum protection was offered by CA and Cur.
Fig. 70  PAGE of native and SNP treated PTPI

Native PTPI (1 μM) was incubated with increasing SNP concentrations 0.05, 1 and 10 mM, for 30 min and subjected to non denaturing PAGE. Gels were silver stained.

Fig. 71  Intrinsic fluorescence analysis of PTPI treated with different concentrations of SNP

1 μM PTPI was incubated with increasing concentrations of SNP for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm. Trace 1 is native PTPI. Trace 2 is 0.05 mM, trace 3 is 1 mM and trace 4 is 10 mM SNP treated PTPI.
<table>
<thead>
<tr>
<th>Lane</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SNP (mM)</td>
<td>-</td>
<td>0.05</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

![Image of gel electrophoresis with lanes labeled a, b, c, d.](image)

![Graph showing fluorescence intensity vs. wavelength (nm).](graph)
**Fig. 72** Effect of natural antioxidants on SNP mediated inactivation of PTPI

Native PTPI (1 μM) was incubated with SNP (1 mM) alone or in presence of curcumin (Cur)/caffeic acid (CA)/quercetin (QE) (final concentration, 20 μM for Cur and 80 μM for CA and QE) for 30 min in a final reaction volume of 1 ml at room temperature and was assayed for its antiproteolytic activity. None of the scavengers used had any effect on PTPI assay. Data are expressed as Mean ± SEM for four experiments. * p < 0.05 compared to PTPI+SNP alone.

**Fig. 73** Intrinsic fluorescence analysis of PTPI treated with SNP in presence of natural antioxidants

1 μM PTPI was incubated with 1 mM SNP in presence of curcumin (Cur)/caffeic acid (CA)/quercetin (QE) (final concentration, 20 μM for Cur, 80 μM for CA and QE) for 30 min in a final reaction volume of 1 ml in 50 mM sodium phosphate (pH 7.5) at room temperature. The excitation wavelength was 280 nm and emission was recorded in range of 300-400 nm with a slit width of 5 nm.
### Graph 1

- **Y-axis:** % Inhibitory Activity
- **X-axis:** Scavengers

- **Legend:**
  - Native PTPI
  - PTPI + 1 mM SNP
  - PTPI + 1 mM SNP + Cur
  - PTPI + 1 mM SNP + CA
  - PTPI + 1 mM SNP + QE

### Graph 2

- **Y-axis:** Fluorescence Intensity
- **X-axis:** Wavelength (nm)

- **Graph Details:**
  - Native PTPI
  - PTPI + 1 mM SNP
  - PTPI + 1 mM SNP + Cur 20 µM
  - PTPI + 1 mM SNP + CA 80 µM
  - PTPI + 1 mM SNP + QE 80 µM
Discussion

Chapter 4
DISCUSSION

Reactive species (ROS/RNS) like superoxide anions, hydroxyl radicals, hydrogen peroxide, and nitric oxide are the intermediates of regular pathway of aerobic metabolism and processes. These reactive species generated from normal metabolism or exogenous insults lead to peroxidation of membrane lipids and damage to cellular macromolecules like DNA and proteins. Aerobic organisms synthesize enzymes devoted to the prevention and repair of oxidative damage and extreme genetic deficiency in either of these two defences can lead to inviability in aerobic environments. The uncontrolled oxidative stress initiates a series of harmful biochemical events associated with diverse pathological processes [Droge, 2002].

There are numerous indications that ROS/RNS play significant role in various pancreatic pathologies including diabetes and its secondary complications like retinopathy, nephropathy and neuropathy, in chronic and acute pancreatitis and in pancreatic stellate cell fibrosis [Chvanov et al., 2005; Pacher et al., 2005; Lenzen, 2008a]. Interestingly, pancreas has been found to be exceptionally liable to oxidative and nitrosative damage as a consequence of its feeble antioxidant defence [Lenzen, 2008a, Neuschwander-Tetri et al., 1997].

Pancreatic thiol proteinases, cathepsin B, H and calpains are involved in activation of zymogen and insulin secretion and action [Thrower et al., 2006; Sreenan et al., 2001]. Their activity is primarily regulated by thiol proteinase inhibitors. In traumatic processes usually the balance between the lysosomal proteinases released by macrophages and neutrophils and their endogenous inhibitors is disturbed. This imbalance may originate from various reasons one of which may be inactivation by radical species viz. H2O2, HOCl, ROS, NO (and RNS).

The present work was thus outlined to deduce the effect of exposure of purified PTPI, in vitro, to photoilluminated riboflavin (mimicking the biological subjection to oxygen radicals [Tsai et al., 1985], non-radical oxidants H2O2 and HOCl and NO (generated from SNP), on its function and structure. The results evinced the susceptibility of PTPI to aforementioned reactive species and the modifications induced included altered molecular weight (aggregation or fragmentation), loss of fluorescence and inhibitory function.

Photosensitized riboflavin produces ROS [Husain et al., 2006] whose damaging effects have been documented in various proteins [Ali et al., 1991; Jazzar and Naseem,
Among the various forms of ROS generated, singlet oxygen is of particular physiologic significance because of its selectively long life in aqueous solution, its ability to cross cell membrane barrier and high reactivity towards biomolecules [Joshi, 1998].

The results reveal the loss of PTPI function on exposure to photosensitized riboflavin. This bears similarity to sheep plasma high molecular weight kininogen (HMWK) [Baba et al., 2004], membrane proteins [Ali et al., 1991], BSA, invertase, lysozyme [Jazzar and Naseem, 1994] and trypsin [Husain et al., 2006; Hasan et al., 2006] damage caused by photosensitized riboflavin. Results obtained with specific ROS scavengers suggested that singlet oxygen and flavin triplet state were predominantly responsible for PTPI damage. Same results are reported for sheep HMWK and catalase [Baba et al., 2004; Gantchev and van Lier, 1995] showing that singlet oxygen and radical species can participate in photosensitizer-induced inactivation of enzymes. Riboflavin at higher concentration (40 and 50 μM) generated aggregate formation. This behaviour has also been reported earlier for sheep plasma HMWK [Baba et al., 2004] and goat brain cystatin [Sumbul and Bano, 2008]. Owing to the absence of free sulphhydryls in purified PTPI [Priyadarshini and Bano, 2009] these aggregates might have formed due to the exposed hydrophobic patches. This is supported by the altered fluorescence of PTPI in presence of photoilluminated riboflavin (a 5 nm blue shift in \( \lambda_{\text{max}} \), from 335 nm for native to 330 nm for the treated PTPI) and enhanced ANS binding to treated PTPI.

The reactive species like \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) have been recognized as hallmark of inflammation [Martinez-Cayuela, 1995]. Macrophages and neutrophils reduce molecular oxygen to superoxide anion, as a part of host defence system to neutralize the invading pathogens [Marnett et al., 2003]. The superoxide produced is rapidly dismutated to hydrogen peroxide. Activated neutrophils release the enzyme myeloperoxidase that reacts with \( \text{H}_2\text{O}_2 \) and chloride ions present to form \( \text{HOCl} \) [Marnett et al., 2003], a reactive oxygen metabolite, that can modify amino acid residues, induce conformational changes in proteins and inactivate enzyme and enzyme inhibitors [Shechter et al., 1975; Wasil et al., 1987; Dean et al., 1997]. \( \text{HOCl} \), a strong oxidant, is known to oxidize many important biomolecules such as DNA, proteins, enzymes and antiproteinases [Jerlich et al., 2000; Whiteman et al., 2003; Khan and Khan, 2004; Szuchman-Sapir et al., 2008].
The results reveal that PTPI, in the presence of HOCl lost its antiproteinase activity rapidly (within 30 min, 90% loss occurred at 5 μM HOCl). Inactivation of proteins like of α2 M, by HOCl has been registered earlier [Khan and Khan, 2004]. Whiteman et al. [2003] reported a 90% loss in activity of isolated α1 antiproteinase in presence of 7 μM HOCl. Protein inactivation by HOCl may occur through multiple mechanisms, like, oxidation of critical amino acid residues e.g. cysteine [Tyagi, 1991], methionine [Moreno and Pryor, 1992]; modification of tyrosines [Feste and Gan, 1981] as well as other residues [Whiteman, 1998]. PTPI inactivation may also be consequent to oxidation/ modification (chlorination) of residues crucial to its activity or due to diminished conformational integrity changing the active site region of the inhibitor.

H₂O₂ was found to be a modest inactivator of PTPI relative to HOCl (and other radicals studied). The treated inhibitor lost only 50% of its antiproteolytic activity even in presence of 500 mM H₂O₂ [Table 12A]. Use of scavengers reflected H₂O₂ as the damaging agent itself with only partial involvement of hydroxyl radicals. Non-radical (molecular reactions) driven damage of proteins (e.g. lens crystallins and α2 M, in vitro) by H₂O₂ has been reported earlier [Khan and Khan, 2004; McNamara and Augusteyn, 1984]. The conformational status of H₂O₂-treated PTPI remained largely unchanged [Fig. 62]. Material with slightly higher mobility appeared at H₂O₂ concentration of 100 mM and above, with a little loss in the band intensity as compared to the parent protein band. Protein fragmentation by H₂O₂ has been shown for BSA by Hunt et al. [1988]. Correspondingly, significantly higher mobility material was observed for HOCl treated PTPI. Glucose and ascorbic acid lodged prevention of protein fragmentation [Fig. 66 and 67]. In case of H₂O₂, marked red shift of 15 nm was observed indicating change in the microenvironment of tryptophan residues towards polar [Fig. 64], and the loss of native folded state of PTPI. While in presence of HOCl, fluorescence of PTPI was quenched completely beyond 5 μM HOCl [Fig. 68] without any change in λ_{max}. In H₂O₂ mediated damage, the higher mobility material consists of fragmentation products, and the unfolding might have facilitated the protein fragmentation [Davies and Delsignore, 1987].

HOCl usually causes aggregation of proteins as has been shown for fibronectin [Vissers and Winterbourn, 1991], apolipoprotein A-I [Bergt et al., 2001], caprine α2 M [Khan and Khan, 2004], ovalbumin [Olszowski et al., 1996], apohaemoglobin and apomyoglobin [Chapman et al., 2003]. Protein fragmentation by HOCl is substantiated from reports on serum albumin, Cu/Zn SOD, and glucose-6-phosphate
dehydrogenase [Hawkins and Davies, 1998; Ullrich et al., 1999; Auchere and Capeillere-Blandin, 2002]. HOCl reacts with amide groups of protein backbone and also with free amino groups of lysine residues of proteins yielding chloramines. These chloramines can then effectuate protein fragmentation. At low HOCl concentrations preferably lysines are modified limiting the rapid backbone fragmentation. Further, extent of fragmentation at low HOCl concentrations is defined by number of lysine residues in proteins [Hawkins and Davies, 1998]. The fluorescence profile of HOCl-treated PTPI reveals complete loss of intensity at concentrations above 5 μM without any change in λ_{max} of emission. There was a HOCl concentration dependent increase in higher mobility material when treated PTPI was subjected to PAGE [Fig. 66] with complete loss of any staining material at 10 μM HOCl. Separate bands were not discernible at any HOCl concentration. This suggests that HOCl even at very low concentrations (protein:HOCl ratio of 1:0.5) causes backbone fragmentation releasing smaller fragments which make up for the appearance of higher mobility material in PAGE. Upto protein:HOCl ratio of 1:5, though fragmentation was induced [Fig. 66], native PTPI might have existed in significant proportions defining the unaltered λ_{max}. Also, HOCl is prejudiced to modify tyrosine, phenylalanine (by chlorination), cysteine, methionine (by sulphotidation and oxy-acid formation) than to oxidize tryptophan [Stadtman and Levine, 2003]. This might also explain the unaltered λ_{max} of PTPI mainly attributable to tryptophan.

NO is a highly reactive free radical gas, which can participate as a cytotoxic effector molecule and/or pathogenic mediator when produced at high rates by inflammatory stimuli induced nitric oxide synthase or over stimulation of constitutive forms of enzyme [Radi, 2004]. In contrast to the protective action of low levels of NO, its excess has injurious outcomes due to exacerbated oxidative damage [Pryor, 2006]. NO also caused extensive loss of PTPI function. Presumably NO enfeebled PTPI in quite a similar fashion as HOCl (by modification or damage of critical amino acid residues). A number of reports catalogue protein inactivation by NO through amino acid modification e.g. goat lung cystatin [Khan et al., 2009], catalase [Sigfrid et al., 2003; Brunelli et al., 2001] and cytochrome P450 [Wink et al., 1993] etc.

NO produced a red shift of 15 nm and PTPI was completely unfolded at higher concentration [Fig. 71]. Such effect has also been reported for goat lung cystatin [Khan et al., 2009]. There were no significant changes in the mobility of NO-treated PTPI when subjected to PAGE. However, increased band intensity was observed as
compared to native PTPI band. This may be due to increased reaction of NO-treated PTPI with silver stain reagent [Davies and Delsignore, 1987]. The drastic change in fluorescence profiles of NO-treated PTPI compared to unnoticeable conformational change on PAGE analysis can be supported to some extent by the initial association of oxidatively generated fragments of PTPI in a conformation similar to that of native protein [Dean et al., 1997]. Similar results were obtained for BSA damaged by hydroxyl radicals, in which initially only very small amount of molecules with changed size were detected [Hazell et al., 1994; Fisher and Stadtman, 1992].

**DIETARY ANTIOXIDANTS AS STRATEGIES AGAINST FREE RADICAL DAMAGE**

Many antioxidant defences, enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (ascorbic acid, uric acid, glucose, glutathione, vitamin E) exist to maintain the balance between reactive species production and neutralization. However, they fail to provide complete protection against conditions of severe oxidative stress [Cesaratto et al., 2004]. Also the non enzymatic oxidants are relatively inefficient as high concentrations are required to prevent oxidative damage. This inefficiency is reinforced by the observation that protein derived radicals can be detected in plasma treated with low concentrations of HOCl even when endogenous antioxidants were present [Hawkins and Davies, 1998; Dean et al., 1997]. Besides, many synthetic antioxidants have shown toxic and/or mutagenic effects. These observations shifted the attention towards naturally occurring antioxidants. Therefore, experiments were also designed to address these issues. Three plant components were chosen, whose protective effects in various pancreatic (and other organ) pathologies have already been registered, viz. Curcumin (Cur), Caffeic acid (CA) and Quercetin (QE) [Kanitkar et al., 2008; Jung et al., 2006; Kim et al., 2007]. These bioflavonoids are known for exerting pleiotropic health benefit through their antioxidant, anti-inflammatory, antimicrobial, anticancer, antidiabetic activities [Aggarwal et al., 2007; Takahama et al., 2009; Sreejayan and Rao, 1997].

Efficacy of these natural antioxidants against the detrimental effects of photosensotized riboflavin, H$_2$O$_2$, HOCl, and NO on PTPI, in vitro, was evaluated. Concentration dependent protective effect was observed for all the three, for all four
radical systems (results not shown). Only the most effective concentration of each, in every radical system is described.

Riboflavin mediated photodynamic damage of PTPI was considerably diminished by QE and CA. Cur provided only partial moderation. QE and CA (350 μM each) caused manifold enhancement in the activity of treated PTPI [Fig. 57B]. Aggregation (observed at 40 μM riboflavin) was also inhibited and tryptophan fluorescence retrieved back. However, Cur failed to show any preventive effects in the latter two cases. Major damage on PTPI by photoilluminated riboflavin was incurred by singlet oxygen and flavin triplet state. High concentration of phenolics has been shown to quench these two radical states [Cardoso et al., 2006]. Such effect was also provided by rutin, catechin and epigallocatechin gallate [Becker et al., 2005].

Direct mitigation of H₂O₂ toxicity in in vitro assay systems finds rare documentation. In the present study, PTPI inactivation (though modest), unfolding and loss in conformational integrity, induced by H₂O₂ was alleviated by 100 μM Cur, 60 μM CA and 50 μM QE. This concurs with the study of Nakayama [1994] in which CA and QE suppressed the H₂O₂ induced cytotoxicity in biological assay systems. In general ROS scavenging on the whole has been shown by Cur [Huang et al., 1988].

HOCl reduced PTPI's antipapain potential, and altered its structural integrity, presumably causing fragmentation and modification of constituent amino acid residues. CA (100 μM) effectuated almost complete reversal of the HOCl damage, followed closely by Cur (100 μM). QE exhibited slightly diminished protection. Diminution of hypochlorite induced damage on human serum albumin by flavonoids has been reported by Firuzi et al. [2004].

In the three radical systems studied, physiological antioxidants like ascorbic acid, glucose etc. were also employed but corresponding protection was attainable at millimolar concentrations corroborating the potency of natural antioxidants.

NO imposed deleterious effects on PTPI function and structure were ameliorated by these natural antioxidants. Cur, an established NO scavenger; completely reverted NO induced PTPI inactivation at 20 μM. 30-50 μM Cur was shown to be potent NO scavenger by Sreejayan and Rao [1997]; Onoda and Inano [2000]; Chan et al. [2005] and Sumanont et al. [2004]. This is also reinforced by the recent study of Khan et al. [2009], showing Cur to prevent NO mediated structural and functional damage of goat lung cystatin. Quite similar effect was shown by CA (80 μM). This is in conjunction with the works of Jung et al. [2006]; Gulcin [2006]; Olmos et al. [2008];
Chung et al. [2006]. 80 μM QE also protected against the NO induced damage. The results draw support from earlier reports [Lapidot et al., 2002; Hirota et al., 2005]. Structural alterations engendered by NO in PTPI were also diminished markedly by the three natural antioxidants.

These results are important as Cur, QE and CA,

a) come out to be potential scavengers of radical sinks viz. NO and HOCl.

b) provide alternate therapeutic strategies against free radical damage.

c) are effortlessly consumed by large proportion of human population and pose lesser or no side effects.
Chapter 5

Drug-PTPI interaction: Effect of pancreatitis causing and antidiabetic agents
3.5 RESULTS

The present set of experiments was devised to investigate the effects of sodium valproate (an antiepileptic drug causing pancreatitis), glimepiride (an antidiabetic drug of sulphonylurea class) and metformin hydrochloride (an antidiabetic drug, of biguanide class) on structure and function of PTPI.

3.5.1 INTERACTION OF PTPI WITH SODIUM VALPROATE

Association of valproate [Werlin and Fish, 2006] and cathepsin mediated trypsinogen activation with pancreatitis provoked the interest in probing the effects of valproate on PTPI, as thiol proteinase inhibitors (cystatins) are prime endogenous regulators of cathepsin activity.

The concentration of sodium valproate (VPA) used in the experiments was well within the therapeutic range [Werlin and Fish, 2006]. Fig. 74 depicts the effect of VPA on PTPI activity. 2 μM PTPI was incubated with increasing concentrations of the drug (2-20 μM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min and its papain inhibitory activity was determined by caseinolytic assay [Kunitz, 1947]. The activity of native PTPI was taken to be 100%. As shown in the figure, ~50% loss in PTPI activity occurred at 2 μM VPA concentration. 67% loss precipitated at 4 μM and at 10 μM only 20% activity was left. Beyond 10 μM the inhibitor was barely active.

Fluorescence and UV-vis absorption spectroscopy are powerful tools for the study of the reactivities of chemical and biological systems since it allows non-intrusive measurements of substances in low concentrations under physiological conditions. 2 μM PTPI treated with increasing concentrations of the drug (2-20 μM) was analyzed spectroscopically by above mentioned techniques. The excitation wavelength was chosen to be 280 nm to assess changes induced in global conformation of the inhibitor on interaction with the drug. The emission range was from 300-500 nm. As in Fig. 75, only mild changes were induced on PTPI. At 2μM drug concentration, ~20% decline in fluorescence intensity was observed. The intensity was slightly enhanced than native at 4 μM VPA. In presence of 6 μM drug, the fluorescence intensity was considerably increased (~40% above native). Between 8-15 μM VPA the intensity was again quenched although remaining faintly higher than the native. At 20 μM VPA concentration, the emission intensity increased 65% above untreated PTPI. However,
Fig. 74 Effects of Sodium valproate complexation on activity of PTPI

Native PTPI (2 μM) was incubated with increasing concentrations of sodium valproate (VPA) (2-20 μM) for 30 min at room temperature. PTPI was assayed for loss of antiproteinase activity by papain caseinolytic assay of Kunitz [1947]. Values are Mean ± SEM for four independent determinations. *Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.

Fig. 75 Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of Sodium valproate (VPA)

The concentration of PTPI was 2 μM. PTPI was preincubated for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing increasing concentration of VPA (2-20 μM). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slit width of 5 nm.
Sodium valproate ($\text{fiM}$)

Native +20 mM VPA

Wavelength (nm)

% Inhibitory Activity

Fluorescence Intensity

Wavelength (nm)
at all the drug concentrations, wavelength of maximum emission ($\lambda_{\text{max}}$) remained unaltered. The UV-vis absorption difference spectra were computed at all the drug concentrations. However, profound changes were noted only for those obtained at 2, 6 and 20 µM VPA [Fig. 76]. The difference spectra obtained for PTPI interacted with 2 µM VPA, shows two distinct positive peaks at 230 nm and 285 nm. In the difference spectra obtained at 6 and 20 µM VPA, broad shoulders were observed at 260 nm and maxima at 255 and 295 nm. Besides, intense negative peaks were noticeable at 210 and 215 nm. On subjection to polyacrylamide gel electrophoresis, the gross conformation of PTPI at all VPA concentrations was found to be unaffected (results not shown).

3.5.2 INTERACTION OF PTPI WITH GLIMEPIRIDE

Glimepiride is an antidiabetic drug belonging to the sulphonylurea class. One of the adverse impacts of glimepiride therapy is the production of ROS in pancreatic cells [Sawada et al., 2008]. Thus the effect of therapeutic concentrations of glimepiride on PTPI activity and structure were examined. 2 µM PTPI was incubated with increasing concentrations of the drug (2-40 µM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min and its inhibitory activity was determined by caseinolytic assay of papain [Kunitz, 1947]. The activity of native PTPI was taken as 100%. On interaction with 2 µM glimepiride, 10% loss of PTPI activity was noticed [Fig. 77]. At 5 µM drug concentration 50% of inhibitor’s activity was compromised. With no significant change in activity of PTPI till 35 µM glimepiride, a drastic decline (85%) was noticed at 40 µM drug concentration and the inhibitor retained only 15% of its original papain inhibition potential.

Effect of glimepiride on the structure of the inhibitor was analyzed by intrinsic fluorescence. The spectra were obtained by exciting the protein at 280 nm and the emission range was 300-500 nm. As shown in Fig. 78 the tertiary structure of the inhibitor remained more or less uninfluenced by the drug with only a little increment in fluorescence till 30 µM concentration. At 35 µM glimepiride, the intensity was slightly quenched below untreated PTPI. However at 40 µM glimepiride, pronounced enhancement in fluorescence intensity was observed. Similar to VPA, no change in $\lambda_{\text{max}}$ was registered. When analyzed by UV-vis spectroscopy, corresponding to fluorescence results no significant changes were observed except at 40 µM glimepiride concentration. The difference spectra obtained at 40 µM drug
Fig. 76  **UV-Absorption difference spectra measured for PTPI-VPA complexes**

PTPI (2 μM) was incubated with VPA (2-20 μM) for 30 min and absorbance difference spectrum was calculated between 200 nm to 320 nm. Results are shown for complexes obtained at 2, 6 and 20 μM VPA.
Fig. 77 Effect of glimepiride complexation on activity of PTPI

Native PTPI (2 μM) was incubated with increasing concentration of glimepiride (2-40 μM) for 30 min at room temperature. PTPI was assayed for loss of antiproteinase activity by papain caseinolytic assay of Kunitz [1947]. Values are Mean ± SEM for four independent determinations.  
*Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.

Fig. 78 Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of glimepiride

The concentration of PTPI was 2 μM. PTPI was preincubated for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing the increasing concentration of glimepiride (2-40 μM). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slitwidth of 5 nm.
Figure 1: Effect of Glimepiride Concentration on % Inhibitory Activity

Figure 2: Fluorescence Intensity vs. Wavelength for Glimepiride Concentrations

- 40 μM Glimepiride
- 5-30 μM Glimepiride
- Native PTPI
- 35 μM Glimepiride
- Glimepiride alone
concentration is shown in Fig. 79. An intense negative peak was observed at 220 nm, a broad shoulder at 260 nm and a small negative peak at 280 nm. The difference spectra obtained at all other glimepiride concentrations showed small peaks in the region of 210-230 nm only. No changes were evidenced in the conformation of PTPI in the presence of drug in PAGE (results not shown).

3.5.3 INTERACTION OF PTPI WITH METFORMIN HYDROCHLORIDE

Another antidiabetic drug, metformin hydrochloride the only representative of the biguanide class, was also analyzed for its interaction with PTPI. 2 μM PTPI was incubated with increasing concentrations of the drug (2-12 μM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min. The effect of drug on antiproteolytic potential of PTPI was determined by inhibition of caseinolytic activity of papain [Kunitz, 1947]. The activity of native PTPI was taken to be 100%. There was a gradual decline in PTPI activity with increasing drug concentration culminating to a 60% loss at 12 μM metformin hydrochloride. Progressive decline of ~20% each in activity of PTPI was observed at 2 μM and 4 μM concentrations of the drug, respectively. Further magnitude of decline was relatively smaller with increasing drug concentration. In contrast to sodium valproate and glimepiride, even at 12 μM metformin hydrochloride the inhibitor retained 40% of its antiproteolytic potential [Fig. 80].

The impact of drug interaction on structure of PTPI was also explored by fluorescence and UV-vis spectroscopy. PTPI treated in similar manner as for the activity measurements was subjected to fluorescence spectroscopic analysis. Fig. 81 illustrates the results obtained. $\lambda_{max}$ of emission remained unaltered at all drug concentrations. There was little increment in fluorescence intensity in comparison to untreated PTPI. In a similar fashion, on subjection to UV-vis spectroscopy and PAGE no significant changes were observed on PTPI (results not shown).
**Fig. 79** UV-Absorption difference spectra measured for PTPI-glimepiride complexes

PTPI (2 μM) was incubated with glimepiride (2-40 μM) for 30 min and absorbance difference spectrum was calculated between 200 nm to 320 nm. Results are shown for complex obtained at 40 μM glimepiride.
Fig. 80  Effect of metformin hydrochloride complexation on activity of PTPI

Native PTPI (2 μM) was incubated with increasing concentration of metformin hydrochloride (2-12 μM) for 30 min at room temperature. PTPI was assayed for loss of antiproteinase activity by caseinolytic assay of Kunitz [1947]. Values are Mean ± SEM for four independent determinations. *Significantly different from native PTPI (control) at p< 0.05 by one way ANOVA.

Fig. 81  Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of metformin hydrochloride

The concentration of PTPI was 2 μM. PTPI was incubated for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) containing the increasing concentration of metformin (2-12 μM). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slitwidth of 5 nm.
3.5.4 INTERACTION OF PTPI WITH INSULIN

Insulin is released from pancreas in response to body glucose levels indirectly regulating protein, carbohydrate and lipid metabolism and acting as a switch between carbohydrate and lipid metabolism. Improper insulin production causes diabetes mellitus/hyperinsulinism. Since, the introduction of insulin in the 1920s as a treatment for diabetes [Banting and Best, 1990], it is considered to be the most effective treatment for Type 1 and Type 2 diabetes.

Antihyperglycemic agent, proteinic in nature, recombinant insulin’s interaction with PTPI was also investigated. 2 μM PTPI was incubated with monocomponent Huminsulin R, in inhibitor to insulin ratios of 1:1-1:10 in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min. The treated PTPI samples were subjected to fluorescence, UV-vis and CD spectroscopy (in the far UV region); to assess changes in the structure of PTPI induced upon complexation with Huminsulin R. The results of fluorescence are shown in Fig. 82. The insulin showed maximum emission at 304 nm and PTPI alone gave a $\lambda_{\text{max}}$ of 335 nm [Priyadarshini and Bano, 2009]. At 1:1 molar ratio of PTPI to insulin, the $\lambda_{\text{max}}$ was red shifted to 345 nm, with considerable enhancement in fluorescence intensity, with respect to PTPI or insulin alone. At a molar ratio of 1:2 of PTPI to insulin, 2 peaks were observed, one specific for insulin at 304 nm and other at 340 nm. At molar ratios lower than 1:1, $\lambda_{\text{max}}$ of 345 nm was observed.

UV absorbance spectra of PTPI, insulin and their complexes are shown in Fig. 83. PTPI and insulin like other proteins showed peaks in regions of 200-210 nm and 278-280 nm. On complexation profound changes were introduced. At 1:1 molar ratio all peaks were abolished with a broad plateau in the range of 200-300 nm, with considerably enhanced absorbance. At 1:2 ratio the peak at 275 nm of lone proteins was shifted to 260 nm. At ratios, 1:4 to 1:10, the peak was again red shifted to 273-275 nm with considerable increment in absorbance.

To examine the effect of complexation on secondary structure of PTPI, the complexes were subjected to CD spectroscopy in the far UV region. For CD experiments, following molar ratios of PTPI:insulin were used, 1:05, 1:0.25, 1:0.5, 1:1 and 1:2. The spectrum of PTPI alone is characterized by a minimum at 222 nm [Priyadarshini and Bano, 2009]. Insulin gave two negative minima at 210 and 222 nm [Fig. 84]. On complexation a gradual increase in ellipticity at 222 nm was observed.
Fig. 82  Intrinsic fluorescence analysis of PTPI on interaction with various concentrations of insulin

The concentration of PTPI was 2 µM. PTPI was incubated for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) with insulin in various molar ratios of PTPI to insulin (1:1 to 1:10). Fluorescence was measured at an excitation wavelength of 280 nm and emission range of 300-400 nm with slitwidth of 5 nm.

Fig. 83  Absorption spectra of PTPI-insulin complexes

The concentration of PTPI was 2 µM. PTPI was incubated for 30 min at room temperature in 50 mM sodium phosphate buffer (pH 7.5) with insulin in various molar ratios of PTPI to insulin (1:1 to 1:10). Absorbance was taken from 200 to 320 nm.
Fig. 84  Far UV-CD Spectra of PTPI in complex with insulin

Far UV-CD spectra of PTPI and insulin alone and insulin-PTPI complexes. The concentration of PTPI was 1.73 µM and PTPI and insulin molar ratios were 1:0.05 to 1:2. The complexes were obtained after incubation at room temperature for 30 minutes in 50 mM sodium phosphate buffer, pH 7.5. Cells of 1 mm path length were used. The unit on the ordinate is mean residue ellipticity.
Insulin: CPTPI: Insulin (1:0.05 to 1:2)
Discussion

Chapter 5
DISCUSSION

Proteins are complex macromolecules that can adopt a large number of slightly different conformations within their native state, called conformational substrates. Even small structural differences between these substrates can lead to drastic changes in functional parameters. In addition, the marginal stability of the native conformation is a delicate balance of various interactions in proteins (van der Waals, electrostatic, hydrogen bonds and disulphide bridges) [Sneppen and Zocchi, 2005] which is affected by pH, temperature or addition of small molecules such as substrates, coenzymes, inhibitors and activators that bind especially to the native state and alters this fragile equilibrium. In this regard, many drugs exert their activity by interaction with proteins.

Drug accumulation at off-target sites in body, lead to unintended off-target adverse reactions [Taniguchi et al., 2007; Scheiber et al., 2008] and drug/ligand induced protein structure conformational alterations [Takeda et al., 1988] are prime problems complicating drug medical therapy.

Numerous drugs have been implicated in the etiology of acute pancreatitis (referred to as drug induced pancreatitis), but reports unravelling its mechanism are sparse [Dhir et al., 2007]. Use of valproic acid (an antiepileptic agent) is associated with an elevated risk estimate for acute pancreatitis besides being incriminated as a potentially fatal hepatotoxic and teratogenic agent [Norgaard et al., 2006; Werlin and Fish, 2006]. Generally, no relationship between the occurrence of pancreatitis and duration of sodium valproate therapy, dosage, serum level, and the use of other anticonvulsants has been found [Werlin and Fish, 2006].

Commonly prescribed antidiabetic drugs are sulfonylureas (glimepiride) and biguanides (metformin), the former act by increasing the insulin release from the β cells of the pancreas and insulin sensitivity in peripheral cells and the latter by decreasing hepatic glucose production and increasing peripheral glucose uptake and use. Sulphonylureas are considered to cause β cell apoptosis, induce nitric oxide generation and reactive species production [Ansar and Ansari, 2006; Sawada et al., 2008]. Synthetic insulin is another commonly prescribed hypoglycemic agent.

Almost all the specific drugs have some side or adverse effects. Thus, study aimed at investigating the effects of above four drugs on purified goat pancreatic thiol proteinase inhibitor. Inclination for this study sprouted up as PTPI is shown to have
anti thiol proteinase activity. Cathepsin mediated trypsinogen activation is one of the initiator points of acute pancreatitis [Thrower et al., 2006; van Acker et al., 2002]. Also, insulin degradation is mediated probably by lysosomes [Sandberg and Borg, 2006]. Thiol proteinase inhibitors /cystatins serve as the main endogenous regulators of lysosomal cathepsins [Turk et al., 2008].

To assess the effect of interaction of these drugs on functional integrity of PTPI, the purified inhibitor was incubated with increasing concentrations of VPA/ glimepiride/ metformin hydrochloride and its antiproteolytic potential was determined. Estimation of protein activity provides a sensitive means to monitor the effect of ligands on the protein as even minute changes in active site region can considerably affect the protein function. VPA diminished the activity of PTPI with almost complete inactivation of the inhibitor at higher concentration [Fig. 74] within a short span of time. Glimepiride induced inhibitor inactivation was less drastic. Considerable activity (~50%) was retained by PTPI till 30 µM drug concentration. Even at maximum concentration of the drug (40 µM) PTPI possessed 15% activity [Fig. 77]. Metformin hydrochloride was milder in inflicting inactivation on PTPI [Fig. 80].

Drug induced inactivation (functional derangement) of proteins has been reported earlier like horse liver alcohol dehydrogenase (ADH) activity was affected differentially by various drugs. Few like barbital, caffeine and diazepam exerted no effect, chlorpromazine, sulpiride, morphine etc. reduced the activity and phenytoin enhanced ADH activity [Roig et al., 1991]. Absence of any major drug induced conformational change in PTPI structure (as discussed later), suggest that inactivation of the inhibitor may be related to subtle changes in the conformation at the active site region induced by the drugs. Similar ligand binding induced functional changes have been shown for isocitrate dehydrogenase [Serry and Farrell Jr., 1990].

Intrinsic fluorescence of proteins provides considerable information about protein structure and dynamics and has been used extensively to study protein folding and association reactions [Lakowicz, 2006]. Various measurable parameters of fluorescence viz. quenching, enhancement of intensity, spectral shift etc. are used for interpretation of related structure-dynamics in proteins [Lakowicz, 2006]. In the present study, fluorescence spectroscopic measurements were undertaken to gain insight into the interaction and complexation of VPA, glimepiride, metformin hydrochloride and insulin with PTPI [Figs. 75, 78, 81 and 82]. In the presence of all these drugs, fluorescence intensity increased (though not profoundly) except for 2 µM
VPA and 35 μM glimepiride. Such a kind of change has been documented earlier. Interaction of ligands (phytohormones, cytokinins, abscisic and gibberellic acids) with wheat germ agglutinin resulted in ~60% increase in fluorescence intensity of native protein [Bogoëva et al., 2004]. Fluorescence enhancement of α1 antitrypsin on interaction with heparin and glucose has also been reported [Finotti and de Laureto, 1997]. Except for PTPI-insulin complexation, no shift in λ_max was noted. A 10 nm red shift was observed on formation of equimolar complex of insulin and PTPI, indicating exposure of aromatic residues to the solvent caused by conformational rearrangement of the two proteins [Monsellier and Bedouelle, 2005; Vivian and Callis, 2001]. When PTPI-insulin ratio was 1:2, there was a blue shift of 5 nm, suggestive of a change in environment of tryptophan residues to non polar.

Absorption spectral measurements of PTPI in the presence of drugs provided information related to their interaction. Difference spectra were computed by subtracting the absorption of PTPI-drug complex from the absorption of PTPI alone, for VPA/glimepiride-PTPI complexes [Fig. 76 and 79]. The intense negative peaks at 210 and 215 nm observed for PTPI-VPA complexes obtained at 6 and 20 μM VPA concentrations, respectively may contain contributions from phenylalanine and histidine residues [Donovan, 1969]. The broad shoulders observed at 260 nm are also due to phenylalanine and may contain contribution form tryptophan [Donovan, 1969]. The peaks observed at 255 and 295 nm indicate the involvement of aromatic residues in interaction. A negative peak noticeable at 275 nm in difference spectra obtained at 6 μM VPA, suggest changes around tyrosine residues [Donovan, 1973a]. For the difference spectra obtained at 2 μM VPA, positive peaks at 230 nm and 285 nm might have the contribution of mainly tryptophan and tyrosine residues [Donovan, 1973a]. The negative peak at 275 nm and the difference peak at 295 nm are characteristic of blue shifts of tryptophan and tyrosine absorption bands and are usually interpreted as arising from increased exposure of these aromatic groups to the solvent [Donovan, 1969]. Difference spectra of glimepiride-PTPI complex at 40 μM glimepiride concentration, showed broad shoulder at 260 nm and a small negative peak at 280 nm, indicative of involvement of phenylalanine and tyrosine in complexation process.

The interaction between insulin-PTPI was studied from UV-vis absorption spectral data. Complex spectroscopic changes were noticed. Equimolar complex of PTPI and insulin showed only a broad absorption band in the region of 200-300 nm, suggesting that the native structure of both the proteins is altered on complexation. The blue shift
observed for 1:2 molar ratio, indicates the changes in the environment of tyrosine residues towards hydrophobic. At higher ratios, shift of spectra towards longer wavelengths was observed. These evidences clearly indicated the interaction and some complex formation between insulin and PTPI [Cui et al., 2004; Hu et al., 2004]. Similar changes have also been shown for UV-vis absorption spectra of horse myoglobin on interaction with phenothiazines promazine and triflupromazine hydrochlorides [Cheema et al., 2008].

Alterations in secondary structure of PTPI on complexation with insulin were also analyzed by CD spectroscopy. Two negative absorbance peaks at 210 and 222 nm for insulin alone and at 222 nm for PTPI alone, are typical peaks of α helix [Fig. 84]. Slight enhancement in ellipticity at 222 nm was observed for all molar ratios of PTPI-insulin, suggesting that binding of insulin to PTPI increased the α helical content of PTPI. Such enhanced ellipticity has also been reported for binding of ferulic acid to cytochrome C [Yang et al., 2007].

When drugs bind to a globular protein, the intramolecular forces responsible for maintaining the secondary and tertiary structures can be altered, resulting in conformational change of the protein [Salvi et al., 2001]. Such conformational changes of varied magnitude have been reported [Guo et al., 2004a; Cheema et al., 2008; Ahmed-Ouameur et al., 2006]. Thus, the results indicate that the UV absorption and fluorescence emission changes on VPA, glimepiride and metformin hydrochloride mediated interaction, are due to minor conformational changes in PTPI and mainly arise from local interactions affecting the chromophoric groups of the protein. While in case of insulin-PTPI interaction, conformational rearrangement might have occurred.

Drug induced changes in enzyme activities as their adverse effects have been an area of continual scientific investigation [Priyamvada et al., 2008]. Limited number of reports is available on effects of drugs on purified tissue proteins which do not function as carriers but are putative targets of the drug induced side effects or whose structural and functional modifications aid in drug mediated damage or are critical for the off-target tissue. This study has tried to explore the effects of few drugs on purified pancreatic thiol proteinase inhibitor. The drugs chosen are either incriminated or prescribed in pancreatic pathologies and thiol proteinase inhibitors are prime regulators of lysosomal cathepsins [Bohley et al., 1978].
On complexation with VPA (a pancreatitis causing drug) PTPI activity was severely challenged, suggesting cathepsin inactivation might be one of the factors in VPA induced pancreatitis. Glimepiride causes β cell apoptosis [Ansar and Ansari, 2006]. These antidiabetic agents are also known to cause nitric oxide and reactive oxygen species production in pancreas [Sawada et al., 2008]. PTPI function was also compromised on interaction with these two drugs though it was less severe with metformin. Functional insulin receptors are known to occur in pancreatic β cells. Studies have shown that exogenous insulin in the presence or absence of nonstimulatory concentrations of glucose evokes exocytosis mediated by the β cell insulin receptor. This autocrine action of insulin diminishes as the concentration of free insulin on cell surface decreases or receptors get blocked [Aspinwall et al., 1999]. Cystatin β has been found to be colocalized with cathepsin in insulin secretory granules in rat pancreas [Watanabe et al., 1988]. In the present work complexation with insulin increased the α helical content of PTPI. Thus it may be speculated that this increase in the native stability of the inhibitor on complexation with (exogenous) insulin, may allow more efficient regulation of cathepsin activity under diseased states and thus preventing insulin degradation.

The possible implications of these results would be on designing therapeautic agents managing VPA induced pancreatitis, understanding of non responsiveness to antidiabetic agents after a period and provide a clue to increase insulin potency.