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SHORT COMMUNICATION

Tryptophan Environment and Functional Characterization of a Kinetically Stable Serine Protease Containing a Polyproline II Fold

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Abstract The single tryptophan residue from *Nocardiopsis sp*. serine protease (NprotI) was studied for its microenvironment using steady state and time-resolved fluorescence. The emission maximum was observed at 353 nm with excitation at 295 nm indicating tryptophan to be solvent exposed. Upon denaturation with 6 M guanidinum thiocyanate (GuSCN) the emission maxima was shifted to 360 nm. Solute quenching studies were performed with neutral (acrylamide) and ionic (I^- and Cs^+) quenchers to probe the exposure and accessibility of tryptophan residue of the protein. Maximum quenching was observed with acrylamide. In the native state, quenching was not observed with Cs^+ indicating presence of only positively charged environment surrounding tryptophan. However; in denatured protein, quenching was observed with Cs^+, indicating charge reorientation after denaturation. No quenching was observed with Cs^+ even at pH 1.0 or 10.0; while at acidic pH, a higher rate of quenching was observed with KI. This indicated presence of more positive charge surrounding tryptophan at acidic pH. In time resolved fluorescence measurements, the fluorescence decay curves could be best fitted to monoexponential pattern with lifetimes of 5.13 ns for NprotI indicating one conformer of the trp. Chemical modification studies with phenyl glyoxal suggested presence of Arg near the active site of the enzyme. No inhibition was seen with soyabean trypsin and limabean inhibitors, while, CanPI uncompetitively inhibited NprotI. Various salts from Hofmeister series were shown to decrease the activity and PPII content of NprotI.

Keywords Serine protease · Fluorescence quenching · Tryptophan · *Nocardiopsis* · Hofmeister series

Abbrevations

Arg	Arginine
CanPI	*Capsicum annuum* pin-II protease inhibitor
CD	Circular dichroism
GdnHCl	Guanidine hydrochloride
GuSCN	Guanidine thiocyanate
PPII fold	Polyproline II fold
Trp	Tryptophan

Introduction

Proteases are the enzymes needed and synthesized by almost all forms of life. They have evolved multiple times, and a completely different catalytic mechanism is used by different class of proteases to perform the same reaction. Serine proteases are extensively studied proteases among different classes of proteases. The interesting property for these enzymes is that although they use same catalytic triad for catalysis, their sequences can be totally different. The serine proteases do vary on the basis of their substrate binding pocket. So, some have broad substrate specificity while some can cleave only particular peptide bond in particular protein.

Extracellular bacterial proteases can have unique structural features in order to survive in harsh environments. These proteases are generally very stable to various denaturing conditions and are called as kinetically stable proteases [1, 2]. In the present study, we have explored biochemical and biophysical properties of a kinetically stable serine protease from *Nocardiopsis sp*. NCIM 5124 (NprotI). In our previous findings the protease was found to be resistant to denaturation by guanidine hydrochloride (GdnHCl), organic solvents,

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proteolytic enzymes and a PPII fold was found to be responsible for this kinetic stability [3]. Also, the enzyme was found to be unusually stable at acidic pH conditions (data communicated elsewhere).

Fluorescence quenching of indole by adding solutes have provided valuable information regarding the structure and dynamics of proteins in solution [4, 5]. We report the structural studies of the NprotI by using steady-state and time resolved fluorescence and CD spectroscopy. The protein has been characterized with respect to tryptophan environment in native, denatured and different pH conditions. Determination of pKa of the amino acids present at active site has been done. Also, amino acid residues probably involved at the active site were studied performing chemical modification studies. Effect of different salts from Hofmeister series was studied with respect to activity and PPII content of NprotI.

Materials and Methods

Materials

Soyabbean trypsin and lima bean inhibitor were procured from Sigma (USA). CanPI 7 was a gift from Dr. Ashok Giri, NCL, Pune. All other reagents, buffer compounds used were of analytical grade. Solutions prepared for spectroscopic measurements were in MilliQ water.

Production and Purification of NprotI

The organism was isolated from an oil contaminated marine site near Mumbai harbor (India). The protocol used for the production and purification of NprotI was as described earlier [6]. Briefly, the culture broth of *Nocardiopsis sp*. NCIM 5124 was obtained by fermentation in a medium containing 1 % starch, 1 % casein, 0.1 % K_2HPO_4, 1 % Na_2CO_3, 0.2 % glucose, pH 10.0 after incubation for 108 h at 30 °C and 200 rpm. NprotI was purified from the cell-free supernatant by two successive cation exchange chromatographic steps at pH 5.0 and at pH 9.0. The purified enzyme was stored at pH 5.0 where it exhibits maximum stability at 2–8 °C.

Enzyme Assay

Protease activity was determined by incubating 3 μg of the enzyme in 300 μl of 1 % casein (substrate) at pH 10.0 at 60 °C for 20 min as described by Dixit et al. [6]. One unit of protease activity is defined as the amount of enzyme which releases 1 μmol of tyrosine per minute in the assay conditions.

Steady State Fluorescence Study

Intrinsic fluorescence of the enzyme was measured using a Perkin-Elmer Luminescence spectrometer LS50B connected to a Julabo F20 water bath. The protein solution was excited at 295 nm and the emission was recorded in the range of wavelength 300–400 nm at 30 °C. The slit widths for the excitation and emission were set at 7.0 nm, and the spectra were recorded at 100 nm/min. To eliminate the background emission the signal produced by the buffer solution was subtracted.

Steady State Fluorescence Quenching

Fluorescence titrations were carried out by adding 3–5 μl of acrylamide (5 M), potassium iodide (5 M) and cesium chloride (5 M) to the protein sample prepared in 20 mM phosphate buffer pH 7.2. Fluorescence intensity was recorded after each addition. The iodide solution contained sodium thiosulfate (200 μM) to suppress triiodate formation. The excitation wavelength was set at 295 nm; the emission spectra were recorded in the range 300 to 400 nm with both the slit widths as 7 nm at a scan speed of 100 nm/min. To eliminate contribution from background emission, the signal produced by buffer solution was subtracted. The steady state fluorescence quenching was done for native (pH 5.0); GuSCN denatured NprotI, as well as, with NprotI at pH 1.0 and 10.0. Following buffers were used in 20 mM concentration; pH 1.0: glycine-HCl, pH 5.0: sodium acetate, pH 10.0: sodium carbonate-bicarbonate.

Time-Resolved Fluorescence Study

Fluorescence lifetime measurements were carried out on Edinburgh Instruments' FLS-920 single photon counting spectrofluorimeter. A pico second pulsed light emitting diode of wavelength 296.8 nm, pulse width 747.8 ps and bandwidth 10.4 nm was used as excitation source and a Synchronization photomultiplier was used to detect the fluorescence. The diluted Ludox solution was used for measuring Instrument Response Function (IRF). NprotI (1 mg/ml) was excited at 295 nm and emission was recorded at 353 nm. Slit widths of 15 nm each were used on the excitation and emission monochromators. The resultant decay curves were analyzed by a reconvolution fitting program supplied by Edinburgh Instruments.

Circular Dichroism (CD) Measurements

The CD spectra of the enzyme were recorded on a J-715 Spectropolarimeter with a PTC343 Peltier unit (Jasco, Tokyo, Japan) at 25 °C in a quartz cuvette. Each CD spectrum was accumulated from five scans at

100 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Far UV CD spectra of the enzyme (250 μg/ml) were collected in the wavelength range of 200–250 nm using a cell of path length 0.1 cm for monitoring the secondary structure. All spectra were corrected for buffer contributions and observed values were converted to mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as

$$MRE = M\theta_\lambda / 10dcr$$

Where, M is the molecular weight of the protein, θ_λ is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ ml and r is the average number of amino acid residues in the protein.

Determination of pKa of Amino Acids at the Active Site Residues

Activity for the enzyme was checked at various pH (pH 7–12) conditions and substrate concentrations. Km and Vmax values were calculated and a plot of log (Vmax/Km) vs pH concentration. The pKa of the amino acids at the active site was obtained from the tangents of bell shaped curve.

Chemical Modification with Phenylglyoxal (PG)

The enzyme in 50 mM Tris–HCl buffer, pH 8.0, was incubated with varying concentrations of PG at 25 °C. Aliquots were removed at suitable intervals and the residual activity was determined under standard assay conditions. Substrate protection and time dependence was also studied in presence of phenyl glyoxal.

Interaction with Soyabean Trypsin, Lima Bean and CanPI Inhibitors

The enzyme was preincubated with different concentrations of inhibitors for 20 min at pH 8.0, Tris–HCl at 25 °C and then the substrate was added and checked for residual activity.

Effect of Various Salts on NprotI

To study the effect on function of the enzyme, NprotI was incubated with NaCl, NH_4Cl, and $MgCl_2$ (0–6 M) in 20 mM sodium acetate buffer, pH 5.0 for 1 h at 25 °C. Suitable aliquot was removed and assayed for enzyme activity. The readings were corrected for blank readings. For CD measurements NprotI in final concentration of 250 μg/ml was incubated in 1 M NaCl, NH_4Cl and $MgCl_2$ in 20 mM sodium acetate buffer at pH 5.0 for 1 h at 25 °C and the spectra were recorded.

Results and Discussion

Fluorescence quenching experiments were carried out on NprotI using Acrylamide (neutral), I^- (negatively charged) and Cs^+ (positively charged) quenchers. Also action of various protease inhibitors was studied. Effect of NaCl, NH_4Cl and $MgCl_2$ on structure and function was studied.

Intrinsic Fluorescence

NprotI contains a single tryptophan residue [6]. Emission maximum at 353 nm was observed with excitation at 295 nm, indicating that the tryptophan residue is exposed to the solvent. The decomposition analysis of trp fluorescence spectra was carried out using PFAST program (http://pfast.phys.uri.edu/pfast/) [7] and it indicated presence of type III tryptophan, i.e. tryptohan is solvent exposed. Emission maximum did not shift in presence of GdnHCl as NprotI is resistant to GdnHCl denaturation [3]. Emission maximum was red shifted by 6 nm in presence of more powerful denaturant GuSCN indicating denaturation of the enzyme (Fig. 1).

Solute Quenching Study

Quenching data for all the quenchers used in these studies were analyzed by the Stern–Volmer eq. (1) as well as by the modified Stern–Volmer eq. (2) [8].

$$Fo/Fc = 1 + K_{sv}[Q] \quad (1)$$

$$F/\Delta F = f_a^{(-1)} + (K_a f_a)^{(-1)}[Q]^{(-1)} \quad (2)$$

Where F_o and F_c are the respective fluorescence intensities corrected for dilution, in the absence and presence of quencher, [Q] is the resultant quencher concentration. K_{sv} is the

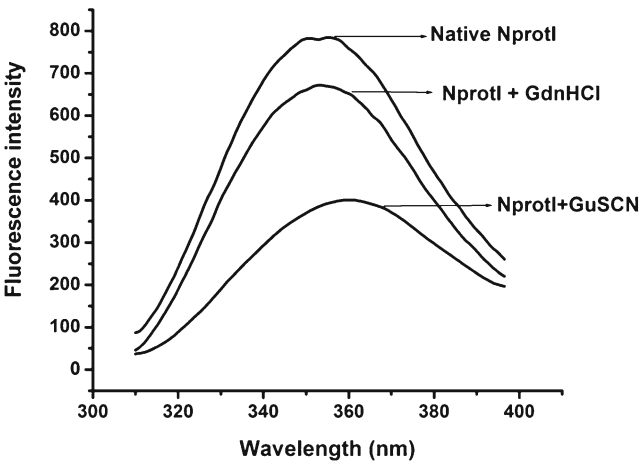


Fig. 1 Steady state fluorescence spectra of NprotI under different conditions

Stern–Volmer quenching constant, f_a refers to the fraction of the total a fluorescence that is accessible to the quencher and K_a is the corresponding quenching constant. Slopes of Stern–Volmer plots yield K_{sv} values, whereas the slopes of modified Stern–Volmer plots give $(K_a\ f_a)^{-1}$ and their ordinates give values of f_a^{-1}.

Stern-Volmer and modified Stern-Volmer plots of quenching of native NprotI, NprotI denatured with GuSCN are shown in Fig. 2 and the various constants are summarized in Table 1.

Solute Quenching Studies of Native NprotI

Fluorescence quenching was not observed with CsCl in native conditions (Fig. 2a). A very high rate of quenching with K_{sv} of 14.017 M^{-1} was observed for $I^{(-)}$ in native conditions. This indicated that tryptophan residue is present in positively charged environment and there are hardly any negatively charged amino acid residues around it. The upward curvature in case of fluorescence quenching of native NprotI with acrylamide indicated presence of both static and dynamic components. The static and dynamic components were then resolved by time dependent fluorescence quenching.

Table 1 Summary of quenching parameters

Quencher and Condition	$K_{sv}(M^{-1})$	$K_s(M^{-1})$	f_a
Acrylamide			
Native	4.80	5.51	1.19
Native+GdnHCl	–		1.594
Native+GuSCN	10		1.039
KI			
Native	14.017		0.899
Native+GdnHCl	10.067		1.111
Native+GuSCN	2.98		0.548
CsCl			
Native	–		–
Native+GdnHCl	–		–
Native+GuSCN	1.34		0.838

The modified Stern-Volmer plot provides the information about fraction accessibility of tryptophan to the quencher. For native protein the single tryptophan, which is surface exposed, was 100 % accessible to neutral quencher acrylamide, while for $I^{(-)}$, it was about 90 % accessible (Fig. 2b).

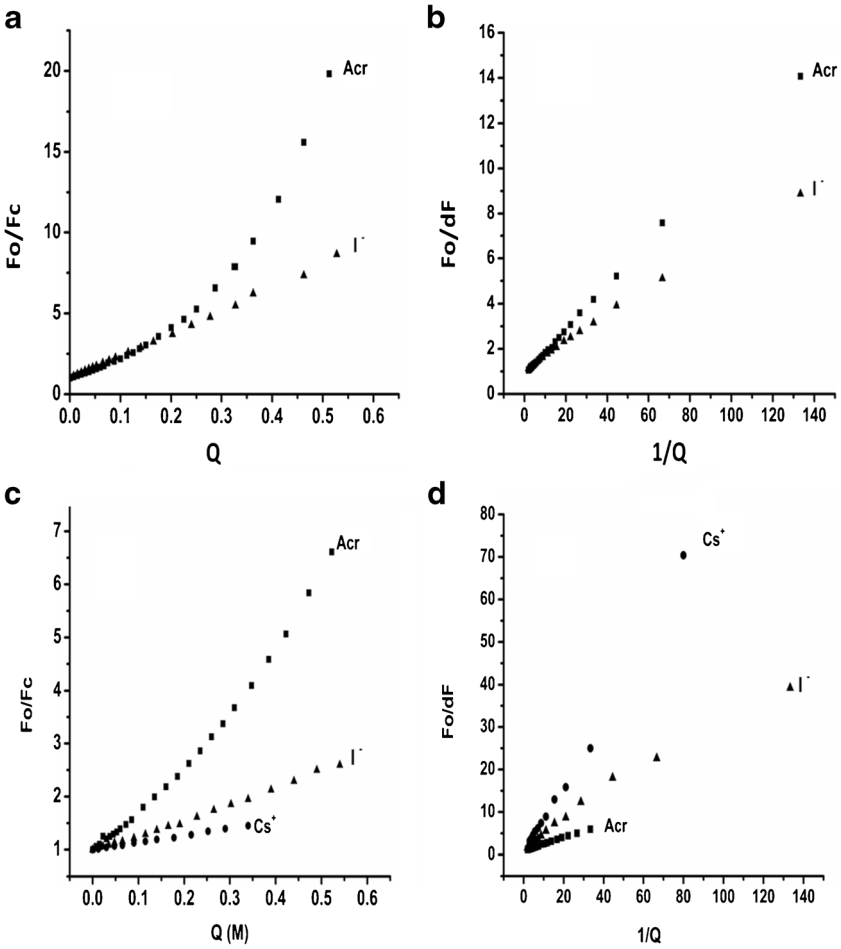


Fig. 2 **a** and **c** Stern-Volmer plot for native and denatured NprotI respectively, **b** and **d** Modified Stern-Volmer plot for native protein and denatured NprotI, respectively

Solute Quenching Studies of Denatured NprotI

As already mentioned, NprotI does not get denatured with GdnHCl; the quenching parameters were almost constant for NprotI treated with 6 M GdnHCl. For the protein denatured with GuSCN only dynamic component was observed with acrylamide and a high K_{sv} obtained indicated higher rate of quenching in the denatured protein. Quenching with CsCl with K_{sv} of 1.34 M^{-1} in denatured protein indicated presence of negative charge environment surrounding tryptophan (Fig. 2c). The K_{sv} (2.98 M^{-1}) for KI was decreased in denatured protein compared to that of native. This suggested charge reorientation in the denatured protein.

The fraction accesiblity for acrylamide in the denatured protein remained same around 100 %. For $I^{(-)}$ it decreased to about 55 % while for $Cs^{(+)}$ it was about 84 % (Fig. 2d). It can be seen here that the accessibility to $I^{(-)}$ was decreased to large extent along with decrease in the quenching rate. But for $Cs^{(+)}$ the quenching rate was not much higher but the accessibility was higher.

Solute Quenching Studies of NprotI in Different pH Conditions

The enzyme was structurally stable in all the pH conditions, while it is most stable at acidic pH (pH 1.0) it is most active at pH 10.0 (Rohamare et al., communicated elsewhere). To investigate the structural changes at these pH conditions, solute quenching studies with ionic quenchers were carried out at pH 1.0, 5.0 and 10.0 (Fig. 3a, b). No quenching with $Cs^{(+)}$ was observed at all the pH, which indicated that still there was no negative charge surrounding tryptophan, which could be due to maintenance of native structure at all the pH conditions and this supported our previous reports. The rate of quenching with KI increased with decreasing pH indicating that in acidic pH the positive charge around tryptophan had increased. Also, at pH 1.0 with increasing additions of KI, the enzyme was found to get denatured. This could be due to presence of very high positive charge on the enzyme at pH 1. Although enzyme at pH 1 was as thermostable as that at pH 5.0, it was found to be resistant to chemical denaturation (Rohamare et al., communicated elsewhere). The observation that NprotI at pH 1.0 gets denatured with addition of KI supported the earlier study.

Time Resolved Fluorescence Study

Events that occur during the lifetime of the excited singlet state can be monitored using time-resolved fluorescence spectroscopy. This time scale can range from a few picoseconds to hundreds of nanoseconds [9]. The fluorescent decay of the tryptophan residue on a nanosecond time scale for NprotI, obtained from time resolved measurements are presented in Fig. 4a. Monoexponential curve could be fitted ($\chi 2=1.13$) to time resolved fluorescence profile indicating one conformer of tryptophan with value of 5.13 ns of decay time for tryptophan. This could be due to presence of tryptophan in a stable environment with fewer fluctuations so that the electron transfer processes with neighboring solvent molecules or amino acid residues are lower [10]. Such monoexponential decay has been observed in case of ribonuclease T1 [11].

Time Resolved Fluorescence Quenching of Native NprotI with Acrylamide

The profile obtained for acrylamide quenching in native conditions showed an upward curvature, indicating dynamic and static components of quenching. The static mechanism involves complex formation, while dynamic mechanism involves collisions with acrylamide during the lifetime of tryptophan in excited state. In such a case, the data can be analyzed using the following eqn. and the dynamic and the static components can be resolved. The parameters obtained for the time resolved quenching with acrylamide are given supplementary table 1.

$$Fo/Fc = (1 + K_{sv}[Q])(1 + K_s[Q]) \quad (3)$$

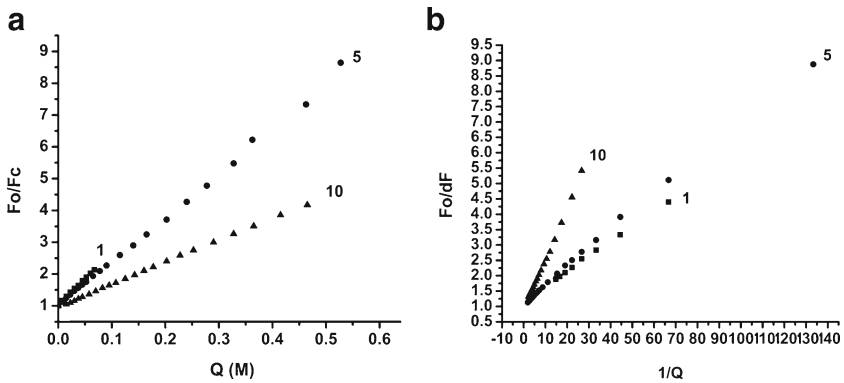
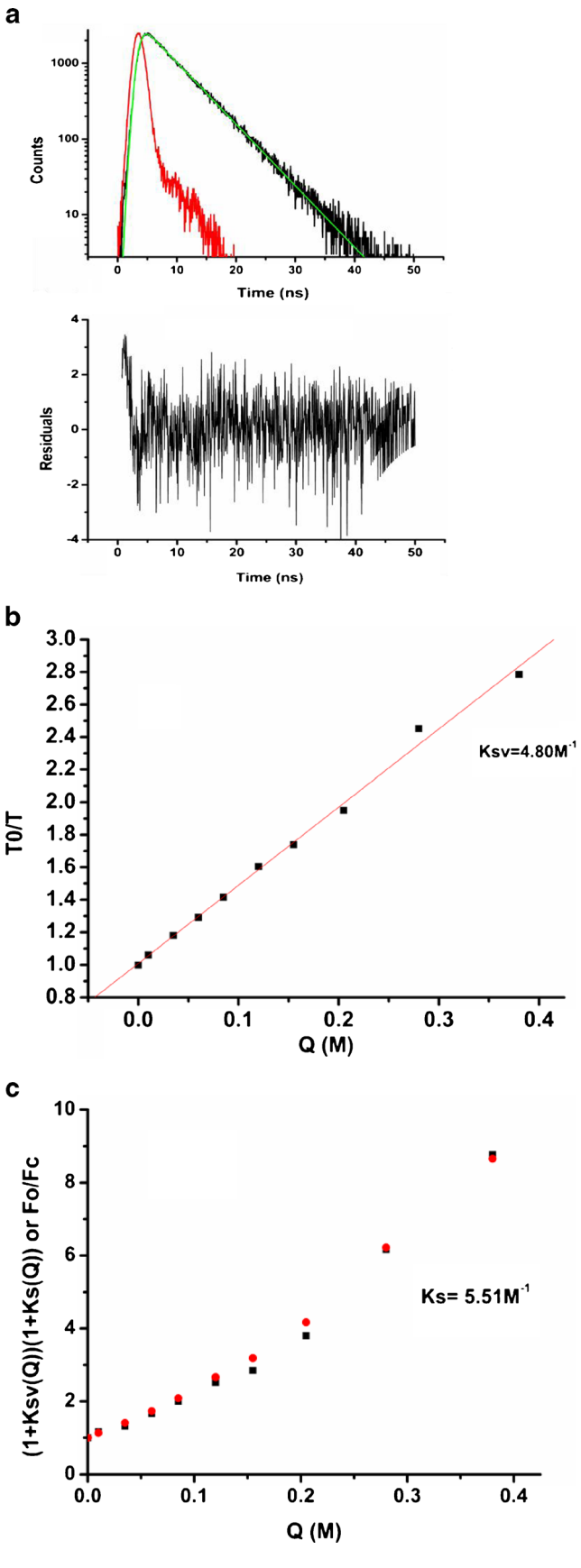


Fig. 3 Quenching with KI pH 1, 5 and 10 **a** Stern-Volmer plot, **b** Modified Stern-Volmer plot. Number on each plot indicates pH of the study



◀ **Fig. 4** Quenching of NprotI fluorescence with acrylamide studied on time-resolved spectrofluorimeter **a** Time-resolved fluorescence decay profile of NprotI **b** The plot of τ_o/τ for the quenching data of NprotI with acrylamide, **c** The plot of Fo/Fc and (1+Ksv[Q])(1+Ks[Q]) against [Q]

Where K_{sv} is the Stern–Volmer (dynamic) quenching constant, K_s is the static quenching constant and [Q] is the quencher concentration. The dynamic quenching constant reflects the degree to which the quencher achieves the encounter distance of the fluorophore and can be determined by the fluorescence lifetime measurements according to the equation:

$$\tau o/\tau = (1 + K_{sv}[Q]) \quad (4)$$

Where τo is the average lifetime in absence of the quencher and τ is the average lifetime in presence of a quencher at a concentration [Q]. The value of K_{sv} obtained for acrylamide quenching of NprotI was 4.8 M^{-1} (Fig. 4b). Putting this value in eq. (3) and plotting a graph of (Fo/Fc)/ (1+K_{sv} [Q]) against [Q], the value of the static quenching constant Ks was obtained as 5.51 M^{-1} (Fig. 4c) and the bimolecular quenching constant, kq was calculated as kq=K_{sv}/τ, and was found out to be 0.9375×10^9 M^{-1} s^{-1}. Incorporating the values of K_{sv} and K_s in the expression (1+K_{sv} [Q]) (1+K_s [Q]), the values obtained were plotted against [Q]. It was observed that the values of Fo/Fc and (1+K_{sv} [Q]) (1+K_s [Q]) match very well (Fig. 4b).

pKa of the Amino Acid Residues at Active Site and Chemical Modification with Phenylglyoxal (PG)

Although the present enzyme is serine protease and the catalytic triad is known, being an alkaline enzyme, we explored

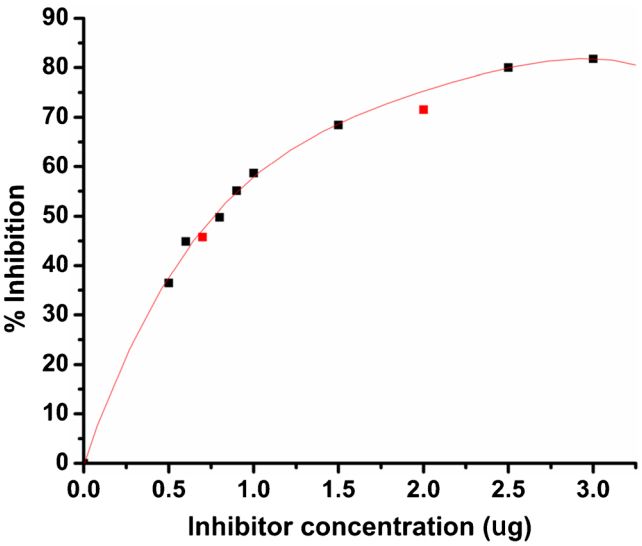


Fig. 5 Inhibition profile with CanPI 7: IC50 determination

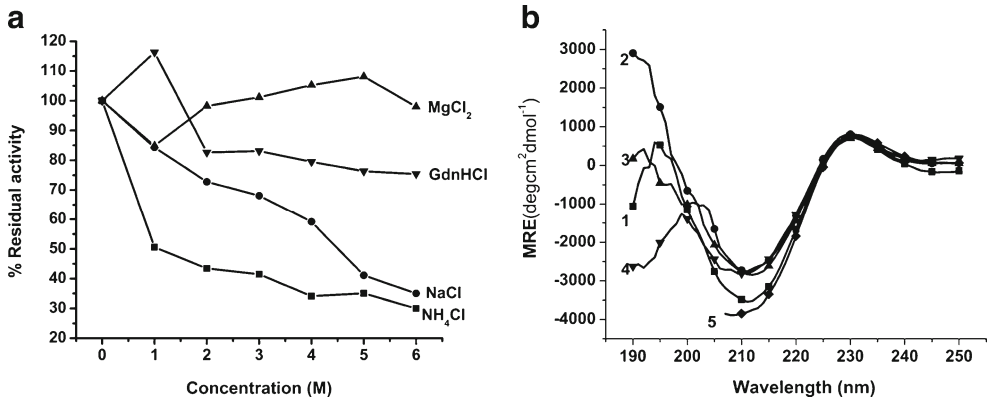


Fig. 6 Effect of different salts on NprotI **a** Activity profile **b** CD profile of NprotI incubated with 1 M of different salts as: 1, native protein; 2, with NaCl; 3, with NH_4Cl; 4, with $MgCl_2$; 5 with GdnHCl. The spectrum no. 5 has been adopted from [3]

the active site residues. The pH activity profile (Supplementary fig. 1) showed that pKa of the amino acids at active site was in the range of 9.4 and 10.3 i.e. on the basic side. Hence, investigation of basic residue at the active site (other than histidine) was carried out with chemical modification studies. Inhibition was observed with phenyl glyoxal (Supplementary fig. 2) which modifies the Arg residue [12]. Then the inhibition was checked for time dependence and substrate protection which was not observed indicating that the Arg residue might not be involved in catalysis but might be responsible for holding the active conformation.

Activity profile of NprotI in presence of soyabean trypsin, lima bean and CanPI 7 inhibitor

The soyabean trypsin, lima bean inhibitors are commercially available serine protease inhibitors. Interestingly, no inhibition was observed in presence of these inhibitors. At much higher concentrations (above 60 μM), the inhibition was achieved (data not shown). Another inhibitor from plant origin *Capsicum annuum* Pin-II PI (CanPI-7) was used in studies. This inhibitor showed inhibition even at lower concentrations. The IC50 value for this inhibitor was 0.8 μg (32.91 nM) (Fig. 5). The Line-Weaver-Burk plot of the data showed an uncompetitive inhibition (Supplementary fig. 3) hence, further kinetics could not be studied. Interestingly, this inhibitor also has PPII fold [13].

Effect of Salts on NprotI and Comparison to Effect of GdnHCl

The enzyme was found to follow the reverse Hofmeister series (Fig. 6a) when the activity was studied in presence of various salts ($MgCl_2$, NaCl and NH_4Cl) from the series. The activity was found to decrease in the order; $Mg^{2+}>Na^{+}>NH_4^{+}$. Long term stability in presence of Gdn^{+}, which is at the extreme end of the series, has been studied previously [3]. It has been studied that protein follow different Hofmeister series at pH above and below its pI [14, 15]. The pI of NprotI is about 8.3 [6] and the protein was incubated with different salts at pH 5.0 as this is the optimum pH.

The content of PPII fold seemed to be decreased in presence of all the cations (Fig. 6b), although the CD spectrum still possessed the PPII character. It was shown in a study by Drake el al that the PPII structure can be disrupted somewhat by the addition of sodium chloride, while still retaining the PPII character [16]. This was also shown by Rucker et al., in homopolymers of lysine [17]. When the lysine peptide was titrated with sodium chloride the PPII content was decreased but the characteristic PPII CD signal was still there. It was speculated that short lysine peptides adopt PPII helical structure as a result of the nature of the backbone rather than as a consequence of electrostatic interactions between side chains.

The ions have ionic radii as NH_4: 175 pm, Na: 116 pm, Mg: 86 pm; hence the lowest activity in NH_4^{+} could be due to the effect of larger sized ammonium ions disrupting the electrostatic interactions in the protein. Interestingly Gdn^{+} (210 pm) showed stabilizing effect on the structure and activity of the enzyme. This effect could be due to ability of Gdn^{+} to increase the structural content of PPII fold, while in the rest of the ions this effect might be lower.

To summarize, the tryptophan residue in NprotI was found to be surrounded by merely positively charged residues at native pH (pH 5.0 as well as at pH 1.0 and 10.0, with more positive charge at pH 1.0. Charge reorientation was seen in denatured protein as indicated by quenching with Cs^{+}. An arginine residue might be responsible for holding active catalytic conformation in NprotI. The enzyme was found to be uncompetitively inhibited by CanPI 7. The activity profile in presence of different salts showed that NprotI followed reverse Hofmeister series.

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