Functional stability and structural transitions of Kallikrein: spectroscopic and molecular dynamics studies

Sayli Dalal\textsuperscript{a}, Anil Mhashal\textsuperscript{b}, Narendra Kadoo\textsuperscript{a} and Sushama M. Gaikwad\textsuperscript{a}\*\*

\textsuperscript{a}Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India; \textsuperscript{b}Division of Physical Chemistry, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

Communicated by Ramaswamy H. Sarma

(Received 3 December 2015; accepted 4 January 2016)

Kallikrein, a physiologically vital serine protease, was investigated for its functional and conformational transitions during chemical (organic solvents, Gdn-HCl), thermal, and pH induced denaturation using biochemical and biophysical techniques and molecular dynamics (MD) simulations approach. The enzyme was exceptionally stable in isopropanol and ethanol showing 110\% and 75\% activity, respectively, after 96 h, showed moderate tolerance in acetonitrile (45\% activity after 72 h) and much lower stability in methanol (40\% activity after 24 h) (all the solvents [90\% v/v]). Far UV CD and fluorescence spectra indicated apparent reduction in compactness of KLKp structure in isopropanol system. MD simulation studies of the enzyme in isopropanol revealed (1) minimal deviation of the structure from native state (2) marginal increase in radius of gyration and solvent accessible surface area (SASA) of the protein and the active site, and (3) loss of density barrier at the active site possibly leading to increased accessibility of substrate to catalytic triad as compared to methanol and acetonitrile. Although kallikrein was structurally stable up to 90 °C as indicated by secondary structure monitoring, it was functionally stable only up to 45 °C, implicating thermolabile active site geometry. In GdnHCl [1.0 M], 75\% of the activity of KLKp was retained after incubation for 4 h, indicating its denaturant tolerance. A molten globule-like structure of KLKp formed at pH 1.0 was more thermostable and exhibited interesting structural transitions in organic solvents. The above results provide deeper understanding of functional and structural stability of the serine proteases at molecular level.

Keywords: Kallikrein; isopropanol tolerance; MD simulation; thermostability; molten globule; serine protease

Introduction

In the last few years, combination of theoretical and experimental approaches has substantiated structural knowledge about proteins particularly with physiological importance, at molecular level (Udgaonkar & Marqusee, 2013). Characterization of protein sequence-structure-function relationship has been reviewed by Ahmad et al. (2013). Depending on solvent conditions, the structural and conformational transitions of proteins are a significant way to elucidate their stability, folding pathways, and intermolecular aggregation behavior. Molecular dynamics (MD) plays a distinctive role in the area of protein folding owing to its simplicity and accuracy in an unbiased way (Rizzuti & Daggett, 2013).

Organic solvents are known to alter enzyme catalysis by improving solubility of substrate, enhancing reaction rates, altering specificity and hydrophobic interactions in a reaction (Serdakowski & Dordick, 2008). Many hydrolases e.g. amylases, cutinases, lipases, and proteases have been studied and modified for their behavior in organic solvents (Castillo et al., 2005; Doukyu & Ogino, 2010). Simulation studies of protein unfolding in presence of co-solvents supplement the experimental data well providing both a framework for data interpretation and a guide for further investigations. Lousa et al. have reported on theoretical basis, the differential stability of pseudolysin and thermolysin in ethanol/ water solutions (Lousa, Baptista, & Soares, 2012, 2013). Studies on trypsin by Meng et al. (2013) have established the weakening of the electrostatic interactions at active site of enzyme by polar solvents that significantly affects the binding strength.

Stable structural intermediates during the unfolding processes have been reported and characterized for various proteins that include a range of proteases like trypsin (Bittar et al., 2003), stem bromelain, papain, streblin, cryptolepain at low pH (Amri & Mamboya, 2012; Haq, Rasheed, & Khan, 2002; Kumar, Tripathi, de Moraes, Caruso, & Jagannadharm, 2014; Prasanna Kumari, Dubey, & Jagannadharm, 2013). Kallikreins are a group of serine proteases in mammals encompassing various plasma kallikreins and tissue kallikreins. Tissue kallikreins (EC 3.4.21.35) and the kallikrein-related peptidases are (chymo)trypsin-like serine proteases.
proteases, belonging to family S1A of clan PA(S) according to the MEROPS classification (Barrett, Tolle, & Rawlings, 2003). Physiological role of various kallikreins in cardiovascular, renal, and central nervous system diseases, as well as their application as cancer biomarkers (Costa-Neto et al., 2008; Paliouras, Borgono, & Diamandis, 2007) is well established. Porcine pancreatic kallikrein (KLKp) is the first enzyme in the family to be purified and biochemically characterized (Zuber & Sache, 1974). Structural studies of KLKp by Bode et al. (1983) have revealed remarkable similarity to trypsin except for deviations in external loops.

In spite of the vast studies, importance and structural information available on KLKp, the protein is unexplored for its folding/unfolding mechanism. With the aim of getting deeper understanding of structural and/or functional elements, KLKp was selected as a model system to perform these studies. Present experimental data concludes the unusual stability of KLKp toward high concentrations of polar organic solvents as examined using biochemical and spectroscopic (CD and intrinsic and extrinsic fluorescence) studies and was also monitored at molecular level by theoretical approaches. The solvents chosen were methanol, ethanol, isopropanol which are polar protic solvents, and acetonitrile and DMSO which are polar aprotic solvents. The protein was also monitored for functional and unfolding transitions during thermal, chemical, and pH-induced denaturation. Molten globule of KLKp at low pH was confirmed and characterized.

Materials and methods

Materials

Porcine pancreatic kallikrein (KLKp) was procured from Sigma-Aldrich (USA). Guanidine hydrochloride (GdnHCl), 1-Anilino-8-naphthalene sulfonate (ANS) were obtained from Sigma Aldrich Ltd., USA. All other reagents including buffer compounds and organic solvents used were of analytical grade. Solutions for spectroscopic measurements were prepared in MilliQ water.

Functional stability of the enzyme in presence of solvents

KLKp (.5 mg/ml) was incubated in 90% (v/v) of methanol, ethanol, isopropanol, acetonitrile (ACN), and dimethyl sulphoxide (DMSO) at pH 8.5 (tris-HCl, 20 mM) for 96 h at 28 °C. The solvents were miscible in the buffer and the enzyme solvent mixtures were incubated in static condition. The incubation mixtures were kept in tightly closed vials to avoid the volatile losses. 50 μl aliquots were removed at regular time intervals of 24 h up to 96 h and assayed for the enzyme activity.

Enzyme assay

The caseinolytic activity was measured by the method of Kunitz (1947), as described by Laskowski (1955) with some modification. The protocol was: Protease activity was determined by incubating 2.5 μg of the enzyme in 300 μl of 1% casein (substrate) and 8.5 (300 μl, 20 mM tris-HCl buffer) at 37 °C for 30 min to make the total volume 600 μl. The reaction was stopped by adding 900 μl of 5% TCA and the reaction mixture was allowed to stand for 30 min. Any precipitate formed was then removed by centrifugation and absorbance of the supernatant was read at 280 nm. One unit of protease activity is defined as the amount of enzyme which releases 1 μmol of tyrosine per minute in the assay conditions.

Circular dichroism (CD) spectroscopy

CD measurements of solvent treated KLKp (.13 mg/ml) samples were recorded using a Jasco J-815–150S (Jasco, Tokyo, Japan) spectropolarimeter connected to a Peltier Type CDFL Cell circulating water bath at 28 °C. Far UV spectra was recorded in a rectangular quartz cell of 1 mm path length in the range of 190–250 nm at a scan speed of 100 nm/ min with a response time of 1 s and a slit width of 1 nm. KLKp at a concentration of .13 mg /ml was used for recording the scan. Each spectrum was recorded as an average of 5 scanned spectra. Conformational transition studies of KLKp were carried out by incubating KLKp (.13 mg/ml) in respective solvents systems (75% v/v) for 72 h.

Results were expressed as mean residue ellipticity (MRE) in deg cm² dmol⁻¹ defined as:

\[
MRE = \frac{M \theta_\lambda}{10 d c r}
\]

where \(M\) is the molecular weight of the protein, \(\theta_\lambda\) 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. The relative content of various secondary structure elements was calculated by using CDPro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html). Low NRMSD values were observed for analysis with CONTINLL.

Steady-state Fluorescence study

Intrinsic fluorescence of the solvent-treated enzyme was measured using a Perkin-Elmer Luminescence spectrometer LS50B connected to a Julabo F20 water bath. The emission scans for KLKp incubated under respective conditions were recorded. The protein solution (40 μg/ml) was excited at 295 nm and the emission was recorded in the range of wavelength 300–400 nm at 28 °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 either buffer solution or buffer containing the appropriate quantity of denaturant or organic solvents was subtracted.

Solute quenching studies by steady-state fluorescence

Fluorescence measurements were performed for KLKp (40 μg/ml) incubated in respective solvent systems (90% v/v for 72 h) titrating with different quenchers like acrylamide (5 M) (neutral quencher), potassium iodide (5 M), and cesium chloride (5 M) (charged quenchers). Small aliquots of quencher stocks were added to protein samples, mixed well, and fluorescence spectra were recorded after each addition. Iodide stock solution contained .2 M sodium thiosulfate to prevent formation of tri-iodide (I\textsuperscript{−3}). Fluorescence intensities were corrected for volume changes before further analysis of quenching data. The data analysis was carried out as described in the supplementary information.

Simulation details

The high resolution X-ray crystal structure of porcine pancreatic kallikrein is reported in Protein Data Bank. This crystal structure (2PKA) was considered as a starting structure for simulations. In the present theoretical investigation, we have studied the effect of organic-water mixture on the conformational stability of the protein. The simulations were performed by taking one test case of the experimental condition of protein stability/activity in the organic/water (75% v/v) mixture. The water mixture of organic solvents such as methanol, ethanol, isopropanol, and acetonitrile were used to perform simulations. The kallikrein protein (2PKA) was solvated with these organic-water mixtures and then subjected to MD simulation. Simulation was also carried out in water (without organic solvent) and termed as reference/control simulation. Therefore, five different simulations (including water and water/organic mixture) were performed using different solvents to understand their effect on the protein conformations.

MD simulations were performed using Gromacs 4.5.5 simulation package (Hess, Kutzner, van der Spoel, & Lindahl, 2008; Van Der Spoel, Lindahl, Hess, Groenhof, & Mark, 2005). The united atom Gromos-53a6 force field was used to model the protein and a Simple Point Charged (SPC) model was used to represent the water molecules (Toukan & Rahman, 1985). The parameters for the organic solvents based on GROMOS-53a6 were taken from Automated Topology Builder (http://compbio.biosci.uq.edu.au/atb) (Malde et al., 2011). The NPT ensemble and periodic boundary conditions were applied to perform the initial 5 ns equilibration run. The protein was position restrained (for equilibration run) with the force constant of 1000 kJ/mol \textsuperscript{−1} which allows efficient equilibration to allow the solvent (water and organic solvent) to penetrate the solvent accessible surface area. The temperature was kept constant at 300 K using V-rescale thermostat with a coupling constant of .1 ps. The isotropic pressure coupling was applied using Berendsen barostat (Berendsen, Postma, van Gunsteren, DiNola, & Haak, 1984) and pressure coupling constant of 2 ps was used to keep pressure constant at 1 bar. The non-bonded interactions, Lennard-Jones potential were taken care with the cut-off of 1.4 nm and the electrostatic interactions were considered in the .9 nm cut-off. A reaction field correction was used for electrostatic interactions using dielectric constants of 67, 68, 68, and 56 for methanol/water, ethanol/water, isopropanol/water, and acetonitrile/water mixture, respectively. The particle meshewald (PME) method was used in protein water simulations to account the electrostatic interactions (Patra, Karttunen, Hyvönen, Falck, & Lindqvist, 2003). The production runs were carried out for 150 ns using same isoenthalpic-isobaric ensemble and simulation trajectories written after every 5 ps of time. These trajectories were then used further for analysis.

Analysis of KLKp structure simulated in respective solvent systems was carried out in iRDP (http://irdp.ncl.res.in) ICAPS module. The structure visualization was carried out using CCP4 mg (McNicholas, Potterton, Wilson, & Noble, 2011).

Thermal denaturation

Activity of KLKp during thermal denaturation was monitored by incubating the enzyme at respective temperature (30–80 °C) for 10 min and then performing the assay as previously mentioned. Peltier unit was used to control the temperature during far UV CD measurements with the ramp rate of 5 °C/min and incubation time set at 10 min after attaining the temperature. Far UV CD Spectra were recorded as mentioned earlier.

GdnHCl-induced denaturation

To study the effect of GdnHCl on the activity of the enzyme, samples of KLKp (.5 mg/ml) were incubated with various concentrations of GdnHCl (1.0–4.0 M), in 20 mM Tris-HCl buffer pH 8.5 for 4 h at 28 °C. Suitable aliquots were removed at regular time intervals of 1 h up to 4 h and assayed for enzyme activity. The readings were corrected for blank samples containing respective concentration of GdnHCl without the enzyme.

pH-induced denaturation

KLKp was incubated in buffers of different pH for 4 h before assaying for the activity (.5 mg/ml) and recording
the far UV CD spectra (.13 mg/ml). The buffers used were glycine–HCl (pH 1.0–3.0), citrate–phosphate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 7.0), Tris–HCl (pH 8.0–9.0), and glycine–NaOH (pH 10.0–12.0); at all 20 mM concentration. For recording near UV CD spectra, KLKp (1 mg/ml) was incubated at pH 1.0. Near UV CD spectra were recorded using a rectangular quartz cell of 5 mm path length in the range of 250–300 nm at a scan speed of 100 nm/ min with a response time of 1 s and a slit width of 1 nm.

I-Anilino-8-naphthalene sulfonate (ANS)-binding assay
The intermediate states were examined by the hydrophobic dye (ANS) binding to KLKp (40 μg/ml) incubated under respective denaturing conditions. The final ANS concentration used was 50 μM, excitation wavelength was 375 nm, and total fluorescence emission was monitored between 400 and 550 nm. Reference spectrum of ANS in each buffer of respective pH and denaturant was subtracted from the spectrum of the sample.

Characterization of molten globule
KLKp (.62 mg/ml) was incubated in 20 mM glycine-HCl buffer of pH 1.0 for 4 h and the sample was used for thermal denaturation studies using far UV CD spectroscopy. To study the effect of organic solvents, acid denatured KLKp was further treated with the respective solvents to final concentration of 75% (v/v) for 24 h. The organic solvents used were methanol, ethanol, isopropanol, and acetonitrile.

Results
Biophysical Characterization of KLKp
As per the available information concerning functional stability and structural transitions of Kallikrein, the protein has seven tryptophan residues. In the present studies, intrinsic fluorescence spectrum of KLKp showed \( \lambda_{\text{max}} \) of 343 nm indicating moderate hydrophilic environment of the tryptophan residues (Figure S1(a)). Decomposition analysis of the spectrum using PFAST software (Shen et al., 2008) showed that all the trp residues belong to either class I or II suggesting possible interaction of indole rings with structured water molecules. The results are supported by the time resolved fluorescence spectrum analysis of KLKp. Bi-exponential fitting of the decay curve obtained from the lifetime measurement of intrinsic fluorescence of KLKp (Figure S1(b)) revealed two decay times \( \tau_1 \) (1.27 ± 0.038 ns) with 38% contribution and \( \tau_2 \) (4.34 ± 1.13 ns) with 62% contribution (\( \chi^2 = 1.015 \)). This shows two populations of trp residues, one with shorter lifetime with less contribution present in the polar environment i.e. on the surface and the other one with longer lifetime with much higher contribution to fluorescence present in the non polar environment. This correlated well with the steady-state fluorescence data.

Native KLKp showed far UV CD spectrum with minima at 204 nm and negative ellipticity at 190 nm (Figure S1(c)), suggesting majority of β-sheet structure and significant content of unordered structure due to which, shift from the ideal β-sheet position (minima at 210–220 nm) toward 204 nm was observed. Analysis of this spectrum with CDPro deduced the secondary structure composition values as 5.5% α-helix, 39.8% β-sheets, 21.7% turns, and 33.0% unordered structure (NRMSD .034) which correlates with that of DSSP analysis of crystal structure available (Table S1).

Stability toward organic solvents
Activity of KLKp
KLKp showed 110% activity (proteolytic or caseinolytic activity) even after 96 h of incubation in presence of isopropanol (90% v/v) (Figure 1(a)). In methanol, at same concentration, 60% loss in activity was observed after 24 h. The residual activity of the enzyme in presence of ethanol was comparable to that of control reaction up to 48 h. Acetonitrile slowly deactivated KLKp with 65% retention of activity even after 48 h. DMSO immediately inactivated the enzyme. KLKp showed similar trend of functional stability in 75% (v/v) solvents as that observed in 90% concentration (Figure S2).

Further structural investigations of the present protein were performed by studying secondary structure, Trp environment, and MD simulations.

Secondary structure
Far UV CD spectra were monitored in presence of 75% solvents since further increase in solvent concentration produced high background noise (Figure 1(b)). Interestingly, KLKp showed considerable reduction in negative ellipticity in presence of isopropanol, as compared to native enzyme, still retaining the minima at 204 nm. This indicated apparent reduction in compactness of the conformation or formation of an altered structure, which might have stabilized the active site geometry and retained the activity of KLKp up to 96 h. The negative ellipticity of KLKp at 190 nm remained unaltered in isopropanol while it turned to positive values in other solvent systems confirming the structural transitions. In 75% methanol, formation of α-helix like structure was induced showing increase in ellipticity and shift in minima to 207 nm in far UV CD spectrum (Table S2). Ethanol caused the shift in the minima of the ellipticity to 207 nm, while shift to 213 nm in presence of 75%
acetonitrile indicated \( \beta \) sheet induction in the structure. However, most of these visible changes were not reflected in secondary structure composition analyzed by CDpro software (Table S2).

**Intrinsic fluorescence and solute quenching studies**

Intrinsic fluorescence spectra and solute quenching parameters of KLKp are shown in Figure 1(c) and Table 1, respectively. The Stern-Volmer plots have been presented in Figure S3.

KLKp in isopropanol showed a blue shift of 4 nm (Figure 1(c)), indicating increased polarity of Trp. Although \( K_{sv} \) for acrylamide was almost similar to that of native protein, the value for KI had increased twofold (1.057–2.57 M\(^{-1}\)) and that for CsCl had increased several folds. Thus, overall charge density with net negative charge around surface Trp had significantly increased.

| Solvent (Dielectric constant) | Activity | Fluorescence \( \lambda_{max} \) (nm) | Acrylamide | KI | CsCl | CD Wavelength of minimum ellipticity (nm) |
|------------------------------|----------|-------------------------------------|------------|----|------|------------------------------------------|
| Water (78.36)                | ++       | 343                                 | 6.246      | 1.057 | .267 | 204                                      |
| Isopropanol (19.92)          | +++      | 339                                 | 7.67       | 2.57 | 5.64 | .70 204 50% Reduced ellipticity          |
| Ethanol (24.55)              | ++       | 343                                 | 6.5        | 2.98 | 1.36 | .74 207                                  |
| Methanol (32.66)             | +        | 354                                 | 14.6       | 7.3  | 2.88 | 1.3 207 Increased ellipticity            |
| Acetonitrile (35.94)         | +        | 343                                 | 11.4       | 7.60 | 3.23 | .51 212                                  |
|                              |          | Increased intensity                 |            |     |      |                                          |
This could have been the ultimate result of stable conformation leading to functional stability of the protein. Ethanol did not cause change in the spectrum, while only minor increase in the negative charge density was observed.

\( \lambda_{\text{max}} \) of the protein in acetonitrile was unaltered, however, considerable increase in fluorescence intensity was observed indicating altered Trp microenvironment. Two-fold increase in \( K_{sv} \) for acrylamide and sevenfold increase in \( K_{sv} \) for KI could be due to the significantly increased fluorescence emission. This could be due to the destabilizing effect of acetonitrile. Significant red shift in \( \lambda_{\text{max}} \) (354 nm) was observed in presence of 90% methanol indicating apparent unfolding leading to inactivation of the protein. Higher rate of fluorescence quenching with acrylamide (14.59 M\(^{-1}\)) as well as with KI (7.25 M\(^{-1}\)) was observed in presence of methanol.

**MD simulation**

MD simulations of KLKp in solvent systems were analyzed over 150 ns (Figure S4). The parameters over last 50 ns were averaged in order to get the numerical values for root-mean-squared (RMS) deviation, radius of gyration (Rg), and solvent accessible surface area (SASA) (Table 2). The RMS deviations of the protein structure equilibrated in respective solvent systems from the initial structures are plotted as a function of simulation time in Figure S1. As compared to control simulation, RMS deviation for the protein backbone atoms was in order of, water (3.35 Å) < isopropanol (4.9 Å) < ethanol (5.5 Å) < acetonitrile (7.1 Å) < methanol (16.3 Å) (Table 2). This clearly indicated that water, isopropanol, and ethanol systems favor the structural stability of KLKp. On the other hand, protein in acetonitrile and methanol shows more structural fluctuation as depicted from the higher RMS values.

The structural compactness of the protein was analyzed from Rg and Solvent accessible surface area (SASA) of the protein. In methanol, significant increase in the Rg and SASA values of KLKp indicated the disordered structure in the protein. This must be the reason for the loss of activity of the protein observed in the experiment. On the contrary, Rg and SASA values remained comparable to that in water (1.73 nm) in isopropanol, ethanol, and acetonitrile (Table 2).

**Active site conformation**

The active site residues within the .5 nm radial distances from the catalytic triad (His57-Asp102-Ser195) were identified and their spatial arrangement in different solvent systems (last frame from MD simulation) was shown as surface representation in Figure 2(a).

KLKp active site in control simulations showed a channel for substrate movement from Ser195 to Asp102 through His57, as seen in the Figure 2(a). The active site conformation remains almost the same in ethanol system, while, in case of isopropanol system, it shows a minor increase in Rg and SASA values, indicating slightly altered pocket conformation that provides its better accessibility for the substrate molecules (Table 2). This justifies the elevated proteolytic activity of KLKp in presence of isopropanol. In case of acetonitrile system, the altered active site conformation of KLKp results in buried catalytic triad residues thus reducing the catalytic efficiency of the enzyme, while in methanol it is disoriented which correlates the loss of activity in these systems.

The analysis of KLKp structures equilibrated in respective solvent systems using iRDP server and the structural superimposition of active site residues of KLKp in water with those in respective solvent systems revealed the structural changes in catalytic triad of the enzyme (Figure 2(b)). In isopropanol, the catalytic triad is more stabilized owing to minor displacement of Asp102 that results in additional hydrogen bond formation between His57-Asp102. Ethanol seems to induce a minor displacement of His57 without considerable change in the conformation of active site residues. Methanol system induced distortion of the active site conformation as seen in Figure 2(b) leading to functional loss of the enzyme.

Analysis of the active site conformation (ASC) supports these conformational changes as RMSD and RMSF values for ASC are in the order of

---

### Table 2. Summary of structural parameters of KLKp simulated in respective solvent systems.

| Solvent  | RMSD (nm) | Rg (nm) | SASA |
|----------|-----------|---------|------|
|          | TS ASC    | TS AS   | TS ASC |
| Water    | .335 ± .015 | .3 ± .01 | 1.73 ± .007 | .91 ± .0075 | 113.5 ± 2.4 | 30.35 ± .7 |
| Isopropanol | .49 ± .06  | .39 ± .021 | 1.8 ± .034 | 1.0 ± .01 | 133.38 ± 2.88 | 34.5 ± 1.04 |
| Ethanol  | .55 ± .047 | .35 ± .023 | 1.86 ± .023 | .965 ± .024 | 135.5 ± 4.8 | 32.41 ± 1.1 |
| Methanol | 1.63 ± .077 | 1.4 ± .065 | 2.19 ± .045 | 1.77 ± .069 | 176.5 ± 4.65 | 44.17 ± 1.52 |
| Acetonitrile | .71 ± .071 | .68 ± .1 | 1.83 ± .031 | 1.114 ± .08 | 130.42 ± 2.99 | 32.63 ± .9 |

Note: TS: Total structure ASC: Active site conformation.
water < isopropanol < ethanol < acetonitrile < methanol (Figure 3(a) and (b)). The increase in the Rg values in ASC of KLKp in methanol (18.3 Å) suggests loss of compact conformation integrity (Figure 3(c)). Increased SASA value in isopropanol validates better accessibility of the active site for the substrate molecules (Figure 3(d), Table 2).

**Thermal transitions of KLKp**

The proteolytic activity of KLKp reduced to 10% at 55 °C in 30 min. However, the residual 10% activity was retained up to 90 °C (Figure 4(a)). The loss in activity was reflected in intrinsic fluorescence spectra, where $\lambda_{\text{max}}$ showed red shift by 7 nm above 60 °C (Figure S5). Sigmoidal fit of the curve indicated that $T_m$ of KLKp as 61.5 °C. Far UV CD spectra did not show significant change in secondary structure even at 90 °C, except reduction in the ellipticity at 190 nm indicating reduction in the ordered structure (Figure 4(b)). The rigidity of secondary structure of KLKp might be responsible for protecting the active site leading to retention of residual activity up to 90 °C.

**GdnHCl induced denaturation**

KLKp expressed 90% and 60% proteolytic activity in presence of 1.0 and 2.0 M GdnHCl, respectively, even after incubation of 4 h (Figure 5(a)). Secondary structure, as monitored by CD spectra, was stable in 1 M GdnHCl for 4 h (Figure 5(b)). Equilibration of KLKp in GdnHCl for 24 h caused gradual red shift in $\lambda_{\text{max}}$ of intrinsic fluorescence of the protein indicating that unfolding of KLKp is a multistep process (Figure 5(c)). Under renaturing conditions, $\lambda_{\text{max}}$ values of refolded samples overlapped with those in unfolding process at respective GdnHCl concentrations (Figure 5(c)) indicating the reversibility of the process.

**pH induced conformational changes**

KLKp is functionally stable in the pH range of 5.0–9.0; although at pH 3.0, the activity reduced to 60% after incubation for 72 h. Immediate loss in activity was observed at pH 1.0 (Figure S6(A)). This could be due to protonation of the amino acid residues and/or change in conformation of the protein. The loss in activity was reflected in intrinsic fluorescence spectra where $\lambda_{\text{max}}$ showed red shift by 3 nm at pH 1.0, 3.0, and 11.0 (Figure S6(b)). There is increase and decrease in the fluorescence intensity observed at pH 11.0 and 1.0, respectively. The deprotonation and protonation of the residues around tryptophans could be changing the microenvironment thereby enhancing or quenching the fluorescence emission of KLKp. Significant ANS binding observed only at pH 1.0 indicated exposure of...
Figure 3. Structural parameters for active site conformation of KLKp simulated in respective solvent system for 150 ns. (a) RMSD of KLKp structure calculated for protein backbone atoms as a function of time (b) RMSF values for active site conformation (c) Radius of gyration (d) Solvent accessible surface area (SASA) for respective solvent systems, Control (1), isopropanol (2), ethanol (3), acetonitrile (4) methanol (5).

Figure 4. Thermal denaturation of KLKp. (a) activity profile (square) and change in $\lambda_{\text{max}}$ (circle) of intrinsic fluorescence spectra during thermal denaturation. (b) Far-UV CD spectra of KLKp (130 $\mu$g/ml) incubated at respective temperature for 10 min as described in materials and methods.
hydrophobic residues on the surface of the protein preceding alteration of tertiary structure (Figure 6(a)). This suggested presence of molten globule at pH 1.0.

The secondary structure of the protein showed noticeable although not significant change in the pH range of 1.0–11.0 as seen in far UV CD spectra (Figure 6(b)). Slight increase in negative ellipticity at pH 11.0 indicated compaction of structure. The near UV CD spectra showed drastic loss in tertiary structure at pH 1.0 in spite of the unaltered secondary structure (Figure 6(c)). This supported the observation of formation of a molten globule-like structure (MG) of KLKp at pH 1.0.

Characterization of the molten-globule-like structure of KLKp

The molten globule-like structure of KLKp observed at pH 1.0 did not show any further change in the secondary structure up to 85 °C (Figure 6(d)). Unlike native KLKp, ellipticity of MG at 190 nm remained unaltered till 85 °C indicating higher thermostability of MG-like structure.

Analysis of secondary structural transitions of KLKp at pH 1.0 in 75% alcohols and acetonitrile (Figure 6(e)) indicated significant α-helix induction in MG-like structure of KLKp. Methanol and acetonitrile were least effective as helix-inducers, whereas ethanol and isopropanol enhanced helix-formation in MG-like structure from 6 to 24% (Table 3). The activity of the enzyme though was not regained in presence of these solvents.

Discussion

Stability of KLKp in organic solvents

The polarity of solvents used in the present study was in the order of Isopropanol < ethanol < Methanol < Acetonitrile < DMSO (Reichardt & Welton, 2011). The functional stability of KLKp in these solvents was inversely proportional to their polarity. Failure of aprotic solvents (acetonitrile and DMSO) to maintain activity of KLKp could be due to different mode of solvation they assume as compared to the protic ones. The instability of KLKp in more polar solvents (methanol) could be due to the stripping of water molecules from the active site leading to unfavorable changes in the conformation.

The intrinsic activation energy of subtilisin catalyzed reactions in organic solvents is strongly dependent on the polarity of the reaction medium (Kim, Clark, & Dordick, 2000). Our results were also supported by the report of trypsin from bovine and porcine pancreas, marine crab and European sea bass, where, enhancement in the activity was observed in presence of propanol (Harpaz, Eshel, & Lindner, 1994; Saborowski, Sahling, del Toro, Walter, & Garcia-Carreño, 2004). The effect of ethanol on trypsin activity was found to be less distinct and methanol was the least activating solvent. As compared to trypsin, chymotrypsin is less stable in presence of polar solvents but showed improved stability when supplemented with 1 M Ca\(^{2+}\) (Kotormán, Laczkó, Szabó, & Simon, 2003). In contrast to trypsin-like proteases, subtilases from *Pseudomonas aeruginosa, Bacillus thermoproteolyticus* and *Beauveria sp.* MTCC 5184 are reported to be more stable in methanol for 24 h (Dalal, More, Shankar, Laxman, & Gaikwad, 2014; Ogino et al., 2007). Earthworm serine protease is reported to be highly stable in 25% organic solvents for prolonged duration up to 100 days (Nakajima, Sugimoto, & Ishihara, 2000). KLKp stands out as a mammalian protease with high organic solvent stability in concentration as high as 90% for 4 days.

Functional stability of KLKp in isopropanol was strongly supported by conformational analysis viz retention of CD minima at 204 nm, similar accessibility of Trp toward neutral quencher and minimum deviation of the structure from that of control simulation. It can be
mentioned that apparent reduction in the compactness of secondary structure as seen in significantly reduced negative ellipticity and intermediate increase in Rg and SASA values as compared to control were also observed. Blue shift in $\lambda_{\max}$ of intrinsic fluorescence spectrum in isopropanol and increased negative charge density around Trp indicated some change in conformation. All these changes could be contributing to the stability and enhanced activity of KLKp after incubation in isopropanol.

In ethanol, the secondary structure, Trp environment, and accessibility to neutral quencher were comparable to those of control. The structural parameters of the protein observed marginally differ from control simulations validating stabilizing effect of ethanol.

Conversely, methanol and acetonitrile changed the conformation of KLKp significantly as seen in CD, fluorescence and MD simulation studies. The alteration of far UV CD spectrum and red shift in $\lambda_{\max}$ of intrinsic fluorescence spectrum suggested structural modification of KLKp in methanol which could be unfavorable. These conformational changes were validated by observation of highest RMSD and RMSF values of the protein in methanol. These structural modifications could lead to

Table 3. Secondary structure analysis of molten globule of KLKp in organic solvents.

| Sample    | Helix (%) | Sheets (%) | Turns (%) | Unordered (%) | NRMSD |
|-----------|-----------|------------|-----------|---------------|-------|
| pH 8.5    | Control   | 5.5        | 39.8      | 21.7          | 33.0  | .034   |
| pH 1.0    | Control   | 6.9        | 37.4      | 21.6          | 32.3  | .049   |
| Methanol  | 7.1       | 39.0       | 21.6      | 32.7          |       | .058   |
| Ethanol   | 23.5      | 23.1       | 20.7      | 32.7          |       | .033   |
| Isopropanol | 24.7   | 23.9       | 22.8      | 34.0          |       | .042   |
| Acetonitrile | 9.0        | 34.3       | 22.8      | 34.0          |       |        |
loss in the activity of KLKp in methanol and acetonitrile.

Analysis of simulation data in iRDP (in silico Rational Design of Proteins) web server (http://irdp.ncl.res.in) revealed that the extent of hydrogen bonding increases considerably in isopropanol as compared to other systems, with additional hydrogen bond formation between His57-Asp102. Additionally, the ionic bond between His57 and Asp102 is maintained only in water and isopropanol system. The ion pair is disrupted in all other solvent systems. As indicated by superimposition of active site residues, ethanol and isopropanol maintain the conformational integrity of active site residues. Burial of active site residues may hinder the catalytic efficiency of KLKp in acetonitrile. In methanol, the drastic change in orientation of active site residues could disrupt the formation of oxyanion hole, which is a rate-limiting step for protease activity. Similar changes in the conformation of active site residues have been documented in case of subtilisin Carlsberg where displacement of Asn155 disrupts formation of oxyanion hole leading to loss of the enzyme in acetonitrile (Cruz, Ramirez, Santana, Barletta, & López, 2009).

Thermal transitions of KLKp
Similar trend of unusual thermostability of KLKp has also been reported for human KLK1, where it retained 25% activity up to 100 °C (Gao et al., 2006). Retention of 20% activity even at 80 °C was also observed in bovine, dog, and rat pancreatic kallikreins (Hojima, Yamashita, Ochi, Moriwaki, & Moriya, 1977). Camel pancreatic kallikrein was partially active till 70 °C, while total deactivation was observed at 80 °C (Fyiad, Khafagy, Paliouras, Borgono, & Diamandis, 2007). The structural basis of the considerable thermostolerance of pancreatic kallikreins is unexplored. The analysis of the KLKp structure [PDB id: 2PKA] in iRDP server revealed presence of five disulfide bridges in the protein which are all buried in the hydrophobic core making it inaccessible for thermal denaturation. This may help in retaining the secondary structure intact as seen in the far UV CD spectra at higher temperatures.

Characterization of molten globule
Higher stability of structural intermediate as observed for KLKp has been reported for GdnHCl-induced molten globule of α-mannosidase from Canavalia ensiformis (Kumar & Gaikwad, 2010). Siddiqui et al. have proposed entropy-driven mechanism for higher thermostability of molten globule of α-amylase (Siddiqui et al., 2010).

Induction of structural transitions in molten globule of KLKp by organic solvents was an interesting observation in the present studies. Dill et al. (1995) have proposed that alcohols stabilize α-helical conformation in unfolded proteins by weakening non-local hydrophobic interactions and enhancing local polar interactions. The hydrogen bonds thus formed result in stabilization of extended helical rods in which the hydrophobic side chains are exposed and polar amide groups are shielded from the solvents. This explains higher α-helix propensity of ethanol and isopropanol which are less polar solvents as compared to methanol and acetonitrile having very low α-helix induction capacity. Similar helix propensity of alcohols was recorded for structural intermediates of stem bromelain and subtilase from Beauveria sp. MTCC 5184 (Dalal et al., 2014; Haq, Rasheed, Sharma, Ahmad, & Khan, 2005).

Conclusion
In spite of several reports on serine proteases being stable in organic solvents, studies describing the structural transitions and their correlation with the functional transitions are scarce. The unusual stability of KLKp under stress like high organic solvent concentration and high temperature makes it an interesting model. The present studies of KLKp bring deeper understanding of the enzyme at molecular level and may help in emerging therapeutic applications of several kallikreins.

Supplementary material
The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2016.1138884.

Disclosure statement
No potential conflict of interest was reported by the authors.

Acknowledgments
The authors are thankful to Dr M. Fernandes and Dr S. Roy, NCL, for allowing the use of CD and MD simulation facility, respectively. SD was supported as SRF by the CSIR, New Delhi, India. AM was supported as SRF from the UGC, New Delhi, India.

References
Ahmad, E., Rabbani, G., Zaidi, N., Khan, M. A., Qadeer, A., Ishtikhar, M., … Khan, R. H. (2013). Revisiting ligand-induced conformational changes in proteins: Essence, advancements, implications and future challenges. Journal of Biomolecular Structure and Dynamics, 31, 630–648.
Amri, E., & Mamboya, F. (2012). Papain, a plant enzyme of biological importance: A review. American Journal of Biochemistry and Biotechnology, 8, 99–104.
Barrett, A. J., Tolle, D. P., & Rawlings, N. D. (2003). Managing peptidases in the genomic era. Biological Chemistry, 384, 873–882.
Rizzuti, B., & Daggett, V. (2013). Using simulations to provide the framework for experimental protein folding studies. *Archives of Biochemistry and Biophysics, 531*, 128–135.

Saborowski, R., Sahling, G., del Toro, M. A. N., Walter, I., & García-Carreño, F. L. (2004). Stability and effects of organic solvents on endopeptidases from the gastric fluid of the marine crab Cancer pagurus. *Journal of Molecular Catalysis B: Enzymatic, 30*, 109–118.

Serdakowski, A. L., & Dordick, J. S. (2008). Enzyme activation for organic solvents made easy. *Trends in Biotechnology, 26*, 48–54.

Shen, C., Menon, R., Das, D., Bansal, N., Nahar, N., Guduru, N., & Reshetnyak, Y. K. (2008). The protein fluorescence and structural toolkit: Database and programs for the analysis of protein fluorescence and structural data. *Proteins: Structure, Function, and Bioinformatics, 71*, 1744–1754.

Siddiqui, K. S., Poljak, A., Francisci, D. D., Guerriero, G., Pilak, O., Burg, D., ..., Cavicchioli, R. (2010). A chemically modified α-amylase with a molten globule state has entropically driven enhanced thermal stability. *Protein Engineering Design and Selection, 23*, 1–12.

Toukan, K., & Rahman, A. (1985). Molecular-dynamics study of atomic motions in water. *Physical Review B, 31*, 2643–2648.

Udgaonkar, J., & Marqusee, S. (2013). Folding and binding. *Current Opinion in Structural Biology, 23*, 1–3.

Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., & Mark, A. E. (2005). GROMACS: Fast, flexible, and free. *Journal of Computational Chemistry, 26*, 1701–1718.

Zuber, M., & Sache, E. (1974). Isolation and characterization of porcine pancreatic kallikrein. *Biochemistry, 13*, 3098–3110.