Application of transverse urea gradient zymography for structural and functional characterization of proteolytic enzymes

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Inactivation of an enzyme as it begins to unfold, along with the conformational perturbations which follow, can provide an insight into dynamics of the unfolding pathway. Urea gradient electrophoresis combined with zymography is a sensitive technique which provides a continuous visual profile of a proteolytic enzyme undergoing denaturation and inactivation simultaneously. Trypsin has been used as a reference protease to validate and standardize the method by correlating inactivation profile generated in zymography with a solution state assay. Stem bromelain, a cysteine endopeptidase was used as a case study to evaluate this methodology. The method highlighted the effect rendered by the substrate on the stability of the proteolytic domain of the enzyme, as it undergoes urea-induced unfolding. Transverse urea gradient zymography combined with molecular modelling of stem bromelain, where the disulphide bonds have been reduced, indicated that the evolutionary retention of Cys_{23}–Cys_{63} could be attributed to localized stabilization imparted by this bond to the catalytic site. This method encompasses various dimensions to extend the understanding of structure–function relationship in denaturant-induced unfolding pathways of proteases.

Keywords: Protein unfolding, stem bromelain, urea gradient, zymography.

TRANSVERSE urea gradient zymography (TUGZ) is an extended protocol based on the incorporation of transverse urea gradient in substrate polyacrylamide gels. Urea gradient electrophoresis provides a qualitative estimate of urea-induced conformational transitions whereas zymography identifies latent proteolytic activity under denaturing conditions[1–6]. Thus, the combination of two standard techniques can provide a comparative analysis of the unfolding behaviour of proteases having complex structures under variable conditions. There are fragmented reports where TUGZ was applied to verify stability of proteases against urea denaturation or to characterize proteolytic activity in a test sample[7,8]. In order to monitor the functional changes resulting from denaturant-induced unfolding, well-studied proteases such as trypsin, chymotrypsin and collagenase were selected which could validate and establish the general applicability of this method[9,10]. Post-standardization, the method was used to evaluate the behaviour of stem bromelain in the presence of urea.

Crude extracts from the stem and fruit of pineapple plant (*Ananas comosus*) are called 'stem bromelain' and 'fruit bromelain' respectively. These bromelains contain a number of cysteine proteases having similar physiochemical properties along with other enzymes like peroxidase and enzyme inhibitors. These cysteine proteases are also called stem bromelain (SB) and fruit bromelain respectively, according to their sources. These cysteine proteases have highest abundance in the source where one component dominates. SB, a cysteine protease of 23.8 kDa, belongs to the ($\alpha + \beta$) protein family[11]. It is the major enzyme present in pineapple stem extract. Some of the minor proteases are likely to be isoforms of stem bromelain while others are distinctly different, e.g. ananain and comosain. SB shares a high degree of sequence homology with papain[12], ervatamin[13] and actinidin[14], indicating the presence of common folding patterns. X-ray diffraction studies indicate a high percentage of hydrophobic and uncharged amino acids with the polypeptide chain folding into two domains which interact through hydrogen bonds, salt bridges, etc.[15].

The sequence of SB contains one sulphydryl group (Cys_{26}), three disulphide bonds (Cys_{23}–Cys_{63}, Cys_{57}–Cys_{96} and Cys_{152}–Cys_{199}) and the catalytic triad Cys_{26}–His_{158}–Trp_{176}. The role of disulphide bonds is an active topic for study since they must provide some distinct advantage to the stability of the molecule, preventing it from being eliminated during evolution[16].

Profiling of stem bromelain using TUGZ was performed and analysed densitometrically to compare with the solution state denaturation profiles. Results revealed distinct visualization of the unfolding transitions of the multiple components present in the proteolytically active fraction of stem bromelain. This method thus provided vital data to simultaneously assess the denaturation and inactivation profiles of stem bromelain. Unlike solution state studies, the profiles generated were continuous ones without missing a point, showing unusual behaviour of the intermediates.

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Materials

SB (EC 3.4.22.32), azocasein, acrylamide, bis-acrylamide, BSA, CHAPS, TEMED, Z-Arg-Arg-NHMec, DEAE-cellulose, CM-cellulose, casein, fibrinogen, thrombin, glycopeptidase A (almonds), trypsin and chymotrypsin (bovine pancreas), collagenase (*Clostridium histolyticum*), agarose, Triton X-100, APS and DTT were from Sigma-Aldrich, USA; urea from Calbiochem; Coomassie Blue R250 and bromophenol blue from Pierce; ampholytes (3–10) from Bio-Rad, Hercules, USA and IPG strip, Sephadex G-50 from GE Healthcare, Uppsala, Sweden. All other reagents were of analytical grade.

Methods

Fractionation of stem bromelain

Crude SB (50 mg/ml) was dissolved in 20 mM Na-phosphate, pH 7.5 (buffer A) and centrifuged at 10,000 rpm for 5 min. The clear supernatant was applied to a sephadex G-50 column (1.2 × 90 cm) pre-equilibrated with buffer A. Flow rate was 15 ml/h and fraction size was 2 ml. Elution of proteins was monitored at 280 nm and assayed for proteolytic activity using azocasein as the substrate[17]. Fractions with appreciable proteolytic activity were pooled and applied to a DEAE-cellulose column (1.6 × 7.5 cm) pre-equilibrated with buffer A. Bound fractions were eluted after a linear gradient of 0–0.5 M NaCl was applied in the same buffer. Elution was continued with an isocratic flow of 0.5 M NaCl in buffer. Flow rate was 1 ml/min and fraction size was 2 ml. The unabsorbed fraction was further applied to a CM-cellulose column (1.6 × 7.5 cm) following similar protocol. Bound and unbound fractions from these two ion-exchange chromatograms were assayed for proteolytic activity as stated. Concentration of bromelain was calculated using $\varepsilon^{1\%}_{280\ nm} = 20.1$ (ref. 18).

Two-dimensional zymography

SB (150 µg, 0.025 ml) was treated with 0.1 ml rehydration buffer (2% CHAPS, 0.2% ampholytes, pH 3–10 and 0.002% bromophenol blue). A nonlinear pH 3–10 IPG strip was rehydrated with this sample for 16 h at 25°C and subjected to isoelectric focusing[19]. Post-run, the strip was equilibrated with 0.05 M Tris-HCl, pH 8.8 containing 30% glycerol and placed on a 12.5% polyacrylamide gel using 1% agarose. After electrophoresis, the gels were washed twice with 2.5% Triton-X 100, each for 20 min followed by incubation in the developing buffer (0.1 M Tris-HCl, pH 7.5 containing 0.01 M $CaCl_2$) for 24 h. The gels were stained with Coomassie R-250 and destained using methanol : acetic acid : water (60 : 10 : 30).

Transverse urea gradient zymography

Electrophoresis was done using a Genei vertical mini gel system (Mumbai, India) with gel size of 8 × 7 cm. TUGZ gels were prepared with a gradient of 0–8 M urea and an inverse acrylamide gradient of 11–7% with 0.15% (w/v) of substrate (BSA, fibrin or gelatin) incorporated in the gel solutions. Gradient solutions with urea (V_1, 3 ml) and without urea (V_2, 3 ml) were prepared as follows: V_1 contained 0.525 ml of 40% acrylamide solution (29 : 1), 1.44 g urea, 125 µl of bovine serum albumin (1.5 mg/ml), 15 µl of 1% SDS, 20 µl TEMED, 10 µl APS (0.1 g/ml) and 1.0 ml of appropriate buffer to make up the volume upto 3.0 ml. V_2 was the same as V_1 except that it was devoid of urea and contained 0.825 ml of 40% acrylamide. TEMED and APS were added prior to casting of the gel. The bottom of the casting chamber was sealed using 10% acrylamide solution. The gradient solutions V_1 and V_2 were poured into the outlet and reservoir chambers of a 25 + 25 ml gradient mixer (Hoefer) holding a magnetic stirrer respectively. The inter chamber valve was opened and urea gradient from 8 to 0 M was poured from bottom to top of the casting chamber. The gel assembly was pre-electrophoresed at 50 V for 60 min in the gel buffer. Electrophoresis of the samples was performed at 12 mA till the dye reached the bottom of the gel. At the completion of the run, the gels were washed twice in 2.5% Triton-X 100 followed by incubation in the developing buffer (0.1 M Tris-HCl, pH 7.5 and 0.01 M $CaCl_2$) for 24 h. The gels were stained in Coomassie R-250. Composition of gel buffer was 25 mM Tris, 192 mM glycine, pH 8.3 and remained unaltered in all sets. The sample to generate unfolding patterns of SB consisted of 25 mM buffer, 20% glycerol, 150 µg SB and 0.002% Bromophenol Blue. Effect of substrate variation on the unfolding of SB was followed using fibrin[20] instead of BSA. Briefly, fibrinogen (0.12%) and thrombin (10 NIH units/ml) were copolymerized with acrylamide and urea with the *in situ* generation of fibrin. Zymograms were scanned densitometrically using Image J software (freely available at http://rsb.info.nih.gov/ij) which gave a measure of the relative density of the selected lanes in terms of arbitrary units. Transverse urea gradient gel electrophoresis (TUGE) was performed under similar conditions as in TUGZ without incorporation of substrate in the gel.

Assay of amidolytic activity

Amidolytic activity of trypsin and SB in presence or absence of denaturants was estimated using Z-Arg-Arg-NHMec as the substrate[21]. The reaction was initiated by the addition of substrate (20 µM) to the protease (50 µg/ml) that was denatured sequentially by 0–8 M urea in 0.01 M Na-phosphate, pH 7.5 at 25°C for 2 h. To determine the catalytic efficiency of SB post-successive

reduction of disulphide bonds, amidolytic activity of reduced SB (250 μg/ml) was measured using the substrate (5–100 μM). Fluorescence of the released NHMec group was followed continuously for 5 min (ex/em: 360/460 nm; slit widths 2.5/2.5 nm) using a Hitachi F4500 spectrofluorimeter attached to a circulating water bath.

Deglycosylation of SB

SB (0.25 mg) was digested with 0.5 mU of glycopeptidase A in 0.1 M Na-citrate, pH 5.2 at 37°C for 5 h. The treated SB was dialysed against 10 mM Na-phosphate, pH 7.5 at 4°C for 12 h. Deglycosylation was verified by estimation of carbohydrate content of treated SB using sulphuric acid-UV method[22]. Proteolytic activity of deglycosylated SB was measured using azocasein as the substrate[16].

Stepwise cleavage of intra-peptide disulphide bonds

SB contains one cysteine and three disulphide bonds. These bonds can be sequentially cleaved using increasing concentrations of denaturant and reducing reagent[23]. First disulphide bond was cleaved after incubating the enzyme in presence of 10 mM DTT and 4 M urea at 0°C for 3 h. Second disulphide bond was reduced under identical conditions except with 7 M urea while the third disulphide bond was reduced by 8 M urea and 10 mM DTT at 60°C for 3 h. Amidolytic activity of the partially or fully reduced SB was estimated. The corresponding K_m and K_{cat} were determined from Lineweaver-Burk plots.

Molecular modelling

In the absence of crystal structure of bromelain, templates based on sequence homology were searched and consequently, 3D molecular models were generated. 'Swiss model' software was used to generate a structure for bromelain based on target-template sequence alignment of 50% sequence identity with the plant cysteine protease ervatamin B (1IWD) isolated from *Ervatamia coronaria*.

MALDI-TOF MS

Mass spectrometric analysis was performed using 4700 MALDI TOF/TOF (Applied Biosystems) operated in positive ion mode. Trypsin was desalted using C_{18} zip tip (Millipore), eluted with 50% acetonitrile/0.1% triflouroacetic acid and applied to the MALDI plate along with the matrix α-cyano-4-hydroxycinnamic acid (CHCA) at a ratio of 1 : 1 (v/v).

Results

Characterizations of TUGZ gels

TUGZ gel is a composite mixture of polyacrylamide, urea, protein substrate and the protease. Therefore, several control gels were run to ensure proper evaluation of the results. Though the theory of zymography depends on copolymerization of the substrate with acrylamide leaving no scope for their electrophoretic mobility, existence of unbound substrate and presence of urea as protein solubilizing agent may leave some of the substrates migrating. To eliminate the possibility of migration of substrate embedded inside the gel to any significant extent, TUGZ was performed without application of any protease. In these gels, substrate concentrations were varied from 0.005 to 0.1% so that partial migration of the substrate, if any, could be detected at relatively low substrate concentrations. In these experiments, duration of electrophoresis varied from 30 to 90 min to follow time-dependent escape of unbound and/or dissociated substrate.

In the case of BSA, post-staining, no distinguishable non-uniformity in the substrate background was observed, supporting the fundamental theory of zymography, where immobilization of the substrate is mentioned. However, depending on the purity of the substrate, acrylamide and the buffer, occasionally a faint blue band was observed which was proteinaceous in nature. This originated from either the degraded products of the substrate or impurities from the reagent which failed to copolymerize with acrylamide. At higher substrate concentration, partial depletion of substrate from the gel due to electrophoretic migration remains undetected as the residual substrate may impart too intense colour after staining. With fibrin, no noticeable migration of substrate was observed which was attributed to the *in situ* generation of this polymer from fibrinogen. Similar results were observed with gelatin as substrate.

Validation of TUGZ profiles

Trypsin served as a reference protease to generate TUGZ profiles for comparison with its reported denaturing properties. Being a monomeric protease, its TUGZ profiles were expected to be free from complications arising from dissociation of subunits affecting electrophoretic motilities, apart from unfolding of the molecule. Further, pure preparations were used. Mass spectrometric analysis revealed a single peak corresponding to 23,302 Da as compared to 23,022 Da obtained from the amino acid sequence (http://web.expasy.org/compute_pi/) (Figure 1 *a*). Therefore, possible interference from contaminating protein bands in the zymogram was avoided. Before applying to TUGZ, trypsin was analysed using TUGE gel to testify the reproducibility and applicability of the method.

In both cases, the profiles showed a single transition between 6 and 7 M urea (Figure 1 *b* and *c*). This agreed with previous studies on stability of trypsin up to 6.5 M urea[24]. The ΔG of unfolding calculated from the zymography gel was -2.00 ± 0.82 RT. This value correlated well with -2.14 ± 0.17 RT, obtained from the transition profile constructed from solution state assay (Figure 1 *d*). These observations provided initial validation of TUGZ to elucidate the conformational and functional changes of a protease in the presence of variable concentration of urea.

Similar attempts were made with other proteases like chymotrypsin and collagenase using BSA and gelatin respectively, as the substrate (Figure 1 *e*–*h*). The TUGZ profile of chymotrypsin showed a major band, the migration profile of which was similar to that of its TUGE (Figure 1 *e* and *f*). Both the bands showed transitions beginning at 5.5 M urea. Another slow migrating band merging into a smear was also visible in the TUGZ profile. Multiple bands were visible in the TUGE of chymotrypsin with diverse migratory patterns. The TUGZ profile of collagenase showed multiple bands where one of them was stable against urea induced unfolding while the rest showed transition at 7–8 M urea. Similar pattern was replicated in the TUGE profile (Figure 1 *g* and *h*). These attempts were made to ascertain the applicability of TUGZ in various proteases.

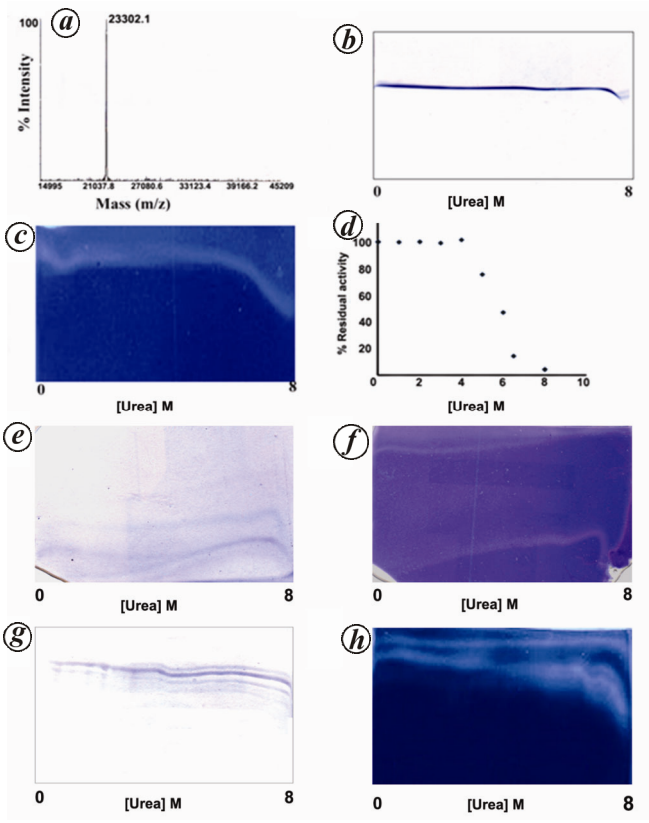

Figure 1. ***a***, MALDI-TOF MS of trypsin under positive ion mode shows 100% abundance of a peak for *m*/*z* 23302.1 Da. ***b***, Transverse urea gradient electrophoresis (TUGE) of trypsin in 0.05 M Tris-HCl, pH 8.8. The linear gradient of 0–8 M urea was superimposed on inverse acrylamide gradient of 11–7%. ***c***, Transverse urea gradient zymography (TUGZ) of trypsin performed under similar conditions as (***b***) along with the incorporation of 0.15% BSA as substrate in the gel. ***d***, Amidolytic assay of trypsin using Z-Arg-Arg-NHMec as the substrate to assess the inactivation of trypsin in presence of urea in solution state. ***e***, ***f***, TUGE and TUGZ of chymotrypsin in 0.05 M Tris-HCl, pH 8.8. ***g***, ***h***, TUGE and TUGZ of collagenase in 0.05 M Tris-HCl, pH 8.8. BSA and gelatin served as substrates for chymotrypsin and collagenase respectively.

Fractionation of bromelain

The crude extract of 'stem bromelain' consists of several cysteine proteases with bromelain being the most abundant one. In order to study the unfolding transitions of bromelain by TUGZ, it was necessary to establish the presence of multiple proteolytic components in the crude extract. Application of 'stem bromelain' to size-exclusion chromatography resolved it into two distinct fractions with the exclusive retention of proteolytic activity by fraction 1 (Figure 2 *a*). This fraction was used as sample for TUGZ. Subsequent application to DEAE-cellulose followed by CM-cellulose indicated the presence of multiple proteolytic components (Figure 2 *b* and *c*). Abundance of alkaline proteases was reflected in the profile of CM-cellulose. It was further confirmed by 2-D zymography that although a majority of components were maximally active at alkaline pH, there were few others all through the pH range (Figure 2 *d*). These experimental attempts provided adequate evidences regarding the presence of multiple proteolytic species which were necessary to gauge the nature of TUGZ profiles. Before application of SB to TUGZ, it was applied to TUGE to elucidate its migration and conformational pattern in the absence of the substrate (Figure 2 *e*). SB showed multiple bands with appreciable stability against urea. At around 7–8 M urea, a major transition was visualized. This gel pattern, when compared with TUGZ, could provide an initial understanding of the role of substrate in urea-induced unfolding and inactivation.

TUGZ of stem bromelain

The presence of partially folded intermediate conformations can be predicted from the plots of mean hydrophobicity of a protein versus mean net charge[25]. Based on the Kyle and Doolittle hydrophobicity scale, it was calculated that SB has a mean hydrophobicity of 0.465 and a mean net charge of 0.0094 at pH 7.0 (http://web.expasy.org/protscale/). This implies that it lies in a phase space where proteins are predicted to unfold with accumulation of partially folded conformers. This creates a possibility of multiple transitions during unfolding of SB.

The TUGZ profiles of SB showed sequential unfolding in the presence of BSA as substrate at 30°C, pH 8.8

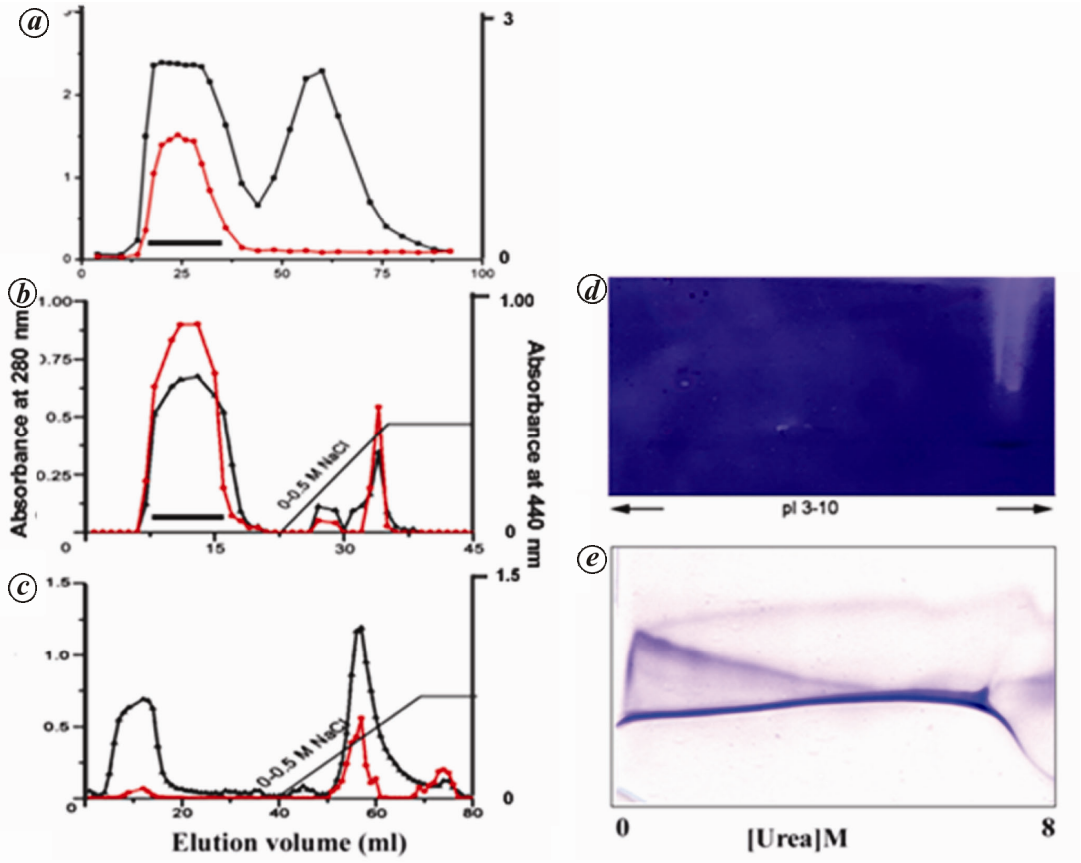

Figure 2. ***a–c***, Chromatographic fractionation of 'stem bromelain' by successive application to Sephadex G-50, DEAE-cellulose and CM-cellulose columns. In all sets, elution of protein was followed at 280 nm (black triangle) while proteolytic activity of the fractions was assayed using azocasein as substrate at 440 nm (red circle). The bars indicate pooled fractions that were applied to the next column. The bound fractions were eluted using a linear gradient of 0–0.5 M NaCl in buffer A. ***d***, 2D-zymography of stem bromelain. The pI range (3–10) used in the first dimension is indicated at the bottom. ***e***, TUGE of stem bromelain in 0.05 M Tris-HCl, pH 8.8.

(Figure 3 *a*). The clear band pattern enabled the calculation of ΔG and $[\text{urea}_{1/2}]^4$. Corresponding values for the first and second transitions were $\Delta G_1 = -5.2 \pm 0.1$ RT, $[\text{urea}]_{1/2} = 3.35$ M; and $\Delta G_2 = -7.3 \pm 0.4$ RT, $[\text{urea}]_{1/2} = 6.1$ M respectively. The TUGZ profiles provided a distinct understanding of conformational changes induced by urea as a two-state unfolding curve or as sequential unfolding with multiple transitions with the overlapping effect on proteolytic activity. Noticeable proteolytic activity was visible even at 8 M urea. This could be attributed to the fact that the TUGZ profiles reflect the unfolding of protease–substrate complex rather than the protease alone. This might provide additional stability to the protease which is reflected at such high concentrations of urea. Also, activity of partially denatured protease may be amplified against a denatured substrate. Thus, although SB maintained a native-like functionality even at 8 M urea, there were certain structural alterations leading to changes in charge and hydrophobicity resulting in different profiles. However, the fact that substrate did interact and formed a complex with the protease was exemplified when the gel pattern was compared with TUGE where the substrate was absent. In TUGE of SB, the migration of protease was faster as compared to TUGZ, probably due to the dense matrix created by the substrate which significantly reduced the migration rate (Figure 2 *e*).

To decipher any selective effect rendered in the TUGZ profile by BSA as a substrate, an unrelated protein like fibrin was incorporated in TUGZ (Figure 3 *b*). Appearance of a different profile with this substrate indicated the role of enzyme-substrate complex as it underwent unfolding. In fibrin zymography, no major transition was observed throughout, except at around 6.5–7 M of urea, which was reflected as the sudden increase in electrophoretic mobility. The substrate acted as a scaffold for the protease to interact with when it enters the gel. The profiles generated were indicative of the varied interaction routes through which these enzyme-substrate complexes unfold in the presence of increasing concentrations of urea as compared to the protease alone. Another prominent feature was the retention of appreciable activity by these complexes in the presence of high concentrations of urea. These observations highlighted the important role played

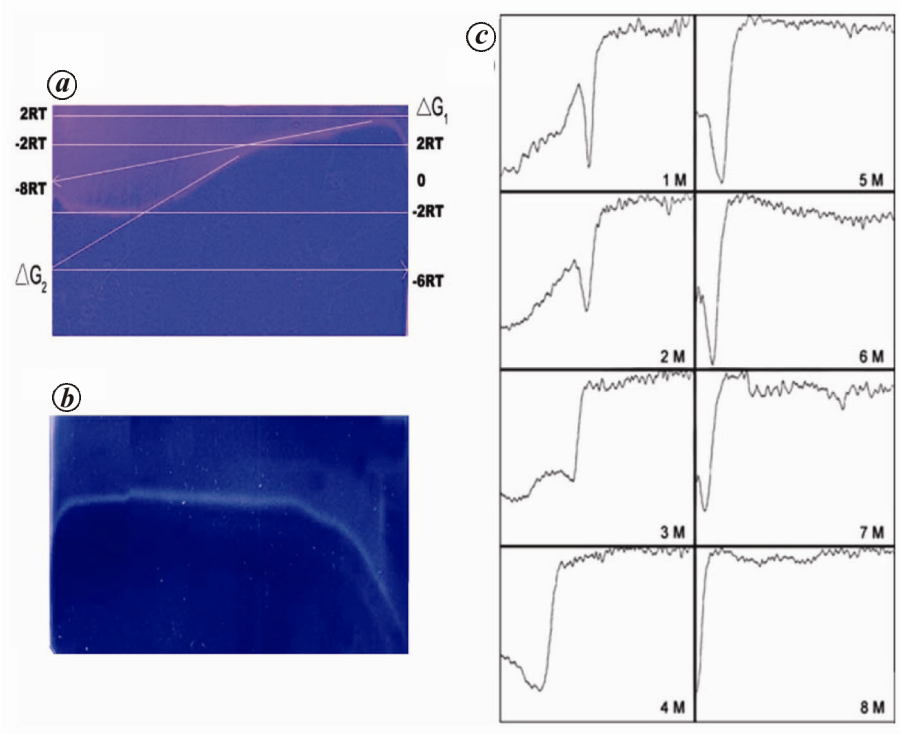

Figure 3. ***a***, Sequential unfolding of SB at pH 8.8, 30°C in TUGZ using 0.15% BSA as substrate. Graphic description of calculation of ΔG for these two transitions; R, universal gas constant (8.314 $JK^{-1} M^{-1}$) and T, absolute temperature. ***b***, TUGZ as of (***a***) except the substrate was fibrin (0.12% w/v). ***c***, Image J densitometric scan of unfolding of bromelain corresponding to profile A. The concentration of urea (1–8 M) corresponding to the vertical lines, that were scanned is mentioned at the bottom corner of each profile.

by the substrate involving its interaction with the enzyme and enhancing the stability of the complex.

Zymograms were densitometrically scanned and analysed using the Image J software. Scanning of the profile supported the information by displaying the shifting of peak region (Figure 3 *c*). The method afforded consistency in the zymography profiles enabling calculation of ΔG and $[\text{urea}]_{1/2}$.

Effect of deglycosylation

SB contains a single asparagine-linked heterosaccharide unit with a carbohydrate composition of mannose, fucose, xylose and N-acetyl glucosamine[26]. To evaluate the role of glycosylation on retention of proteolytic activity, SB was deglycosylated using glycopeptidase A, which cleaves β-aspartylglycosylamine linkages[27]. The deglycosylated sample was applied to TUGE, which resulted in a profile with three distinct bands showing no clear transitions (Figure 4 *a*). Application to TUGZ resulted in a profile with two closely migrating bands with less intensity than glycosylated SB (Figure 4 *b*). The differential pattern shown in TUGE and TUGZ profile reiterated the role of substrate–enzyme complex in TUGZ. Influence of depletion of carbohydrate on catalytic efficiency was reflected with a significant loss in activity as estimated from the enzyme assay using azocasein as the substrate (Figure 4 *c*). Comparison of these two results indicated the sensitivity of TUGZ towards the assessment of a sample which has approximately one tenth of its original activity. To establish that the glycopeptidase was free from peptidases, the enzyme was incubated with BSA for 16 h at 37°C and applied to an SDS-PAGE. Absence of any degradation pattern confirmed that glycopeptidase A was free from proteases (Figure 4 *c*, inset). It may be noted that Figure 4 *a* shows a characteristic edge effect due to current leakage on the sides of the gel. Since it does not have any detrimental effect on the interpretation of data, it has been excluded in all other representative images.

Role of disulphide bridges of bromelain

To estimate the effect of sequential reduction of three disulphide bonds on the activity and conformational stability of SB, enzymes reduced by DTT were prepared and examined through TUGZ. TUGE was also performed under identical conditions for a comparative analysis of the structural variations with catalytic activity. As a general function, disulphide bonds provide either conformational stability by decreasing the flexibility and entropy of the unfolded state or they destabilize the folded state entropically by reducing the number of water molecules ordered in the unfolded state. Apart from these, they have to provide some crucial, selective advantage which explains

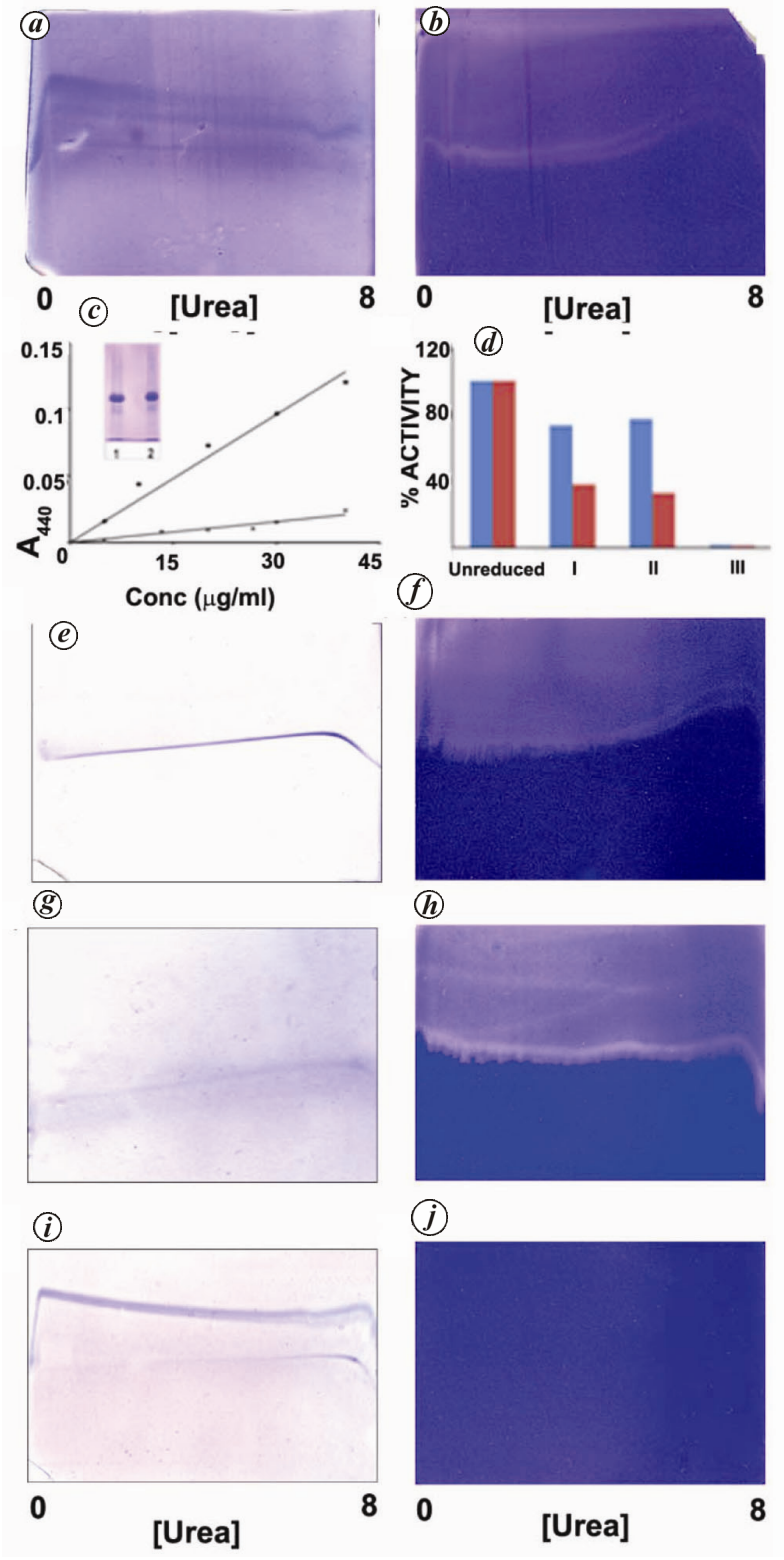

Figure 4. ***a***, TUGE of deglycosylated stem bromelain at pH 8.8, 30°C. ***b***, TUGZ of deglycosylated stem bromelain at pH 8.8, 30°C. ***c***, Comparison of residual proteolytic activity of glycosylated (■) and deglycosylated (•) SB estimated using azocasein as substrate. (Inset) SDS-PAGE profile of BSA treated with glycopeptidase A for 16 h at 37°C. Lane 1, untreated BSA as control; Lane 2, BSA after treatment where proteolytic degradation of BSA could not be detected. ***d***, Amidolytic activity of SB in solution state; untreated or modified after reduction of one, two and three disulphide bond/s respectively (I–III). Blue and red bars indicate dialysed and undialysed samples respectively. ***e–j***, TUGE and TUGZ of SB post successive reduction of one; two and three disulphide bonds respectively.

their retention under evolutionary selection pressure. Amidolytic assay was performed on the reduced enzymes and the effect of disulphide bond reduction on activity was estimated based on the calculated reaction rates (Figure 4 *d*). The corresponding K_{cat}/K_m values for SB under unreduced and one, two disulphide bonds reduced conditions were 337.04e6, 28.19e6 and 24.08e6 ($s^{-1}\,M^{-1}$) respectively. These values clearly indicate substantial loss in enzyme efficiency upon disulphide bond reduction but it is more or less maintained even after reduction of the second bond. SB with partially reduced disulphide bonds showed about 50% inactivation when the samples were not dialysed. Prolonged presence of the reducing agent and the denaturant probably reduced a portion of the essential disulphide bond required for maintenance of the catalytic site. Under equivalent conditions, up to the reduction of two bonds, TUGE and TUGZ profiles were distinctly dissimilar (Figure 4 *e–h*). TUGE profiles showed a single band, whereas TUGZ profiles showed multiple bands with substantial retention of activity. Since the sample contains several proteases of similar pI and molecular mass, TUGE possibly could not resolve them into different bands. However, in TUGZ depending upon individual interaction with the substrate, multiple bands were observed. Upon reduction of three bonds, TUGE profile showed two bands (Figure 4 *i*). The reduced enzyme resolved into these bands indicating the possibility of rapid thiol-disulphide interchange, resulting in disulphide mismatches since DTT was added to the sample buffer and not to the gel. In TUGZ profile, the activity was completely lost (Figure 4 *j*). These results were consistent with reaction rates obtained from amidolytic assay done on the reduced enzyme. Thus, it implied the vital role played by the most stable bond in retaining the functionality of bromelain. Only one amongst these three bonds is essential in maintaining the structural integrity of the catalytic site.

Urea decomposes to ammonia and isocyanic acid under gentle heating. The latter may react with reactive side chains of enzymes leading to inactivation. To verify whether such reactions occurred with bromelain, the following sets were prepared. Bromelain (0.05 mg/ml) was incubated with 20 mM Na-phosphate, pH 7.5 at 4°C for 4 h serving as control or with 8 M urea in the same buffer for the same duration either at 25°C or 37°C serving as test samples. The residual activities of the enzymes were measured with azocasein as substrate. Considering the activity offered by the control as 100%, urea treated samples were active by $90 \pm 4\%$. Assuming a low level of irreversible denaturation by urea in either case, no detectable inactivation of the enzyme could be detected due to carbamylation.

Correlation between activity and 3D molecular model of SB

Papain and chymopapain are model cysteine proteases, but the positioning of disulphide bonds in bromelain exhibits maximum homology to ervatamin B. Based on the model generated by Swiss Model, it was observed that the disulphide bond between the residues Cys_{23}–Cys_{63}, might play an essential role in maintaining the structural integrity of the molecule with respect to its activity because of its proximity to active site Cys_{26} residue. The ultimate cleavage of this bond probably leads to complete loss of activity as observed in TUGZ profiles. Thus, disulphide bridges in SB are not just present to provide localized stabilization to the molecule, but play a vital role in preserving its catalytic efficiency. Disulphide bonds with low or intermediate stability could not be predicted from the 3D model since there was no change in stability of the generated structure on cleavage of other two disulphide bonds.

Discussion

Existence of a fine balance between native and denatured states of a protein relies on the unequivocal contributions of electrostatics, hydrophobicity, temperature, etc. which have complementary impact on structural and functional stability[28]. This study explores TUGZ as a method to gain an insight into the structure–function relationship of proteases as they undergo urea-induced unfolding. Results obtained clearly supported the observations that the active site containing domain was very stable and resistant to urea-induced denaturation for SB[29]. This method provides valuable information regarding structural changes which cannot be captured by circular dichroism and solution stated fluorescence emission measurements. TUGZ also aided in separating the contribution of disulphide bridges on catalytic efficiency.

To test the general applicability of this methodology, three other proteases, belonging to different superfamilies, i.e. trypsin, chymotrypsin and collagenase were studied with encouraging results. For heterogeneous proteases like collagenases, separation into multiple components as well as varied mobility patterns could be visualized. Paradoxically, in order to study the effect of denaturation on catalytic activity in a single frame, substrate is incorporated which reflects the effect of urea on the enzyme–substrate complex whilst providing a means to investigate the stabilizing effect of protein–protein interaction during denaturation. One of the limitations of this method is that, once inactivated upon unfolding, the enzyme profile is likely to remain invisible against dark staining of the substrate. Still, there exist sound evidences which favour this method as a future prospect for characterization of proteases in multiple dimensions.

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