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Structural stability of commercial ficin under different denaturing conditions

[Ticari fisinin yapısal stabilitesine farklı denature edici koşulların etkisi]*

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ABSTRACT

Aim: To study the conformational stability of commercial ficin (CF) in the presence of guanidine hydrochloride (GdnHCl), urea, ethanol or at acidic pH and compare it with that reported for the major ficin fraction (MFF) obtained by purification of CF.

Methods: Far-UV and near-UV CD spectral signals, intrinsic fluorescence, acrylamide quenching and enzymatic activity were used to study the effects of chemical denaturants and acidic pH on CF. The data were analyzed using two-state hypothesis, if required.

Results: GdnHCl produced complete loss of secondary and tertiary structures of the protein. Loss of all enzymatic activity was observed at 4 M GdnHCl. CF showed structural resistance against 9 M urea and 50 % ethanol. Significant differences in emission maximum, acrylamide quenching, denaturation transition and enzymatic activity were noted between CF and MFF treated with different denaturants. CF showed greater stability at acidic pH than MFF.

Conclusion: We conclude that CF is more structurally resistant than MFF against chemical and acid denaturations.

Key Words: commercial ficin, structural stability, chemical denaturation, major ficin fraction

Conflict of Interest: The authors declare no conflict of interest of any kind.

ÖZET

Amaç: Bu çalışmanın amacı ticari fisinin (CF) yapısal stabilite çalışmalarını, guanidin hidroklorür (GdnHCl), üre, etanol varlığında veya asidik pH'da yapmak ve CF saflaştırılması ile elde edilen major fisin fraksiyonu (MFF) ile karşılaştırmaktır.

Metot: Uzak-UV ve yakın-UV CD spektral sinyalleri, içsel floresan, akrilamid su verme ve enzimatik aktivite metotları denatüre edici kimyasalların ve asidik pH'nın CF üzerindeki etkilerini incelemek için kullanılmışlardır.

Bulgular: GdnHCl proteinin ikincil ve üçüncül yapıların tam kaybına sebep oldu. Enzimatik aktivite tam kaybı 4 M GdnHCl gözlemlendi. CF, 9 M üreye ve% 50 etanole karşı yapısal direnç gösterdi. Maksimum emisyonda, akrilamid su vermede, denatürasyon geçişinde ve enzimatik aktivitedeki önemli farklılıklar, farklı denatüranlar ile muamele edilen CF ve MFF arasında kaydedildi. Daha asidik pH da CF, MFF'Den daha fazla stabilitesini korudu.

Sonuç: CF'nin kimyasal ve asit denatürantlara karşı MFF'den daha dayanıklı bir yapıya sahip olduğu sonucuna varıldı.

Anahtar Kelimeler: commercial ficin, structural stability, chemical denaturation, major ficin fraction

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Although enzymes have become important molecules in biotechnology industry, their stability and its enhancement, remain the subjects of intensive research to meet industrial demand [1-3]. Insight on the role of intrinsic factors that govern enzyme stability has been derived from use of different denaturants [4-7]. Such studies are important because temperature variations, extremes of pH, chemical denaturants, high ionic strength, high pressure and organic solvents are integral to several industrial processes [8-10]. A detailed analysis has suggested that proteins are marginally stable and that the free energy of stabilization ($\Delta G_D H_2O$) is 5 to 15 kcal/mol for stable, globular proteins [11].

Both commercial and purified protein preparations have been used to study protein denaturation [12-18]. Purification of a commercially available protein, leads to removal of contaminants such as aggregates, isoforms, conformers *etc*., which co-exist with this protein under *in vivo* conditions, thus, leaving only a single major protein. However, commercial proteins with their contaminants are thought to resemble the *in vivo* environment of the protein. Since a large number of denaturation studies have used both commercial and purified protein fractions, it remains unclear whether presence of these contaminants contributes to any significant variation in the results and to what extent protein stability differs between these two preparations.

Ficin (E.C. 3.4.22.3), an endopeptidase of the sulfhydryl protease class, has been widely used for industrial meat tenderization, alcohol production, photographic industries *etc*. [19]. It belongs to the papain superfamily because of its many properties and primary structural similarity to papain [20]. It consists of several active components in the latex of fig plants [21-24] that may arise from different ways of folding of the same polypeptide into different conformers. These have recently been fractionated by ion exchange chromatography on a SP-Sepharose column [25] and both acid and chemical denaturation studies have been performed on MFF [15, 26]. We have shown that the denatured states of CF produced in 9M urea and 6M GdnHCl are significantly different, the enzyme being more denatured by the latter [27]. In order to understand the stabilizing/ destabilizing effects of the conformers present in CF, we have studied its behavior on GdnHCl, urea, ethanol or acid denaturations and compared our results with published results on MFF.

Materials and Methods

Materials

CF from fig tree latex, 2 × crystallized (Lot 058K7019), GdnHCl (Lot 078K5425), urea (SigmaUltra) (Lot 127K0106), minimum 99 % acrylamide (Lot 059K1523), Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA), N-acetyl-L-tryptophanamide (NATA) and 2-mercaptoethanol were purchased from Sigma-Aldrich Inc., USA. Dimethyl sulfoxide was purchased from Merck, Germany, whereas ethanol (95 % containing 5 % additives) was purchased from Systerm, Malaysia. All other chemicals were of analytical grade. CF was used after dialysis against 0.1 M sodium phosphate buffer, pH 7.0.

Analytical procedures

CF concentration was determined spectrophotometrically on a Shimadzu double beam spectrophotometer, model UV-2450, using a specific extinction coefficient, $E^{1\%}_{280nm}$ of 21.0 [20]. Stock concentrations of GdnHCl and urea solutions were prepared as described by Pace *et al.* [28]. Enzyme activity was measured using BAPA as the substrate [27].

CD spectroscopy

CD measurements were performed on a Jasco spectropolarimeter, model J-815, after calibration with (+)-10-camphorsulfonic acid. All measurements were made at 25°C with a thermostatically-controlled cell holder, attached to a water bath under constant nitrogen flow. Far-UV (200–250 nm) CD measurements were recorded at a protein concentration of 10 μM using a 1 mm path length cell, whereas near-UV (240–320 nm) CD measurements were recorded at a protein concentration of 15 μM using 10 mm path length cell. The scan speed, band width and response time were set at 100 nm / min, 1 nm and 1 s respectively. Each spectrum represented the average of three scans, corrected with suitable blanks. The results were expressed as mean residue ellipticity, MRE in deg. cm^2. $dmol^{-1}$ which can be defined as:

$$MRE = \theta_{obs} / (10 \times n \times C_p \times l) \quad (1)$$

where θ_{obs} is the observed ellipticity in millidegrees; n is the total number of amino acid residues (210); C_p is the molar concentration and l is the path length in cm [4]. MRE data at 222 nm obtained at different denaturant concentrations were transformed into relative MRE by taking the MRE value of native protein at 222 nm as 100.

Fluorescence spectroscopy

Intrinsic fluorescence measurements were made on a Hitachi fluorescence spectrophotometer, model F-2500, equipped with a data recorder. The excitation and emission slits were fixed at 10 nm each and a 1 cm path length cell/cuvette was used. Fluorescence spectra (300–400 nm) of protein (0.6 μM) were recorded upon excitation at 280 nm and corrected by subtracting the fluorescence contribution of blank solution (containing similar denaturant concentrations in the buffer) from the fluorescence of protein solution at respective wavelengths.

Acrylamide quenching

Acrylamide quenching was performed as reported earlier [29]. Sodium phosphate buffer (0.1 M, pH 7.0) was used to prepare acrylamide, NATA and protein stock solutions. Aliquots of stock acrylamide solution (5 M) were added to 2.5 ml of stock NATA or protein solution (3.6 µM) in a cuvette to achieve the desired acrylamide concentration (0–0.5 M) in a total volume of 3.0 ml. The fluorescence spectra (300–400 nm) of NATA and protein solutions were recorded upon excitation at 295 nm to excite Trp residues only. The decrease in fluorescence intensity at emission maximum was analyzed according to the Stern-Volmer equation [30]:

$$F_o / F = 1 + K_{sv}[Q] \quad (2)$$

where F_o and F are the fluorescence intensities at emission maximum in absence or presence of quencher (acrylamide) respectively; K_{sv} is the Stern-Volmer constant and [Q] is the concentration of the quencher. The data were plotted as F_o / F versus acrylamide concentration. K_{sv} values were obtained from the slopes of the initial linear parts of the Stern-Volmer plots.

Enzyme assay

The enzymatic activity of CF was measured in absence or presence of denaturant. Protein solutions with desired denaturant concentrations were incubated for 6 h at 25°C. To 0.5 ml of CF solution (3.0 µM) with and without denaturant taken in different tubes, 0.28 ml of 0.1 M sodium phosphate buffer, pH 7.0, 0.01 ml of 0.2 M 2-mercaptoethanol and 0.01 ml of 0.01 M EDTA were sequentially added, mixed thoroughly and incubated at 37ºC for 1 h. This was followed by addition of 3.0 ml of stock BAPA solution (prepared fresh by dissolving 22 mg of BAPA crystals in 0.5 ml of dimethyl sulfoxide and diluting with 0.1 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA to a final volume of 50 ml and warmed to 37ºC) and the mixture was incubated further for 1 h at 37ºC. The reaction was terminated by addition of 0.2 ml of glacial acetic acid. Enzyme and substrate blank solutions were prepared in the same way but without enzyme and substrate, respectively. Enzyme activity was determined from the absorbance of p-nitroaniline (ε_M = 8800 M^{-1} cm^{-1}) at 410 nm. Absorbance values were corrected by subtraction of the values of blank solutions. One unit of enzyme activity was defined as the quantity of ficin required to catalyze the hydrolysis of BAPA at the rate of 1.0 µmole/min. Enzyme activity data were then transformed into percentage activity by taking the activity of native protein as 100.

Denaturation experiments

Solutions of GdnHCl, urea and ethanol were prepared in 0.1 M sodium phosphate buffer, pH 7.0. To 0.5 ml stock protein solution (concentration = 100 µM / 150 µM / 6.0 µM / 36 µM and 30 µM for far-UV CD / near-UV CD / intrinsic fluorescence / acrylamide quenching and enzymatic activity measurements, respectively), different volumes of buffer and stock denaturant solutions (10 M urea / 6.67 M GdnHCl / 95 % ethanol) were added to achieve the desired concentration of denaturants. The final solution mixture (5.0 ml) was incubated for 6–12 h at 25°C prior to spectral / enzymatic activity measurements. Blank solutions containing similar concentrations of denaturants but without protein were also analysed.

Data analysis

Denaturation data were analyzed by assuming a two-state mechanism [29]. The denaturation curve was normalized to the apparent fraction of denatured form, F_D, using the following relationship:

$$F_D = (Y - Y_N) / (Y_D - Y_N) \quad (3)$$

where, Y indicates observed variable spectral signal at a given denaturant concentration and Y_N and Y_D are the variable characteristics of the native and denatured states respectively, obtained by linear extrapolation of pre- and post-transition regions. The apparent equilibrium constant $K_{D,}$ was calculated using values of F_D ranging from 0.20 to 0.80 and substituting them into the following formula:

$$K_D = F_D / (1 - F_D) \quad (4)$$

Free energy change (ΔG_D) was calculated from K_D values using the following equation:

$$\Delta G_D = - RT \ln K_D \quad (5)$$

where R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature. The free energy of stabilization $\Delta G_D H_2 0$ was determined using least squares analysis of the plot of ΔG_D versus denaturant concentration, [D] to fit the data to the following equation:

$$\Delta G_D = \Delta G_D H_2 0 - m [D] \quad (6)$$

where '*m*' is the slope of the linear plot and is a measure of dependence of ΔG_D on denaturant concentration. $\Delta G_D H_2 0$ values were obtained from the intercept on Y-axis.

Acid denaturation

A constant volume (4.5 ml) of different buffers with similar molarity (20 mM) but different pH in the range of pH 1.0–7.0, *i.e.* KCl-HCl mixture (pH 1.13, 1.28, 1.41, 1.50 and 1.84), Gly-HCl buffer (pH 2.11, 2.22, 2.32, 2.41, 2.53, 2.60, 2.68, 2.71, 2.80, 3.06, 3.20 and 3.41), sodium acetate buffer (pH 3.76, 4.01, 4.58 and 5.08) and sodium phosphate buffer (pH 6.01, 6.57 and 7.0) was added to 0.5 ml of stock protein solution (100 µM) and incubated for 3 h at 25°C. Far-UV CD spectra were recorded for each protein solution and data were transformed into MRE at 222 nm.

Results and Discussion

CD spectral characteristics

Figure 1 shows the effects of 6 M GdnHCl, 9 M urea, and 40 % ethanol on far-UV and near-UV CD spectra of CF. Far-UV CD spectrum was characterized by the presence

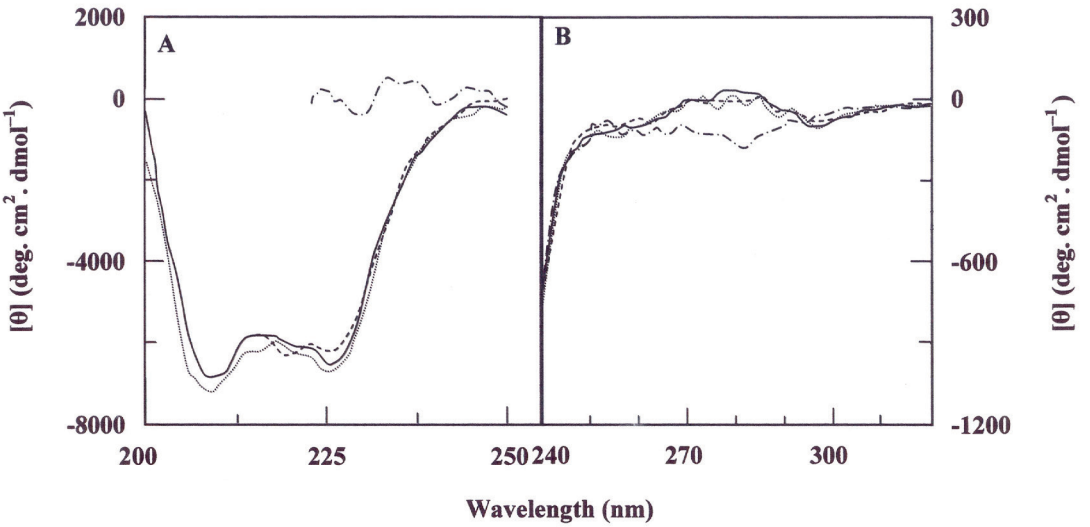

Figure 1. Effect of denaturants on far-UV CD (A) and near-UV CD (B) spectrum of CF in 0.1 M sodium phosphate buffer, pH 7.0, at 25ºC. The former were recorded at 200–250 nm and protein concentration of 10 µM, and the latter at 240–320 nm and protein concentration of 15 µM. CF without denaturants (▬), with 6 M GdnHCl (– · –), 9 M urea (-----) and 40 % ethanol (•••••).

of two minima at 208 and 225 nm (Fig. 1A), reflecting the characteristics of α-helical structure [31]. Decrease in the ellipticity value at 222 nm with increasing denaturant concentrations has been successfully used to probe protein denaturation from loss in the protein's secondary structure [29, 32].

GdnHCl (6M) resulted in total loss of the CD spectral features, indicating complete denaturation of CF, and was in agreement with the reported GdnHCl-induced denaturation of MFF [26]. CF retained its secondary structural characteristics with minimal alteration in either 9 M urea or 40 % ethanol (Fig. 1A), in agreement with the reported changes (~13 %) in MRE_{222nm} of MFF by 8 M urea [26]. The complete far-UV CD spectrum (in the range, 200–250 nm) of CF in presence of 6 M GdnHCl or of **9** M urea could not be obtained because of high signal to noise ratio at lower wavelengths. Similarly, data could not be collected at ethanol concentrations > 40 % because of turbidity from significant protein precipitation. The retention of secondary structural characteristics in presence of 9 M urea or 40 % ethanol suggested a remarkable stability/resistance of the protein to these denaturants. Several other members of the 'sulfhydryl protease' family have also been stable in 8 M urea [33, 34].

Near-UV CD spectra (in the range, 240–320 nm) reflect changes in protein tertiary structure by these denaturants. As seen from Fig. 1B, near-UV CD spectrum of CF was characterized by two positive signals around 278 and 284 nm, a negative signal around 295 nm, and a shoulder at 291 nm, reflecting the asymmetric environment of the aromatic amino acid residues. These spectral features were completely abolished by 6 M GdnHCl, which produced a negative band around 282 nm. However, 9 M urea or 40 % ethanol did not perturb the near-UV CD spectrum significantly, suggesting three-dimensional stability of CF towards these denaturants. These results agree with published results on urea and GdnHCl denaturation of MFF, purified from the commercial Sigma preparation [26].

In view of the differential effects of these denaturants on the stability of CF, the far-UV CD spectral signal (MRE_{222nm}) at increasing denaturant concentrations (0–6 M GdnHCl / 0–9 M urea / 0–40 % ethanol) were determined and transformed into relative MRE_{222nm}. A plot of these values against denaturant concentration is shown in Fig. 2. Addition of increasing concentrations of urea or ethanol resulted in minimal change (~3 %) to the MRE_{222nm} value at these concentrations, whereas the plot of relative MRE_{222nm} against GdnHCl concentration showed a typical denaturation curve. The change in relative MRE_{222nm} was smaller at 0–1.5 M and at 4.0–6.0 M and more pronounced at 1.75–4.0 M GdnHCl concentrations. The denaturation curve revealed a two-state, single-step transition, starting at 1.5 M and completed around 4.0 M with a mid-point at around 2.75 M GdnHCl. Published results on MFF have shown a similar transition with GdnHCl but with a mid-point of the transition at 2.4 ± 0.1 M [26] versus ours at 2.75 M GdnHCl concentration (Table 1). This is suggestive of relatively higher stability of CF than of MFF. Following transformation into F_D and ΔG_D values, the plot of ΔG_D against GdnHCl concentration (inset of Fig. 2) produced a $\Delta G_D H_2O$ of 5.2 cal/mol, which is similar to the previously published value [26] and agrees well with the other $\Delta G_D H_2O$ values of 5−15 kcal/mol, reported for several globular proteins including ficin [11, 26].

Fluorescence characteristics

The fluorescence spectra of CF (upon excitation at 280 nm) with increasing concentrations of GdnHCl (0.25–6.0 M), urea (0.5–9.0 M) and ethanol (2–50 %) are

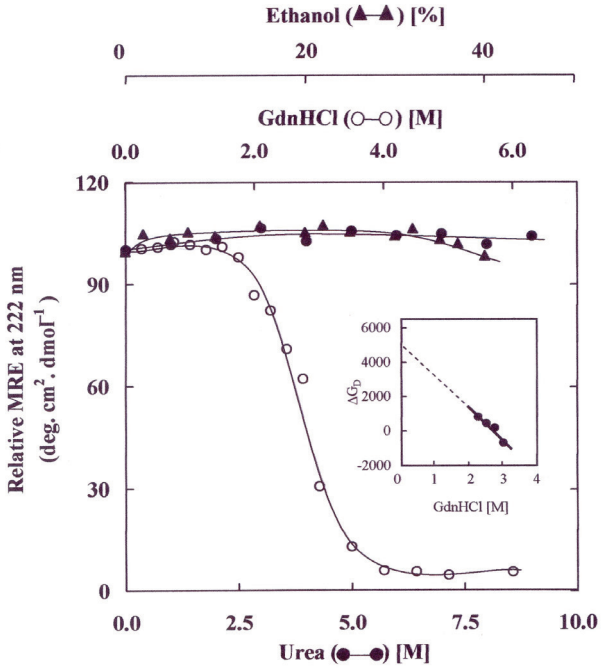

Figure 2. Effect of denaturants on relative MRE at 222 nm of CF in 0.1 M sodium phosphate buffer, pH 7.0, at 25ºC. Values of MRE were transformed into relative MRE by taking the MRE values of CF at 222 nm as 100. Inset shows the plot of ΔG_D, obtained from the normalized transition curve for GdnHCl-induced denaturation of CF, against GdnHCl concentration. GdnHCl (○), urea (●) and ethanol (▲).

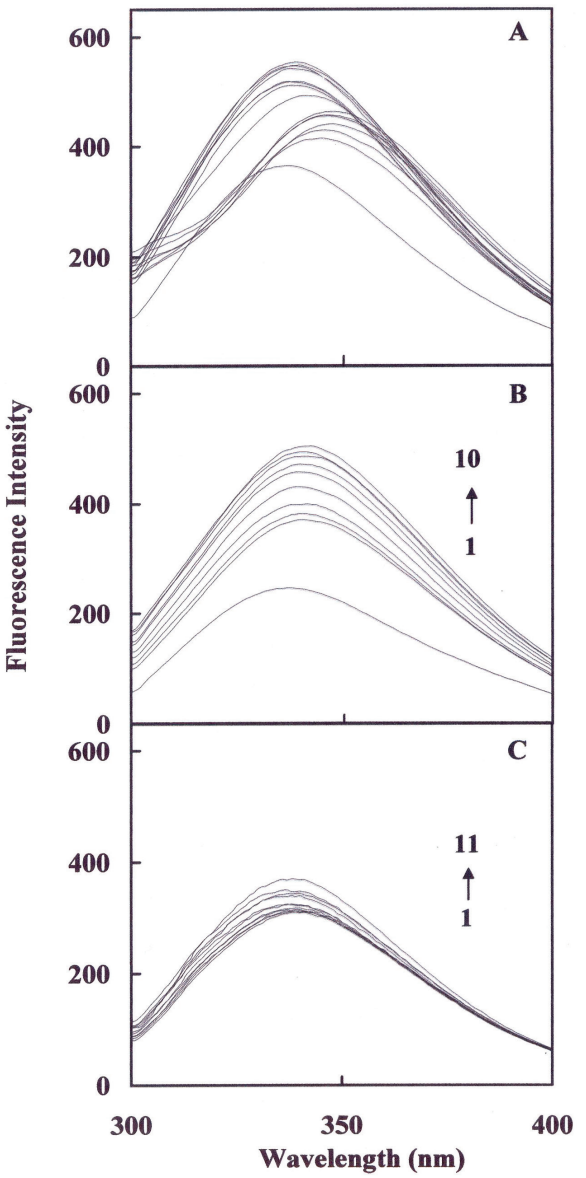

Figure 3. Effect of denaturants on the fluorescence spectrum of CF in 0.1 M sodium phosphate buffer, pH 7.0, at 25ºC upon excitation at 280 nm. (A) From bottom to top, GdnHCl concentrations at 0, 3.0, 3.5, 5.0, 4.0, 4.5, 6.0, 2.5, 0.25, 2.0, 0.75, 1.25 and 1.0 M, respectively. (B) 1–10, urea concentrations at 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 M, respectively. (C) 1–11, ethanol concentrations at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 %, respectively. Some spectra are omitted for clarity.

shown in Figs. 3A, B and C, respectively. The spectrum at 300–400 nm showed an emission maximum at 338 nm, suggestive of the presence of Trp residues [35], was significantly lower than that of 347 nm reported for MFF [15] (Table 1). The lower emission maximum of CF indicated a more hydrophobic environment for the Trp residues. GdnHCl or urea significantly affected the fluorescence intensity, whereas ethanol produced less alteration in the spectrum. A significant red shift in the emission maximum was noted with increasing GdnHCl concentrations, which was smaller or absent with urea or ethanol. The fluorescence intensity at 338 nm was transformed into relative fluorescence intensity by taking the value for native CF as 100 and plotted against GdnHCl / urea / ethanol concentration (Fig. 4A). The change in emission maximum at different denaturant concentrations is shown in Fig. 4B.

The effects of GdnHCl, urea and ethanol on the fluorescence spectrum of CF are shown in Fig. 4A and B. Although GdnHCl and urea produced an initial increase (50 % and 57 %, respectively) in intensity at up to 1.25 M GdnHCl / 1.5 M urea, there was a significant decrease (41 %) at 1.25–3.0 M GdnHCl, followed by a slight increase (8 %) at up to 6 M GdnHCl (Fig. 4A). An earlier report on MFF [26] showed no change at up to 1.8 M GdnHCl followed by continuously increased intensity at up to 6 M GdnHCl (Table 1). Increasing concentrations of urea (>1.5 M) produced a continuous slow increase in intensity up to a value of 196.7 (~97 % increase) at 8 M urea (Fig. 4A). These results differ from those on MFF where the increase in intensity noted at 8 M urea was only ~13 % [26] (Table 1). Ethanol (2–50 %) produced only a small increase (~4 %) in intensity at >25 % concentration (Fig. 4A). A difference in the shift in emission maximum was also found with these denaturants (Fig. 4B). A significant red shift was only observed with GdnHCl with a typical transition curve (Fig. 4B). These results resemble those of MFF [26] in terms of the nature of the curve, while the red shift shown in MFF [15] was 7 nm against ours

Table 1. Characteristics of CF and MFF monitored by different probes

Protein state	Probe	CF	MFF*
Native	Intrinsic fluorescence (Emission maximum)	338 nm	347 nm
	Acrylamide quenching (K_{sv}, M^{-1})	3.4	8.6
GdnHCl-denatured	Far-UV CD data (Transition curve)		
	- Mid-point	2.75 M	2.4 ± 0.1 M
	Intrinsic fluorescence (Fluorescence intensity)	0–1.25 M: 50 % ↑ >1.25–3 M: 41% ↓ >3–6 M: 8% ↑	0–1.8 M: No change >1.8 M: ~33% ↑
	(Emission maximum)		
	- Red shift	10 nm	7 nm
	Acrylamide quenching (K_{sv}, M^{-1})	8.2	12.5
	Enzymatic activity	100% ↓ at 4 M	100% ↓ at 3 M
Urea-denatured	Intrinsic fluorescence (Fluorescence intensity)		
	- % change at 8M urea	~97 % ↑	~13 % ↑
	(Emission maximum)		
	- Red shift	5 nm	0 nm
	Enzymatic activity	73 % ↓ at 9 M	100 % ↓ at 6 M
Acid-denatured	Far-UV CD data (First transition)		
	- Start-point	pH 3.2	pH 4.0
	- End-point	pH 2.5	pH 3.0
	- MRE_{222nm}	68 % ↓	31 % ↓
	(Second transition)		
	- MRE_{222nm}	52 % ↑	5 % ↑

* Data from Devaraj *et al.* [18, 38]

↑ and ↓ represent increase and decrease respectively

10 nm with 6 M GdnHCl (Table 1). Ethanol produced no significant change in the emission maximum, whereas urea produced only a small fluctuation (5 nm) (Fig. 4B). No red shift was produced in the emission maximum of MFF by urea (Table 1) [26].

Changes in fluorescence intensity and emission maximum of CF produced by these denaturants indicate alteration in the protein's three-dimensional structure. The intrinsic fluorescence is sensitive to the environment around Tyr and Trp residues [36]. However, Tyr fluorescence is either very weak or masked by the greater signal from Trp [37]. Ficin contains six Trp and fifteen Tyr residues [19]. The emission maximum at 338 nm in the fluorescence spectrum of CF agrees well with that of class 'B' proteins which contain Trp residues [38]. Denaturation usually results in a red shift accompanied by a decrease in intensity due to the change in the non-polar microenvironment of Trp residues to a polar one [4, 12]. However, we observed an increase in the intensity in presence of these denaturants (see Fig. 4A). This may reflect the release of fluorescence quenching due to the proximity of fluorophores and quenchers, like disulfide bonds and carbonyl groups, and to the quenching that results from the energy transfer between Trp and Tyr residues [39]. Local disordering may have disrupted these interactions in presence of denaturants, and led to increase in the intensity. However, perturbation in the microenvironment of Trp residues towards polar surroundings could be responsible for the significant decrease in fluorescence intensity at 1.25–3.0 M GdnHCl, supported by the red shift observed in the fluorescence spectra [40]. Differences in fluorescence behaviour of CF in presence of GdnHCl or urea can be attributed to the net result of the different mechanisms described above.

Acrylamide quenching of Trp fluorescence

The effects of 6 M GdnHCl, 9 M urea and 50 % ethanol on the acrylamide quenching of CF Trp fluorescence are shown by the Stern-Volmer plots in Fig. 5. Results for acrylamide quenching of the Trp analogue, NATA are also included in this figure. Stern-Volmer constants (K_{sv}) were obtained from the slope of linear parts of the Stern-Volmer plots and are shown in Table 2. This constant, also known as the dynamic quenching constant, indicates the extent to which the quencher achieves the encounter distance of the fluorophore [39]. The quenching of NATA had a K_{sv} value of 23.0 M^{-1} against 3.4 M^{-1} obtained for CF (Table 2). This differs from the K_{sv} of 8.6 M^{-1} reported earlier [15] (Table 1). The smaller value of K_{sv} that we found indicated mark shielding of Trp residues in CF. This K_{sv} value did not change significantly with 9 M urea (3.3 M^{-1}) or 50 % ethanol (3.6 M^{-1}), suggesting a significant degree of shielding and low collision frequency in presence of these denaturants. However, 6 M GdnHCl increased the K_{sv} value to 8.2 M^{-1}. Several other proteins have produced K_{sv} value similar to ours with 6 M GdnHCl [13, 29]. These results accord with our CD data in which GdnHCl was very effective while urea and ethanol were ineffective for denaturation of CF. MFF had a K_{sv} value of 12.5 M^{-1} in presence of 6 M GdnHCl [15]. These differences in K_{sv} value (Table 1)

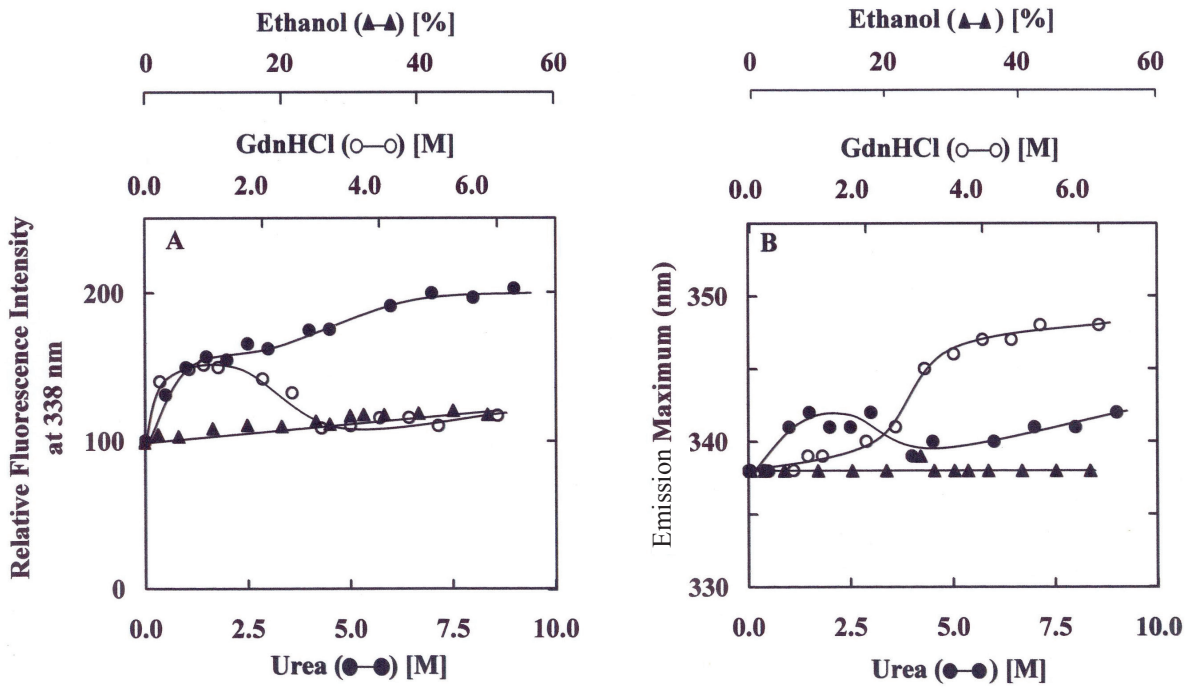

Figure 4. Effect of denaturants on relative fluorescence intensity at 338 nm (A) and emission maximum (B) of CF against increasing GdnHCl (○), urea (●) and ethanol (▲) concentrations obtained from Fig. 3. Values for fluorescence intensity were transformed into relative fluorescence intensity by taking the fluorescence intensity of CF at 338 nm as 100.

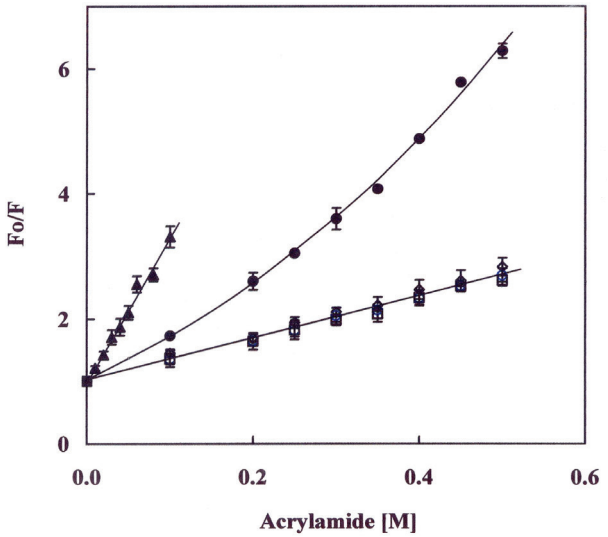

Figure 5. Effect of denaturants on the acrylamide quenching of Trp fluorescence of CF in 0.1 M sodium phosphate buffer, pH 7.0, at 25ºC. Stern-Volmer plots with no denaturant (o), + 6 M GdnHCl (●), 9 M urea (□) or 50 % ethanol (◊). Stern-Volmer plot with NATA (▲) as the reference standard is also included.

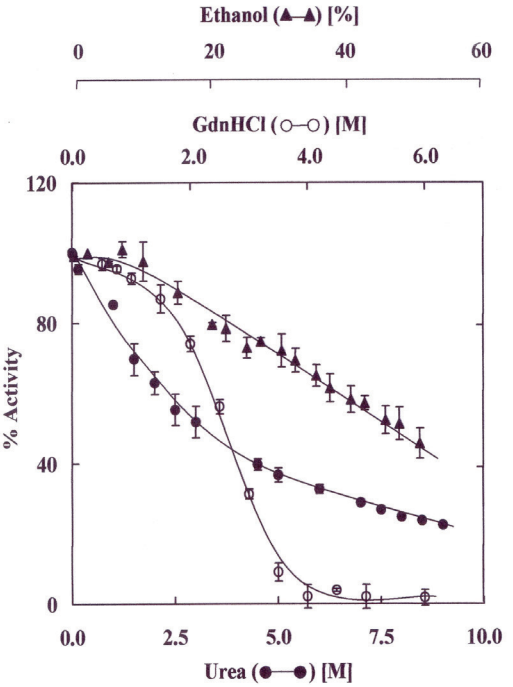

Figure 6. Effect of denaturants on the enzymatic activity of CF (3.0 µM) in 0.1 M sodium phosphate buffer, pH 7.0 containing 1.0 mM EDTA at 37ºC. Incubation was for 6 h at 25ºC before measurement. Activity was determined at 37ºC using BAPA as substrate. Different denaturant concentrations used were: [GdnHCl (○), urea (●) and ethanol (▲)] GdnHCl – 0, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 6.0 M; urea – 0, 0.15, 1.0, 1.5, 2.0, 2.5, 3.0, 4.5, 5.0, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0 M and ethanol – 0, 2, 5, 7, 10, 15, 20, 22, 25, 27, 30, 32, 35, 37, 40, 42, 45, 47 and 50 %.

Table 2. Effect of denaturants on Stern-Volmer constants for acrylamide quenching of CF fluorescence

Protein state	K_{sv} (M^{-1})
Native CF	3.4
CF + 6.0 M GdnHCl	8.2
CF + 9.0 M Urea	3.3
CF + 50 % Ethanol	3.6

can be attributed to the presence of different isomers in the commercial preparation which may have contributed to decreased exposure of Trp residues.

Enzymatic activity

The effects of increasing concentrations of GdnHCl, urea and ethanol on enzymatic activity of CF are shown in Fig. 6. There was complete loss of activity at GdnHCl concentrations ≥ 4 M, while 73 % and 53 % loss of activity at 9 M urea or 50 % ethanol, respectively (Table 1). MFF loses activity completely at 3 M GdnHCl and 6 M urea [26] (Table 1). Retention of significant activity of CF at 9 M urea, 50 % ethanol and at 3 M GdnHCl indicates greater stability of CF than of MFF.

Acid denaturation

The effect of acid (pH 7.0–1.1) on ellipticity measurement at 222 nm is shown in Fig. 7. Decrease in pH from pH 7.0 to pH 3.2 did not affect the MRE_{222nm} value. A drastic decrease was noted between pH 3.2 and pH 2.5, the lowest value being at pH 2.5. Between pH 2.5 and pH 1.4, there was a significant increase in MRE_{222nm} value. These results are similar to those with MFF [15]. However, with MFF, the transition started at pH 4.0 and

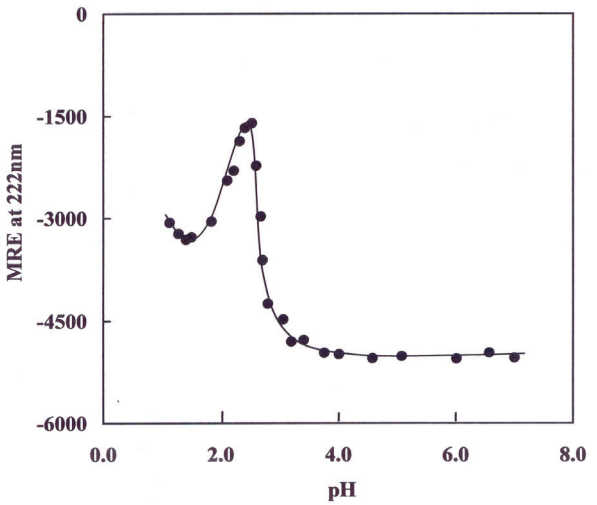

Figure 7. Effect of pH on MRE at 222 nm of CF at 25ºC. Data were obtained with a protein concentration of 10 μM.

ended at pH 3.0 against pH 3.2 and pH 2.5, respectively, observed with CF (Table 1). Also, decrease in MRE_{222nm} value at the end point of transition of MFF was 31% against our 68 % with CF (Table 1). An increase of 52% in MRE_{222nm} was seen with CF when pH was lowered from pH 2.5 to pH 1.4, whereas with MFF, there was a 5% increase in MRE_{222nm} between pH 2.0 and pH 1.0. These results indicate a higher stability of CF than of MFF towards acid denaturation.

From these results we conclude that GdnHCl possesses strong denaturing potential on CF compared to urea or ethanol by disrupting both secondary and tertiary structures and that CF has greater conformational stability than MFF. Hence, CF can be a better choice for industrial use than MFF, thus making further purification unnecessary.

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