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# Denatured States of Ficin Induced by Urea and Guanidine Hydrochloride

## [Fisin'in Üre ve Guanidin Hidroklorür ile Gelişen Denatüre Evreleri]

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**ABSTRACT**

**Objective:** To compare states of ficin denatured by 9 M urea and 6 M guanidine hydrochloride since a few proteins have shown different unfolded states upon denaturation with these agents.

**Methods:** Intrinsic fluorescence, tryptophan fluorescence, far-UV spectral signal and enzyme activity were used to characterize native and different denatured states of ficin.

**Results:** Treatment produced different denatured states when probed by intrinsic and tryptophan fluorescence. Both states differ significantly in magnitude of change in fluorescence intensity and extent of red shift. Red shifts of 12 and 17 nm were observed in the emission maximum with guanidine hydrochloride, and of 3 and 10 nm with urea using intrinsic and tryptophan fluorescence respectively. There were significant differences in magnitude of hypochromism and extent of red shift of far-UV absorption spectra. Guanidine hydrochloride produced total loss and urea 65% loss of enzyme activity.

**Conclusion:** Denatured states of ficin produced by urea and guanidine hydrochloride were significantly different and guanidine hydrochloride was more effective than urea. These results may be useful in determining the structure-activity relationship of ficin.

**Key Words:** denaturation, denatured states, ficin, fluorescence, guanidine hydrochloride, urea.

**ÖZET**

**Amaç:** 9 M üre ve 6 M guanidin hidroklorür ile denature olan fisinin denatürasyon evrelerini karşılaştırmaktır. Az sayıda protein bu maddelerle denatüre olduğu zaman farklı açılma evreleri sergiler.

**Metod:** Fisinin doğal ve farklı denatürasyon evrelerini tanımlamak için içsel floresans, triptofan floresansı, uzak-UV spektral sinyali ve enzim aktivitesi kullanıldı.

**Bulgular:** İçsel ve triptofan floresansı bulgularına göre farklı denatürasyon evreleri oluşmuştur. Her iki evre floresans yoğunluk değişiminin büyüklüğü ve kırmızı kaymanın derecesi bakımından farklıydı. Sırasıyla içsel ve triptofan floresansı kullanılarak guanidin hidroklorür ile emisyon maksimumunda 12 ve 17 nm, üre ile 3 ve10 nm kırmızıya kayma gözlenmiştir. UV absorpsiyon spektrumunlarında hipokromizmin büyüklüğü ve uzak kırmızı kayma farkları anlamlıydı. Enzim aktivitesinde guanidin hidroklorür %65, üre ise tam kayba yol açtı.

**Sonuç:** üre ve guanidine hidroklorürün fisinde ortaya çıkardığı denatürasyon evresi anlamlı derecede farklıdır ve guanidin hidroklorür üreden daha etkilidir. Bu sonuçlar fisinin yapı aktivite ilişkisini tesbitte yararlı olabilir.

**Anahtar kelimeler:** denatürasyon, denatürasyon evreleri, fisin, floresans, guanidin hidroklorür, üre

## Introduction

A protein's function depends on its ability to acquire its native structure [1, 2]. How a protein folds into a well-ordered native conformation is important not only for understanding the many phenomena resulting from the misfolding of proteins which render them non-functional, but also useful in its applications in biology, medicine and biotechnology [3-5]. Denaturation has long been a useful means for study of the properties and characteristics of protein folding and has given a better insight into the mechanism of the folding process [6-8]. Exposure of proteins to denaturing agents such as extremes of pH, temperature variation, high ionic strength, chemical denaturants and organic solvents has been employed to study the unfolding/folding process [9-11].

Chemical denaturants like urea and guanidine hydrochloride (GdnHCl) at their highest concentrations of 8-9 M and 6 M respectively, perturb protein structure by binding directly to peptide groups and weakening internal hydrogen bonds or by upsetting the hydrophobicity of the protein by changing the structure of water around hydrophobic groups [12-15]. There has been increased interest in the use of urea and GdnHCl in the study of protein folding because of their high denaturing potential. Denaturation induced by these agents usually gives rise to the same unfolded state of proteins [16-18], despite the difference in concentration required to give the same unfolded state [19]. However, some proteins, such as papain, cytochrome $c_{551}$ and stem bromelain show different unfolded states with these agents [20-22].

Ficin (E.C. 3.4.22.3), an endopeptidase belongs to the class of sulfhydryl proteases. It is classified under the papain super family based on its many properties and structural similarity to papain. Being a commercial enzyme, ficin is known as a good meat tenderizer and is utilized in many industries like photographic, baking, alcohol production etc [23]. Recently, a few studies describing thermal and acid stability of ficin have been published [24, 25]. However, no information is available on urea and GdnHCl denaturation of this enzyme. This has prompted us to investigate its denatured states obtained in the highest concentration of urea and GdnHCl. Since protein solution in 9 M urea is easily prepared and has already been used [15], we preferred to use for our studies. We here report our studies on the denatured states of ficin using intrinsic fluorescence, tryptophan fluorescence, UV-absorption measurements and enzymatic activity.

## Materials and Methods

### *Chemicals*

Ficin (F-4125, Lot 114K7775), ultra pure urea (U0631, Lot 086K0196), guanidine hydrochloride (GdnHCl) (≥99%) (G4505, Lot 096K5437), Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA) and 2-mercaptoethanol were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Dimethylsulfoxide was the product of Merck, Germany. All other chemicals used were analytical grade reagents.

### *Analytical procedures*

Protein concentration was determined spectrophotometrically on a Shimadzu double beam spectrophotometer model UV-2450, using specific absorption coefficient as 21.0 at 280 nm [26]. Concentrations of urea and GdnHCl stock solutions were determined from the data in [27] and [28] respectively, as described in [29]. Enzyme activity was measured using BAPA as the substrate [30].

### *Fluorescence spectroscopy*

Fluorescence measurements were made on a Hitachi fluorescence spectrophotometer model F-2500 equipped with a data recorder. The excitation and emission slits were set at 10 nm each and the path length of the sample cuvette was 1 cm. Intrinsic fluorescence and tryptophan fluorescence spectra were recorded in the range 300–400 nm after exciting the sample at 280 and 295 nm, respectively at a protein concentration of 0.6 μM.

### *Absorption spectroscopy*

Absorption measurements were carried out at 25ºC on Shimadzu double beam spectrophotometer model UV-2450 using a quartz cuvette of 1-cm path length. Spectra were recorded in the absence and in the presence of 9 M urea or 6 M GdnHCl in the range 190–250 nm against 0.1 M sodium phosphate buffer, pH 7.0 at a protein concentration of 3.0 μM. Blank solutions containing 9M urea or 6M GdnHCl were prepared using 0.1 M sodium phosphate buffer, pH 7.0 and their spectra were recorded in the same range.

### *Denaturation*

All solutions for denaturation experiments were prepared in 0.1 M sodium phosphate buffer, pH 7.0. To 0.5 ml stock protein solution (6.0 μM and 30 μM for intrinsic/Trp fluorescence and absorption measurements, respectively), 4.5 ml of stock denaturant solutions (10 M urea or 6.67 M GdnHCl) were added to give the desired concentration (9 M urea or 6 M GdnHCl). The final mixture was incubated for 12 h at 25°C before measurements were made. Blank solutions that contained the appropriate concentrations of denaturants were also used.

## Results and Discussion

Figure 1A shows the intrinsic fluorescence spectra of ficin (solid lines) in the absence and in presence of urea and GdnHCl upon excitation at 280 nm. Native ficin gave emission maximum around 338 nm which was indicative of tryptophan fluorescence [31, 32]. Fluorescence spectra in the presence of denaturants showed an increase in fluorescence intensity and red shift in emission maximum. An additional peak at 313 nm appeared in the

fluorescence spectrum with GdnHCl. Protein-free denaturant solutions showed significant fluorescence signals in the same range (300–400 nm) when excited at 280 nm (as shown by dotted lines in Fig. 1A). An emission maximum of GdnHCl solution was found at 309 nm with significant fluorescence intensity, whereas urea solution produced a small peak at 311 nm. We consider the appearance of an additional peak at 313 nm in the fluorescence spectrum of ficin in GdnHCl to represent the 309 nm GdnHCl signal. Therefore, we corrected the fluorescence spectra of ficin with these denaturants by subtracting the contribution of denaturant solution at each wavelength. Figure 1B shows these corrected fluorescence spectra of ficin. Note that the additional peak at 313 nm (Fig. 1A) disappeared with the correction. The increase in fluorescence intensity was more pronounced (~ 102%) with urea than with GdnHCl (31%). On the other hand, extent of red shift was found higher (12 nm) with GdnHCl than with urea (3 nm).

These changes in fluorescence intensity and emission maximum suggested that a gross conformational alteration in the protein was induced by these denaturants. Similar increases in fluorescence intensity with these denaturants have been reported [33-36]. The increase in the fluorescence signal suggested release of quenching in the native protein caused by energy transfer between Trp residues and fluorophore proximity to disulfides [33, 37]. However, the release of quenching between the Trp residues was higher with urea than with GdnHCl. Treatment of ficin with urea probably caused the Trp residues to be more separated from each other than with GdnHCl. The red shift in the fluorescence spectrum indicated exposure of Trp residues from hydrophobic to aqueous environment [48, 49]. Since the increase in fluorescence intensity and the extent of red shift with these denaturants were significantly different, it appears that urea and GdnHCl produce different denatured conformations of the protein.

Tryptophan fluorescence spectra of ficin (solid lines) upon excitation at 295 nm are shown in Fig. 2A. Because of the significant fluorescence exhibited by denaturant solutions with a peak at 330 nm (as shown by dotted lines in Fig. 2A), fluorescence spectra of ficin were corrected by subtraction of denaturant fluorescence (Fig. 2B). This figure shows that addition of urea or GdnHCl shifted the emission maximum from 334 nm (for native ficin) to a higher wavelength side with a change in fluorescence intensity. A red shift of 10 and 17 nm in the emission maximum of ficin was observed with urea and GdnHCl respectively. Whereas fluorescence intensity increased (~ 31%) with urea, GdnHCl decreased (~ 24%) fluorescence intensity. The decrease in fluorescence intensity and red shift observed with GdnHCl suggested exposure of Trp residues of the protein to the aqueous environment. However, the exposure of Trp residues was not as marked with urea as with GdnHCl since a lesser red shift accompanied the increase in fluorescence intensity with urea.

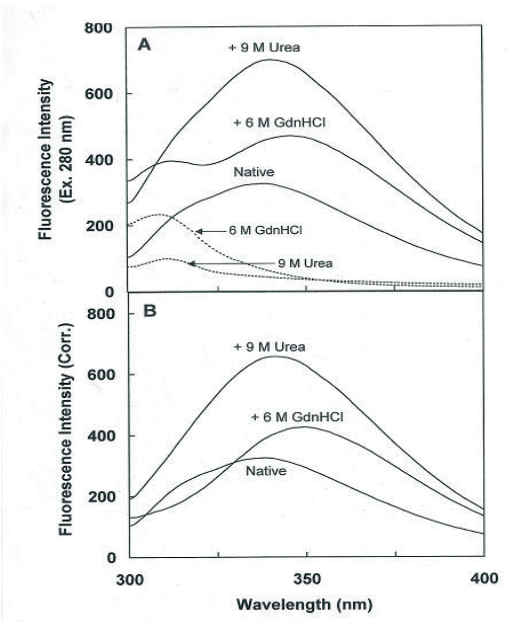


**Figure 1.** Fluorescence spectra of native and denatured states of ficin in 0.1 M sodium phosphate buffer, pH 7.0 at 25ºC upon excitation at 280 nm. (A) Spectra (solid lines) of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea). Fluorescence spectra of 6 M GdnHCl and 9 M urea in absence of protein are shown by dotted lines. (B) Corrected fluorescence spectra of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea) obtained by subtraction of the fluorescence spectrum of denaturant (dotted lines) from the spectrum of denatured protein (solid lines).

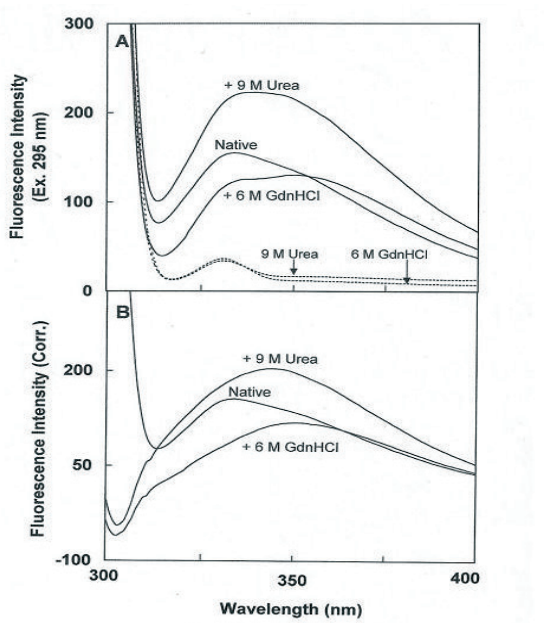


**Figure 2.** Fluorescence spectra of native and denatured states of ficin in 0.1 M sodium phosphate buffer, pH 7.0 at 25ºC upon excitation at 295 nm. (A) Spectra (solid lines) of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea). Fluorescence spectra of 6 M GdnHCl and 9 M urea in absence of protein are shown by dotted lines. (B) Corrected fluorescence spectra of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea) obtained by subtraction of the fluorescence spectrum of denaturant (dotted lines) from the spectrum of denatured protein (solid lines).

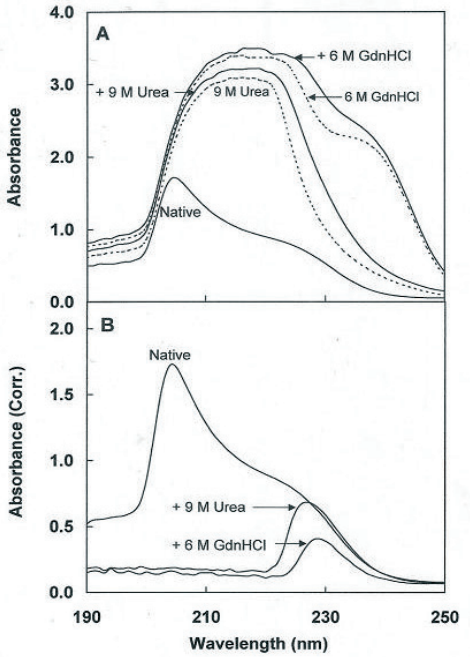


**Figure 3.** Far-UV absorption spectra of native and denatured states of ficin in 0.1 M sodium phosphate buffer, pH 7.0 at 25ºC. (A) Absorption spectra (solid lines) of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea). Absorption spectra of 6 M GdnHCl and 9 M urea in absence of protein are shown by dotted lines. (B) Corrected absorption spectra of protein in buffer (Native) and in denaturant solutions (+ 6 M GdnHCl; + 9 M urea) obtained by subtraction of the absorption spectrum of denaturant (dotted lines) from the absorption spectrum of denatured protein (solid lines).

The behaviour of ficin in urea and in GdnHCl seems to be different when studied by intrinsic fluorescence and Trp fluorescence. Ficin contains 15 Tyr and 6 Trp residues [30]. Although the fluorescence intensity produced by Tyr is frequently neglected because of its relatively weak signal and the quenching effect of neighbouring Trp residues [40, 41], the results in Fig. 1B suggest contribution of Tyr towards the fluorescence signal. This was evident from the smaller red shift (3 nm) observed in urea versus the 12 nm red shift produced in GdnHCl. Since Trp residues are also exposed in urea as shown by the significant red shift (10 nm) upon excitation at 295 nm, a smaller red shift obtained in urea upon excitation at 280 nm suggested changes in the environment of Tyr. Similar results were found with GdnHCl where the extent of red shift upon excitation at 280 nm was smaller (10 nm) than upon excitation at 295 nm (17 nm). These results accord with those in Fig. 1B and suggest a more unfolded conformation with GdnHCl than with urea.

Far-UV absorption spectra of ficin (solid lines) are shown in Fig. 3A. For native ficin the spectrum was in the range 200–250 nm with a maximum around 204 nm. With urea and with GdnHCl there was a significant red shift and hyperchromism. Denaturant solutions produced absorption spectra in this range (as shown by dotted lines in Fig. 3A). The absorption spectra of ficin were corrected by subtracting the absorbance of denaturant solutions. Corrected absorption spectra of ficin are shown in Fig. 3B. Both urea and GdnHCl produced a marked red shift and hypochromism (decrease in absorbance). The extent of the red shift (25 nm) and hypochromism (~ 76%) were higher with GdnHCl compared to 23 nm and ~ 60% respectively with urea. The absorption maximum of native ficin at around 205 nm indicated absorption by the peptide bonds in the protein [42]. Any change in the spectral characteristics of native ficin upon addition of urea or of GdnHCl suggests helix-coil transitions in ficin that can be attributed to gain in polarity of the solvent which is more likely to happen upon opening up of protein helices [43].

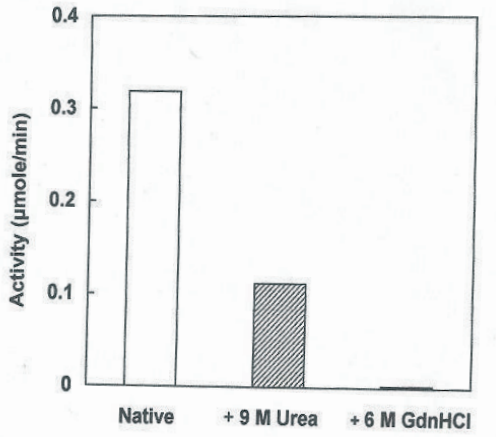


**Figure 4.** Effect of 9 M urea and 6 M GdnHCl on the protease activity of ficin at 37ºC. Ficin solution (3.0 µM) was incubated in the absence or in presence of denaturant solutions (6 M GdnHCl or 9 M urea) for 1 h followed by measurement using BAPA as substrate.

Addition of urea or of GdnHCl to ficin markedly decreased the enzyme activity as shown in Fig. 4. Urea produced a loss of about 65% of enzyme activity whereas GdnHCl produced complete loss of activity. These results showed that GdnHCl denatured the protein more completely than urea. These results accord with those obtained with intrinsic fluorescence, Trp fluorescence and far-UV absorption measurements.

Taken together all these results suggest that GdnHCl markedly affects the protein conformation than urea as reflected from the marked changes in the intensity and red shift in the fluorescence spectra, hypochromism and red shift in the UV absorption spectra and complete loss of enzyme activity. We conclude that GdnHCl denaturation of ficin is more effective in removing non-covalent interactions than urea.

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