Denaturation of the high molecular weight protein fraction of mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*) by urea or guanidinium hydrochloride

A. GURURAJ RAO* and M. S. NARASINGA RAO

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570 013

* Present address: Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland 44106, Ohio, USA.

Abstract. Urea and guanidinium hydrochloride dissociate the 12S protein of mustard and rapeseed to 1.8 S protein and the extent of dissociation depends on the concentration of the denaturant. Mustard (*Brassica juncea*) protein is more readily dissociated than the rapeseed (*Brassica campestris*) protein. The reagents denature the protein as evidenced by increase in viscosity, appearance of difference spectra and quenching of fluorescence. Rapeseed protein is denatured more readily than the mustard protein. Analysis of visctosity, spectral and fluoresence data suggests that the first event in the denaturation reaction is the perturbation of the aromatic amino acid residues followed by their exposure to the solvent medium and unfolding of the protein molecule.

Keywords. Mustard protein; rapeseed protein; denaturation; urea; guanidinium hydrochloride.

Introduction

Mustard and rapeseed proteins consist essentially of two fractions, a low and a high molecular weight protein. The high molecular weight protein, the 12 S protein, has been isolated to homogeneity (Gururaj Rao and Narasinga Rao, 1981). The protein from mustard (*Brassica juncea*) and rapeseed (*Brassica campestris*) differ in amino acid composition, subunit structure, susceptibility to proteolysis and seconddary structure (Gururaj Rao and Narasinga Rao, 1981). In order to delineate further the differences between the two proteins the effect of denaturants, urea and guanidinium hydrochloride (GndHCl), has been studied. The results are reported in this communication.

Materials and methods

Mustard and rapeseed

Mustard seed (*B. juncea*) variety RT 590 and rapeseed (*B. campestris*) variety yellow sarson T 151 were obtained from Haryana Agricultural University, Hissar, India. Urea from British Drug House and guanidinium hydrochloride (GndHCl)

Abbreviations used: GndHCl, Guanidinium hydrochloride; RTIC, rotor temperature indicator and control; SDS, sodium dodecyl sulphate; S_{app}, apparent sedimentation coefficient; $n_{red,}$ reduced viscosity; CD, circular dichroic; UV, ultra-violet.

from Sigma Chemical Co., St. Louis, Missouri, USA, were used. Urea was recrystallized once from 50% ethanol. All the other reagents were of analytical reagent quality.

Isolation of the 12 S protein

It was isolated by the procedure reported earlier (Gururaj Rao and Narasinga Rao, 1981).

Protein concen tration

This was determined by measuring the absorbance of the solution at 280 nm. The values of $E_{1\,cm}^{1\%}$ used were 9.9 for mustard protein and 10.8 for rapeseed protein (Gururaj Rao, 1980).

Ultracentrifugation.

This was performed at room temperature (~ 27° C) in a Spinco Model E Analytical Ultracentrifuge equipped with rotor temperature indicator and control (RTIC) unit and phase plate schlieren optics using 1% protein solution in the appropriate solvent. The runs were made at 59,780 rpm. From the photographs taken at different intervals of centrifugation, apparent sedimentation coefficient, (S_{app}), was calculated. Enlarged tracings of the photographs were used to calculate the proportion of the protein components in the denaturing solvent.

Viscosity was measured at 30±0.1° C using an Ostwald visco-meter having a flow time of 184 s with deionized water. Measurements were made with 1% protein solution at different denaturant concentrations:

Difference spectra produced by the addition of different concentrations of denaturant to the protein were recorded in the range 240–300 nm using a Perkin-Elmer Model 124 recording spectrophotometer. Protein solutions having an absorbance of 1.8 at 280 nm were used.

Fluorescence spectrum

The emission spectrum was measured in the range 300–400 nm after excitation at 280 nm. Protein solution having an absorbance of 0.05 at 280 nm was used. The blank for the measurement contained the appropriate concentration of the denaturant. Fluorescence measurements were made with Perkin-Elmer Model 203 fluorescence spectrophotometer at room temperature.

Unless otherwise measured, all the measurements were made in 0.025 M Tris-glycine buffer of pH 8.3.

Results and discussion

The value of the measured parameter in the presence of the denaturant was time-dependent. In urea solutions constancy of values was obtained at 48 h and in GndHCl solutions at24 h Hence the solutions were left at room temperature for 24 h in GndHCl and for 48 h in urea, and the rneasurement made.

The addition of urea to mustard 12 S protein dissociated it into 1.8 S protein; the extent of dissociation depended on urea concentration (figure 1). In 8 M urea solution the protein had completely dissociated into the 1.8 S component (figure 2A). GndHCl also dissociated the protein. The dissociating effect of GndHCl appeared to be greater than that of urea (figure 2 A, B). For example at 2 M concentration, in urea the protein dissociated to the extent of 20% and in GndHCl it dissociated to 65%; at 4 M urea the dissociation was 80% and in 4 M GndHCl it was 100% (figure 2).
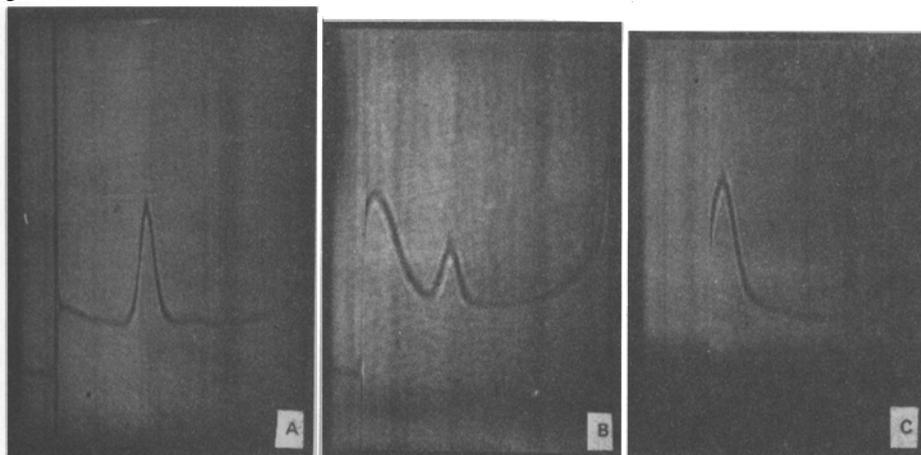

Figure 1. Sedimentation velocity pattern of mustard 12 S protein (in 0.025 M Tris-glycine buffer, pH 8.3).
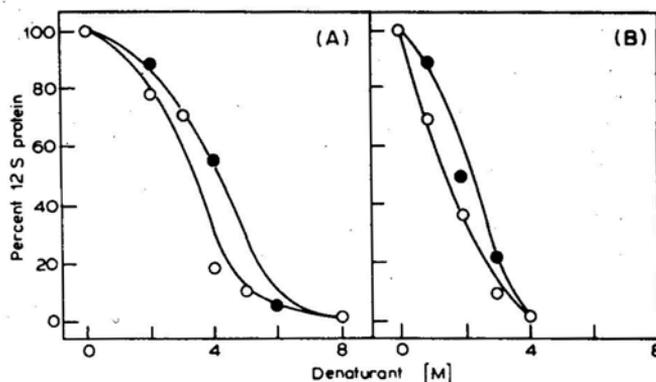 **A.** Mustard 12 S protein; **B.** Mustard 12 S protein+ 4 M urea; C. Mustard 12 S protein+8 M urea.

Figure 2 The per cent proportion of 12 S protein as a function of denaturant concentration.
  A. In urea,(O), mustard protein;(●), rapeseed protein. **B.** In GndHCl,(O), mustard protein;(●), rapeseed protein.

In the case of rapeseed 12 S protein also urea or GndHCl caused dissociation of the protein to 1.8 S protein and the extent of dissociation increased with the denaturant concentration. However, either in urea or GndHCl rapeseed protein

appeared to be dissociated to a lower extent than the mustard (figure 2 A, B). In 4M urea, the extent of dissociation of the mustard protein was 75–80% as against 55% with rapeseed protein (figure 2A). Similarly, in 2 M GndHCl, the dissociation of the mustard protein was 65% and that of the rapeseed protein 50% (figure 2B). Although much quantitative significance cannot be attached to the values, the trend appeared that the mustard protein dissociated more readily than the rapeseed protein.

Thus urea and GndHCl dissociated the 12 S protein of mustard/rapeseed directly to the 1.8 S protein without the formation of any intermediates. Sodium dodecyl sulphate (SDS) also dissociated the 12 S protein directly to the 1.8 S protein (Gururaj Rao and Narasinga Rao, 1980, 1983). This behaviour of the 12 S protein from *Brassica* species contrasts with that of other vegetable proteins. In the case of sunflower, 11S protein SDS directly dissociates if to the 2 S protein, whereas urea or GndHCl dissociates it to an intermediate 7 S protien (Rahma and Narasinga Rao, 1981). The 11 S protein of guar seed also behaves similarly (Nath and Narasinga Rao, 1983). In the case of sesame α-globulin SDS and urea (and GndHCl) dissociate the 14 S protein to 2 S protein through intermediates, namely 7 S and 4 S proteins (Prakash and Nandi, 1975,1977). This statement appears to be valid when the results obtained with 1% protein solution are compared. However, with a 3% protein solution, mustard and rapeseed 12 S protein also showed the presence of a 7 S protein at intermediate urea or GndHCl concentrations. This would suggest that the formation of the intermediate(s)is considerably less with mustard and rapeseed 12 S protein.

The reduced viscosity (n_{red}) of the mustard 12 S protein (measured at 1% protein concentration) had a value of 5.1 cc/g which is close to its intrinsic viscosity of 5.4 cc/g (Gururaj Rao and Narasinga Rao, 1981). Addition of urea increased n_{red} and at 8 M urea concentration it was 22 cc/g (figure 3). Even at this concentraton of urea, a plateau value was not obtained, although the observed value was close to
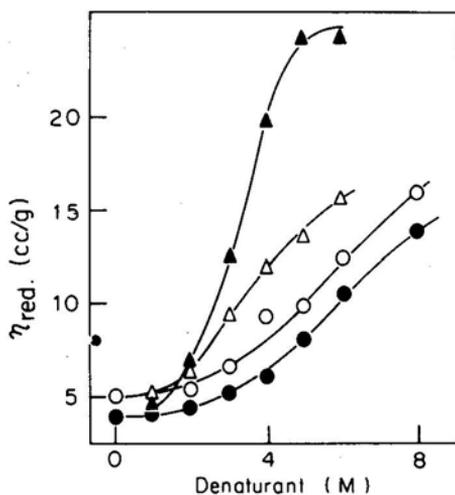

Figure 3.   Reduced viscosity of the protein as a function of denaturant concentration (in 0.025 M Tris-glycine buffer, pH 8.3).(○), Mustard in urea;(●), rapeseed protein in urea; (Δ), mustard protein in GndHCl; (▲), rapeseed protein in GndHCl.

the value of 23 cc/g reported for proteins in random-coil conformation (Tanford, 1970). Addition of 2-mercaptoethanol did not alter the values significantly.

Rapeseed 12 S protein had a value of n_{red} = 4.2 cc/g close to its intrinsic viscosity of 4.5 cc/g (Gururaj Rao and Narasinga Rao, 1981). Addition of urea increased the value and at 8 M urea concentration the value was 14 cc/g (figure 3). This was much lower than that of the mustard 12 S protein at the same urea concentration; further even at other urea concentrations, the values were lower in the case of the rapeseed protein.

In GndHCl solution the mustard 12 S protein reached a value of 16 cc/g at a concentration of 6 M (figure 3) and no plateau value was reached. However, the rapeseed 12 S protein attained a constant value of 25 cc/g in the region of 4 to 6 M GndHCl concentration.

These results indicated that the rapeseed 12 S protein was more resistant to denaturation by urea; further with both the proteins a fully random-coil conformation was not obtained even in 8 M urea solutions. However, in GndHCl rapeseed protein was more readily denatured than mustard protein and at 4 to 6 M GndHCl concentration the protein attained a random-coil conformation. Mustard and rapeseed 12 S protein contain little or no α-helix; they consist of β-structure and random coil (Gururaj Rao and Narasinga Rao, 1981). However, in circular dichroic (CD) spectrum the mustard protein gives a value of–3940 deg cm^2 /d for [θ] at 210 nm and the rapeseed protein a value of— 4900 deg cm^2 /d mol, suggesting that the latter has a more-ordered structure. The rapeseed 12 S protein was more resistant to hydrolysis by papain than the mustard protein. On the other hand chymotrypsin hydrolysed the rapeseed protein more readily than the mustard protein (Gururaj Rao and Narasinga Rao, 1981). Thus the responses of the two proteins to different treatments seemed to vary. The observed difference between the effect of urea and GndHCl on the mustard and rapeseed 12 S protein could be due to possible differences in their conformational stability.

The addition of urea to the mustard 12 S protein generated a difference spectrum in 260-300 nm region with troughs at 295, 287 and 278 nm (figure 4). The
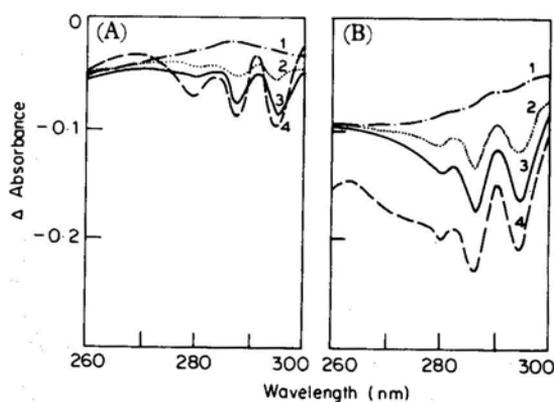

Figure 4 Differnce spectra (in 0.025 M Tris-glycine buffer, pH 8.3) recorded against protein blank.

  A.(1) Mustard protein+2 M urea; (2) mustard protein+ 4 M urea; (3) mustard protein+6 M urea; (4) mustard protein+8 M urea.

  B.(1) rapeseed protein +2 M urea; (2) rapeseed protein +4 M urea; (3) rapeseed protein+6 M urea; (4) rapeseed protein +8 M urea.

intensity of the troughs increased with urea concentration. Similar difference spectra were obtained with the rapeseed 12 S protein. However, the following differences were observed between the mustard and rapeseed protein: (i) in the case of the mustard protein the intensity of the troughs was less than with the rapeseed protein and (ii) intensity of the 294 nm trough was more than that of the 287 nm trough in the case of mustard 12 S protein and the converse was true in the case of rapeseed protein.

The minima at 278, 287 and 294 nm are due to the perturbation of tyrosine and tryptophan residues (Donovan, 1969). The mustard 12 S protein has 9 tryptophan residues and 16 tyrosine residues per 100,000 g and the rapeseed protein has 9 tryptophan and 14 tyrosine residues (Gururaj Rao and Narasinga Rao, 1981). Thus the increased intensity observed with the rapeseed protein cannot be correlated with its tyrosine and tryptophan content. Similarly in the case of the mustard 12 S protein, the greater intensity of the 294 nm peak than that of the 287 nm peak cannot also be correlated with the tyrosine and tryptophan content of the protein.

The blue shift observed can be ascribed to the binding of the denaturants to these chromophores or denaturation of the protein (Donovan, 1969). It is not clear which cause is responsible for the observed differences.

Similar difference spectra were generated by the addition of GndHCl. In this denaturant the intensities of the troughs were greater than in urea solution; the ΔA values at 287 nm are given in figure 5. Also the values with the rapeseed protein were higher than with the mustard protein.

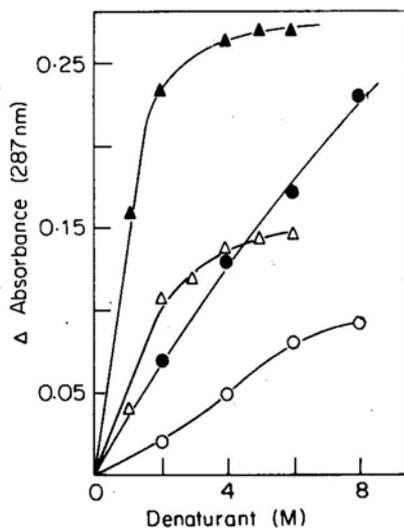The mustard and rapeseed 12 S protein give fluorescence emission spectrum

Figure 5.   Value or absorbance change at 287 nm as a function of denaturant. Concentration; (O), mustard protein in urea;(●), rapeseed protein in urea;(Δ), mustard protein in GndHCl;(▲), rapeseed protein in GndHCl.

with a maximum at 325 nm. The addition of the denaturant caused quenching of fluorescence; the extent of quenching increased with increase in denaturant concentration. Also at higher denaturant concentrations a red shift in the emission maximum occurred. Representative spectra obtained with the rapeseed 12 S protein in GndHCl solution are given in figure 6.
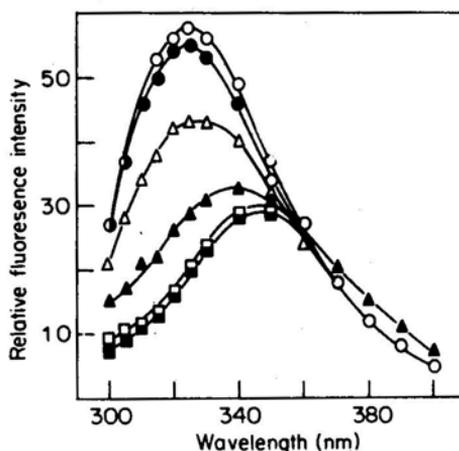
Figure 6. Fluorescence emission spectrum of rapeseed 12 S protein (in 0.025 M Tris-glycine buffer pH 8.3) in GndHCl solution;(O), protein;(●), protein +1 M GndHCl; (Δ), protein + 2 M GndHCl;(▲), protein + 3 M GndHCl,(□), protein+ 4 M GndHCl;(■), protein +6 M GndHCl.

A plot of the ratio of the fluorescence intensity at 325 nm as a function of the denaturant concentration showed that the extent of quenching was greater in GndHCl than in urea, although the limiting value was the same in both the solvents (figure 7). Further with the same denaturant the extent of fluorescence quenching of mustard and rapeseed protein was the same, within the limits of experimental error.
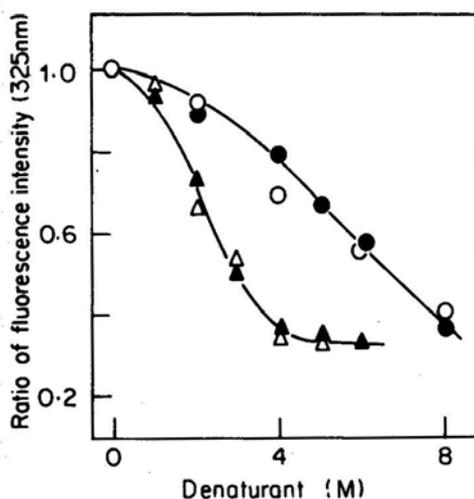

Figure 7. Ratio of fluorescence intensity at 325 nm as a function of denaturant concentration (in 0.025 M Tris-glycine buffer, pH 8.3(O), mustard protein in urea;(●), rapeseed protein in urea;(Δ), mustard protein in GndHCl;(▲), rapeseed in GndHCl.

The sedimentation velocity experiments suggest that in either urea or GndHCl solution the mustard 12 S protein is more readily dissociated than the rapeseed protein, although it is not possible to attach quantitative significance to the observed differences. In viscosity measurements the rapeseed 12 S protein was

less readily denatured by urea than the mustard protein and more readily by GndHCl. Fluorescence data did not indicate any difference between the rapeseed and mustard protein. On the other hand, the difference spectral data showed that rapeseed 12 S protein was more readily denatured by both urea and GndHCl than the mustard protein. Thus apparently conflicting conclusions would emerge from the three types of measurements.

Possibly the three techniques measured different facets of the conformational changes occurring due to the action of the denaturant. From the value of the measured parameter by any single technique as a function of denaturant concentration it is possible to calculate the fraction of the protein denatured as a function of denaturant concentration. If such values, obtained by diverse techniques fit the same curve it is usually taken as an indication that all the techniques are measuring the same conformational change (Tanford, 1970).

For such calculations, the value characteristic of the native protein and that of the fully denatured protein must be known. The value of the native protein can be obtained by measurement in the absence of the denaturant. The value of the denatured state is usually obtained from the plateau values at high concentrations of the denaturant. Reference to figures 3, 5 and 7 shows that plateau values were not always obtained. But the rapeseed 12 S protein in GndHCl solution gave plateau values in viscosity, difference spectra and fluorescence quenching measurements. Using these data, the fraction of protein denatured was calculated as a function of GndHCl concentration (Tanford, 1968).

The data of figure 8 show that the values obtained by·different techniques did not fit the same curve; changes in ultra-violet (UV) spectra occur at lower GndHCl
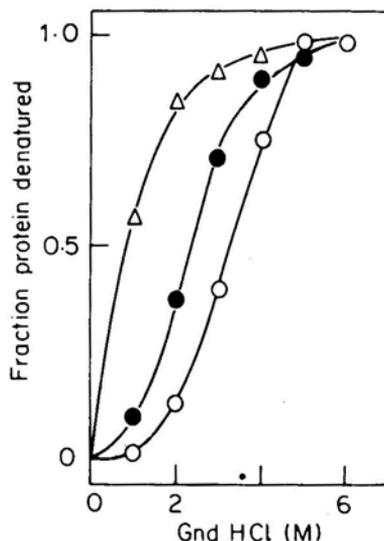

Figure 8. Fraction of rapeseed 12 S protein denatured as a function of GndHCl concentration:(O), n_{red} data;(Δ), A data;($\bullet$), fluorescence data.

concentrations, followed by fluorescence and viscosity changes. The appearance of difference spectra is due to the perturbation of tryptophan and tyrosine residues; the 287 nm peak is due to tyrosine perturbation (Donovan, 1969). The

325 nm fluorescence emission is due to the buried tryptophan residues (Teale, 1960); quenching of the fluorescence is due to the exposure of the buried tryptophan residues. Thus the first event in the denaturation reaction appears to be perturbation of the aromatic amino acid residues. This is followed by the exposure of the buried aromatic amino acids and finally by a conformational change leading to an increase in hydrodynamic volume.

Such an analysis of the observed data is, however, complicated by the dissociation of the oligomeric protein by the denaturant. If dissociation and denaturation of an oligomeric protein can be considered as equivalent phenomena (Lapanje, 1978) then the above analysis would have validity.

This analysis does not still answer the question why the two proteins which show only minor differences in amino acid composition and secondary structure, behave differently towards the denaturants. Urea and GndHCl act as bifunctional reagents and proteins bind one molecule of denaturant per two peptide residues and through aromatic amino acid residues (Robinson and Jencks 1965; Nozaki and Tanford, 1970; Prakash *et al.,* 1981). Denaturation of proteins by these reagents occurs by breaking of hydrogen and hydrophobic bonds. The hydrophobicity and fractional charge units per residue of the mustard and rapeseed 12 S protein was calculated by the procedure outlined by Bigelow (1967) using data of amino acid composition of the two proteins (Gururaj Rao and Narasinga Rao, 1981). The hydrophobicity value was 808 cals/residue for mustard protein and 721 cals/residue for rapeseed protein. Similarly[1] the fractional charge per residue was 0.375 charge unit for mustard protein, and 0.386 charge unit for rapeseed protein. These differences are not significant and may not explain the differing behaviour of the two proteins towards urea and GndHCl. Prakash *et al.* (1981) did not find any correlation between protein hydrophobicity and the number of denaturant molecules bound per protein molecule.

The subunit composition of the two proteins as determined by SDS-polyacrylamide gel electrophoresis, shows significant differences (Gururaj Rao and Narasinga Rao, 1981), The amino acid compostion of the subunits and their geometry in the oligomeric protein may play a role in the denaturation of these proteins.

Acknowledgement

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