Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 334-341

VISCOMETRIC CHARACTERIZATION OF PENNISETIN FROM PEARL MILLET

M.N. Sainani, A.H. Lachke, N.A. Sahasrabudhe and P.K. Ranjekar

Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411008, India

Received August 21, 1989

SUMMARY: Pennisetin, the alcohol soluble storage protein of pearl millet (Pennisetum americanum), was isolated in a homogeneous state. The intrinsic viscosity $[\eta]$ of this protein was found to be in the range of 16.5-17.7 ml/g in 70% (v/v) aqueous ethanol. The $[\eta]$ changed marginally when temperature was increased from 20 to 70°C and also in the presence of 10 mM NaCl. The data indicated that pennisetin was a rigid, rod shaped asymmetric hydrodynamic particle with molecular dimensions in the range of 301 x 14.4 Å - 317.7 x 14.2 Å . During denaturation with guanidine hydrochloride (Gdn.HCl), the intrinsic viscosity of pennisetin increased from 16 to 25ml/g with a mid point at 3.6 M of the denaturant. The native protein structure was unfolded in 6 M Gdn.HCl as shown by the exposure of aromatic amino acid residues buried in the native state and this transition was found to be reversible. The intrinsic viscosity of pennisetin in 5.9 M Gdn.HCl corresponded to Mr 25,000 which was comparable to that determined by SDS-PAGE.

In pearl millet, the alcohol soluble protein (prolamin) is termed as pennisetin. This storage protein is the major nitrogenous fraction of the endosperm and accounts for 40% of the total nitrogen at grain maturity (1). It is rich in glutamine, proline and hydrophobic amino acids but deficient in basic and acidic amino acids making it soluble in aqueous solutions of alcohol. It is nutritionally poor due to deficiency in lysine and tryptophan (2). Based upon the knowledge of its sequence and structure, it is possible to improve the nutritional value of prolamin as well as its functional properties such as viscoelasticity and film formation. In this regard, characterization of C hordein from barley and A gliadin from wheat has been reported earlier (3-6). In the present study, we have described the molecular dimensions and conformational changes in native as well as under denaturing conditions of pennisetin by viscometry.

MATERIALS AND METHODS

Preparation of pennisetin: Pearl millet prolamin (pennisetin) was prepared from defatted meal after removal of water soluble and salt soluble fractions, using 70% ethanol at 60°C. The prolamin containing supernatent

0006-291X/89 $1.50

was mixed with equal volume of 1.5 M NaCl and kept overnight at 4°C. The precipitated prolamin was recovered by centrifugation and lyophilized. It was then purified on Octyl Sepharose as described by Mawal et al. (7).

SDS polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed according to Robert et al. (8) using 14% acrylamide (w/v) gel. The gel was stained with 0.05% Coomassie brilliant blue R-250 for 6 h and destained in a mixture of water:ethanol:acetic acid (50:40:10).

Determination of protein concentration: Protein concentrations were measured by Kjeldahl's method using a factor of 5.7 for the conversion of nitrogen to protein for solutions in 70% ethanol.

Viscometry: Viscosity measurements were made in an Ubbelöhde capillary viscometer with a flow time of 155 s for water at 25°C. The temperature of the viscometer was controlled using a constant temperature water bath. Several flow times for solvent (t_0) and for protein solutions (t) were recorded. Density of solutions was found using a 10 ml pycnometer bottle at 25°C. Partial specific volume ($\bar{v}_2$) of pennisetin was calculated from the amino acid composition. Solutions of pennisetin in 70% ethanol, 70% ethanol + 10 mM NaCl and different concentrations of Gdn.HCl were filtered through sintered glass filter (G_3) before use. Dilutions of the protein solutions were made directly inside the viscometer.

All denaturation and renaturation experiments were done at 30°C. The protein solutions in Gdn.HCl were incubated for a minimum period of 1 h. Each solution was transferred to the viscometer gently to avoid shearing and foaming of the protein solution. For renaturation experiments, the pennisetin was first denatured with 7 M Gdn.HCl and then diluted with 70% ethanol to get the required Gdn.HCl concentration.

Fluorescence measurements: Fluoroscence spectra were recorded at excitation wavelength of 274 and 295 nm using Aminco SPF-500 spectrophotometer. Protein solution in 70% ethanol having an absorbance below 0.1 at 280 nm was used. The solution of pennisetin was incubated with 6 M Gdn.HCl for minimum 1 h prior to assessing the fluorescence spectra of denatured pennisetin.

RESULTS AND DISCUSSION

The homogeneity and molecular weight of pennisetin were examined on 14% (w/v) acrylamide gel under reduced condition. The protein migrated as a single band indicating that it consisted of a single polypeptide chain. The molecular weight of pennisetin was found to be 22,000 by comparing its relative mobility with that of standard proteins (Figure 1).

Intrinsic viscosity of native pennisetin: The reduced viscosities (η_{sp}/c) with/without NaCl in 70% aqueous ethanol against various protein concentrations are shown in Figure 2. The intrinsic viscosities [η] obtained by extrapolation to zero concentration are given in Table I. The values of [η] in the temperature range 20-70°C and with 10 mM NaCl vary between 16.5-17.7 ml/g and are significantly higher than those reported for proteins possessing a compact globular structure (3.3-4.0 ml/g) (9). This indicates that the pennisetin exists as either highly solvated randomly

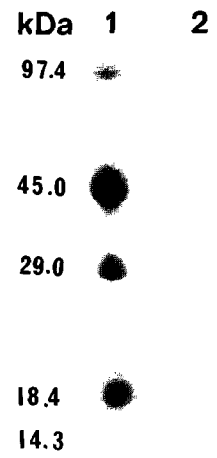

Figure 1. Molecular weight of pennisetin in SDS-PAGE. Standard molecular weight proteins are phosphorylase b, ovalbumin, carbonic anhydrase, β-lactoglobulin and lysozyme (lane 1). Pennisetin (lane 2).

coiled chain or relatively rigid asymmetric particle. Increase in temperature from 20° to 70° C has shown almost no effect on intrinsic viscosity of pennisetin solutions. This insensitivity of [η] at various

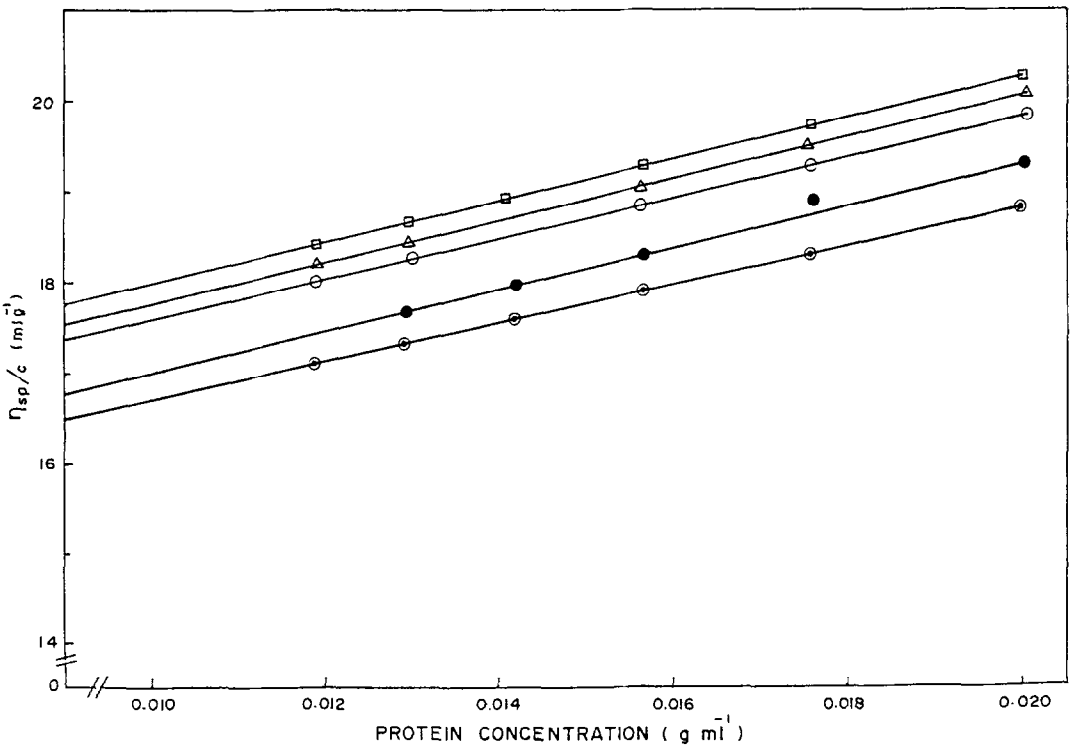

Figure 2. Reduced viscosities ($\eta_{sp/c}$) of pennisetin in 70% (v/v) aqueous ethanol with and without NaCl in the temperature range 20-70°C (●, 22°; △ 30°; □ 50°C; ○, 70°; ○, 30° + 10 mM NaCl).

Table I: The intrinsic viscosity, molecular dimensions and radius of gyration

Protein	Solvent	Temperature (0°C)	[η] (ml/g)	Molecular dimensions, Å			Radius of gyration, Å		
				Length (L)	Diameter (d)	L/d	1[a]	2[b]	3[b]
Pennisetin (native)	70% (v/v) aqueous alcohol	22	16.8	306.6	14.4	21.3	88.5	43.7	49.3
		30	17.5	315.6	14.3	22.1	91.1	44.2	50.0
		50	17.7	317.7	14.2	22.3	91.7	44.4	50.1
		70	16.5	301.0	14.4	20.9	86.9	43.4	49.0
	70% (v/v) alcohol + 10 mM NaCl	30	17.2	308.5	14.4	21.2	88.1	43.6	49.2
Pennisetin (under denaturing conditions)	Gdn.HCl								
	1 M	30	16.5	301.0	14.4	20.9	88.9	43.4	49.0
	2 M	30	17.5 (16.5)[c]	315.6	14.3	22.1	91.1	44.2	50.0
	3 M	30	18.4 (17.5)	326.7	14.0	23.3	94.3	45.0	50.8
	4 M	30	22.0 (23.0)	376.1	13.5	27.8	108.6	47.7	53.9
	5 M	30	24.5 (24.8)	396.4	13.2	30.0	114.3	49.5	55.9
	5.9 M	30	25.0	398.7	13.2	30.3	115.0	49.6	56.0

[a] assuming a rod shaped molecule.
[b] assuming a random coil conformation in ideal and non-ideal solvents, respectively.
[c] Values in parentheses represents the [η] during renaturation.

temperatures suggests that pennisetin is an asymmetric hydrodynamic particle rather than its occurrence in the form of a random coil (10). No polyelectrolyte effect on [η] value was observed in the presence of 10 mM NaCl.

The molecular dimensions of pennisetin (native state): The intrinsic viscosity of a protein depends on its shape and hydration for rigid molecules. The dimensions were calculated on the assumption that pennisetin is an asymmetric hydrodynamic particle. The Simha shape factor V was determined using the equation 1 (11):

$$[\eta] = V (\bar{v}_2 + \delta_1 v_1^\circ) \qquad [1]$$

($\bar{v}_2$, partial specific volume of pennisetin; v_1°, the specific volume of the solvent and δ_1 the effective solvation of the macromolecule; taken as 0.2 g/g dry protein) (12).

Thus, the viscosity increment V was found to be in the range of 17.0-18.3 ml/g which corresponded to an axial ratio a/b of the prolate ellipsoid between 11.6-12.2. The values of a and b were calculated using Tanford's equations [2] and [3].

$$V_h = 4/3 \pi ab^2 \qquad [2]$$

$$V_h = M/N (\bar{v}_2 + \delta_1 v_1^o) \quad [3]$$

(V_h, the hydrodynamic volume and M, the unsolvated Mr as determined by SDS-PAGE). The length and diameter of pennisetin was further computed since L = 2a and d = $(2/3)^{\frac{1}{2}}$. (b/a).L for the rod shaped molecule. The dimensions of pennisetin thus determined vary between 301 x 14.4 Å to 317.7 x 14.2 Å under different experimental conditions (Table I). This data indicates that pennisetin is a rod shaped molecule similar to C hordein. Although both the prolamins exist as rod shaped, C hordein has larger dimensions (355 x 17.2 Å, L/d = 20) than those of pennisetin (3)

Radius of gyration of pennisetin (R_G): The values of R_G were measured using equation [4] and [5], considering pennisetin as a rod shape molecule and assuming a flexible coil conformation in ideal and non-ideal solvents (Table I).

$$R_G = L/\sqrt{12} \quad [4]$$

$$R_G = \sqrt[3]{(3 M [\eta]/10\pi N \xi^3)} \quad [5]$$

(ξ = 0.875 for ideal solvents and 0.775 for poor solvents).

Denaturation-renaturation of pennisetin: The Gdn.HCl was used as a denaturing agent as it brings out significant changes in physico-chemical properties of proteins with minimum electrostatic interactions. Since this denaturation is not an instanteneous reaction, the extent of denaturation with respect to time was determined. Complete denaturation of pennisetin which required about 1 h was determined by measuring the viscosity of pennisetin in Gdn.HCl at various time intervals. The transition of pennisetin from native to denatured state at different concentrations of Gdn.HCl is shown in Figure 3. The transition starts above 1 M and appears to be completed at 5.9 M Gdn.HCl where the intrinsic viscosity is 25 ml/g. This result indicates that 5.9 M Gdn.HCl is required for the complete unfolding of pennisetin. Further, significant amounts of secondary and tertiary structure are present at low Gdn.HCl concentrations. During unfolding with 5.9 M Gdn.HCl, the molecular dimensions of native pennisetin vary from 317.7 x 14.2 Å to 398.7 x 13.2 Å. The denaturation of the protein is, however, completely reversible as exhibited during renaturation process (Figure 3). During renaturation, the intrinsic viscosity at 2 M Gdn.HCl is same as that for the native protein indicating that protein regains its native conformation after denaturation with Gdn.HCl. The values of [η] obtained during renaturation are not parallel to those obtained during denaturation.

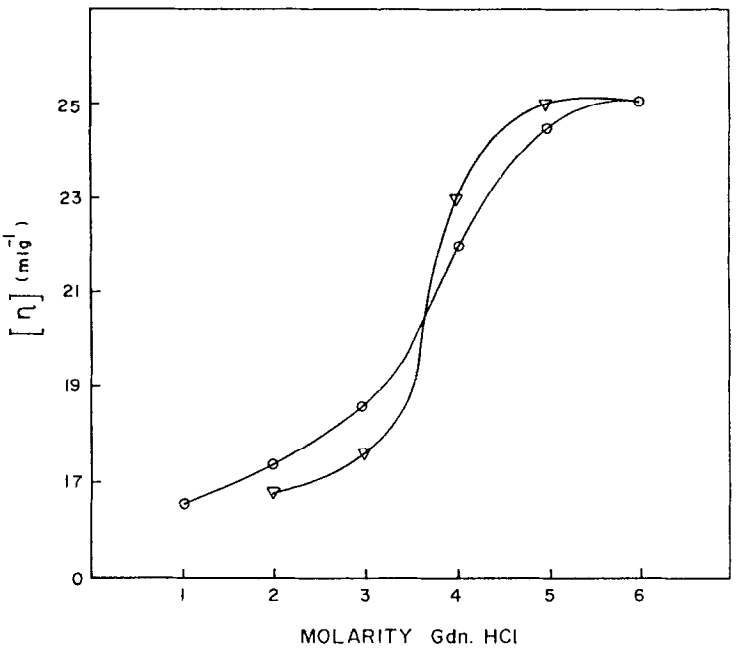

Figure 3. Intrinsic viscosity of pennisetin as a function of Gdn.HCl concentration under denaturing (○) and renaturing (△) states.

The Mr of pennisetin was calculated from intrinsic viscosity in 5.9 M Gdn.HCl by equation [6] which corresponded to a molecular weight of 25,000.

$$[\eta] = K (n)^a \qquad [6]$$

(n, number of amino acid residues; K and a are constants, where K = 0.716 and a = 0.66 as determined by Tanford et al.)(12).

Fluorescence of pennisetin: As an alternative evidence to demonstrate the denaturation of pennisetin, its fluorescence was measured. This technique was found to be sensitive for monitoring conformational changes of pennisetin during denaturation. Excitation of native pennisetin at 274 and 295 nm resulted in an emission spectrum with a single emission peak at 335 nm. The denaturation by 6 M Gdn.HCl caused a decrease in the relative fluorescence intensity and a shift in the emission peak from 335 to 344 nm at both excitation wavelengths (Figure 4a, b). However, for tyrosine a shoulder at 305 nm was observed. This shift of the peak is typical of a protein that unfolds during denaturation thereby exposing tyrosine and tryptophan residues from the hydrophobic interior to collisional quenching by the solvent.

In summary, our results based on measurements of intrinsic viscosity have shown that pennisetin in 70% ethanol has high intrinsic viscosity and

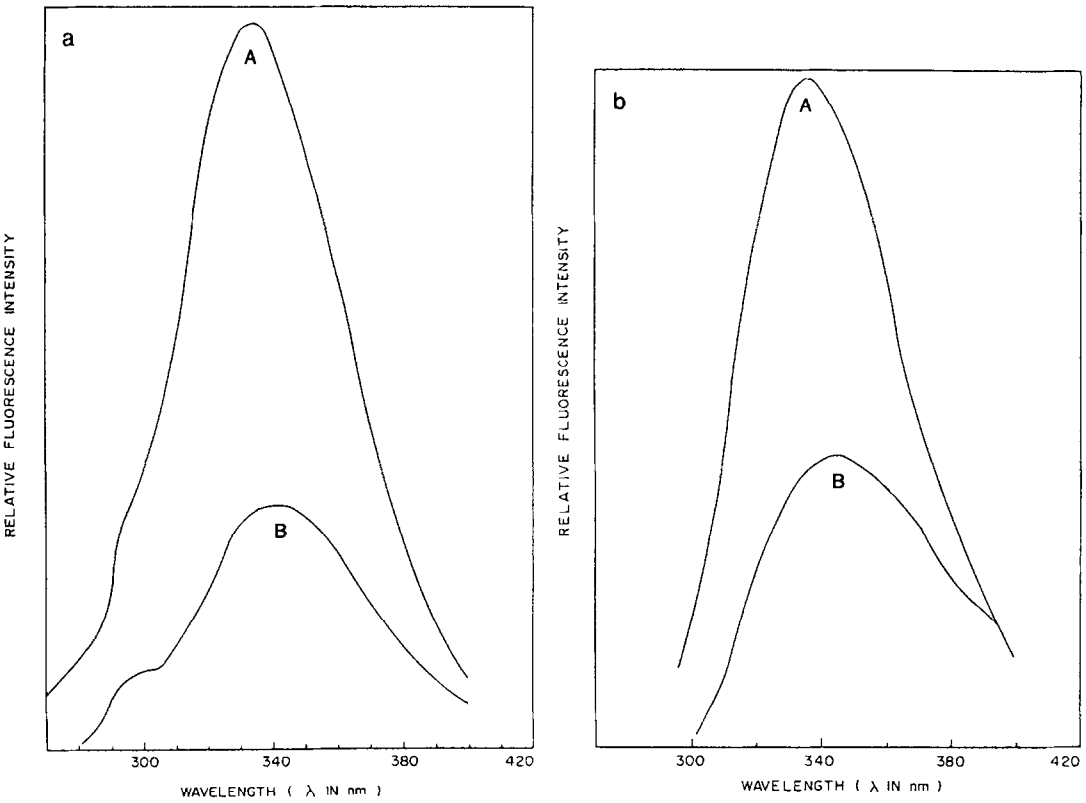

Figure 4. a and b. Fluorescence emission spectra of native (A) and denatured (B) pennisetin at excitation wavelength: 274 nm (a) and 295 nm (b)

axial ratio which are clearly incompatible with globular proteins. Complete unfolding of pennisetin is observed in 5.9 M Gdn.HCl and this process is mostly reversible. Our's is the first report on the structure of pennisetin and attempts are being made to obtain and characterize the genomic as well as c-DNA clones coding for this protein.

REFERENCES

1. Okoh, N.P., Nwasike, C.C. and Ikediobi, C.O. (1985) J. Agric. Food Chem. 33, 55-57.
2. Nwasike, C.C., Mertz, E.T., Pickett, R.C., Glover, U.D., Chibber, B.A.K. and Van Scoyoc, S.W. (1979) J. Agric. Food Chem. 27, 1329-1331
3. Field,J.M., Tatham, A.S., Baker, A.M. and Shewry, P.R. (1986) FEBS Lett. 200, 76-80.
4. Cole, E.W., Kasarda, D.D. and Lafiandro, D. (1984) Biochim. Biophys. Acta 787, 244-251.
5. Wu, Y.V. and Dimler, R.J. (1963) Arch. Biochem. Biophys. 103, 310-318
6. Beckwith, A.C., Wall, J.S. and Jordan, R.W. (1965) Arch. Biochem. Biophys. 112, 16-24.
7. Mawal, Y.R., Mawal, M.R. and Ranjekar, P.K. (1989) Biotechnol. Techniques 3, 167-172.

8. Robert, L.S. Nozzolillo, C. and Altosaar, I. (1983) Cereal Chem. 60, 438-443.
9. Tanford, C. (1961) Physical Chemistry of Macromolecules, p.394. Wiley.
10. Ahmad, F. and Salahuddin, A. (1974) Biochemistry 13, 245-249.
11. Simha, R. (1940) J. Phys. Chem. 44, 25-34.
12. Tanford, C., Kawahara, K. and Lapanje, S. (1967) J. Am. Chem. Soc. 89, 729-736.