ELSEVIER

International Journal of Biological Macromolecules
29 (2001) 273–280

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

Analysis of equilibrium dissociation and unfolding in denaturants of soybean agglutinin and two of its derivatives

Manjir Ghosh, Dipak K. Mandal *

Department of Chemistry, Presidency College, 86/1 College Street, Calcutta 700 073, India

Received 6 June 2001; accepted 9 August 2001

Abstract

The equilibrium denaturation of tetrameric soybean agglutinin (SBA) in urea and guanidine hydrochloride (GdnHCl) has been examined by steady-state fluorescence and size-exclusion chromatography. The denaturation of SBA reveals two distinct and separable transitions: dissociation (native tetramer↔tertiary monomer) and unfolding (tertiary monomer↔unfolded monomer). The urea denaturation curves of *N*-dimethyl and acetyl derivatives of SBA are also similar to unmodified lectin but the midpoints, $[D]_{1/2}$, are shifted to lower denaturant concentrations. The free energy of stabilization of tertiary structure ($\Delta G_{u,aq}$) of SBA is estimated to be 4.5–4.6 kcal mol^{-1}, which shows a decrease by $\sim$10–15% for both *N*-dimethyl SBA and acetyl-SBA. The free energy term ($\Delta G_{d,aq}$) for the relative stability of the quaternary structure of SBA and its derivatives shows that the decrease in stability relative to SBA occurs by $<$10% for *N*-dimethyl SBA while for acetyl-SBA, this occurs by $\sim$30%. However, the *m* values depicting the dependence of free energy on denaturant concentration for SBA and its derivatives are similar for dissociation as well as unfolding, which suggest similar denaturation pathways of unmodified and modified SBA.

Keywords: Soybean agglutinin; Denaturation curve analysis; Chemical modification

1. Introduction

Lectins are proteins which bind to specific carbohydrate structures of glycoproteins and glycolipids that are present on the surface of cells. The lectin-carbohydrate interactions have been demonstrated to be involved in a variety of biological recognition processes including adhesion [1], metastasis [2,3], apoptosis [4], and host-pathogen interactions [5]. Lectins are widely distributed in nature, and are found in plants, microorganisms and animals [6]. Soybean agglutinin (SBA) is a member of the legume family of plant lectins with important biological properties, including its ability to induce mitogenicity in lymphocytes [7], and to localize carbohydrate receptors on the surface of normal and transformed cells [8]. The X-ray crystal structures of SBA cross-linked with biantennary oligosaccharides [9,10] as well as many members of this family have been determined. The results demonstrate that this family of proteins has very similar tertiary structures [11], but they often differ in their quaternary structures, particularly among tetrameric lectins such as concanavalin A, the peanut agglutinin and SBA. As a consequence of their multivalent quaternary structure, lectins binding to cells often lead to cross-linking and aggregation of specific glycoprotein and glycolipid receptors which, in many cases, is associated with signal transduction effects [12,13]. In this regard, many legume lectins have been shown to bind and cross-link with specific branched-chain oligosaccharides to form unique, homogeneous cross-linked lattices [14]. These lectins have also been shown to form specific cross-linked complexes with glycoproteins [15,16], and this unique specificity of interaction of lectins is mediated, in part, by the effects of the specific quaternary structures to which these proteins fold and assemble.

Abbreviations: SBA, soybean agglutinin, lectin from soybean (*Glycine max*); GdnHCl, guanidine hydrochloride; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PBS, 0.01 M sodium phosphate buffered with 0.15 M sodium chloride, pH 7.2.

* Corresponding author. Tel.: +91-33-241-3893; fax: +91-33-512-3156.

E-mail address: dkmm@cal2.vsnl.net.in (D.K. Mandal).

0141-8130/01/$ - see front matter
PII: S0141-8130(01)00175-1

Despite extensive studies of lectin-carbohydrate interactions, little information is available on the protein stability and the problem of 'dissociation/association and unfolding/refolding' of multimeric lectins. Here we present the thermodynamic investigations of subunit dissociation and unfolding of SBA under equilibrium conditions in urea and GdnHCl. This is the first report in relation to the energetics of protein folding of a tetrameric lectin from the denaturation curve analysis. There is also considerable interest in determining how small changes in chemical structure can alter the protein stability. We have examined the effects of chemical modification, on the stability of SBA at both tertiary and quaternary levels, by reductive methylation and acetylation reactions which are often used to label proteins in various studies including multivalent lectin-carbohydrate cross-linking interactions.

2. Materials and methods

2.1. Materials

Untoasted soybean meal was purchased from a local chemical company. Cross-linked guar gum matrix was prepared by cross-linking guar gum (Sigma) with epichlorohydrin by slight modification of the published procedure [17]. Sephadex G-100 and Sephacryl HR 100 were obtained from Pharmacia. Guanidine hydrochloride (GdnHCl) ($>99\%$), sodium cyanoborohydride, trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma. Urea (AR, E. Merck, India) was further crystallized from hot ethanol to remove possible contamination by cyanate ions [18]. The concentrations of stock solutions of urea and GdnHCl were determined on dry weight basis. All other reagents used were of analytical grade. Absorption spectra were recorded on Hitachi U 3210 UV-VIS spectrophotometer using Sigma cuvette (volume: 2 ml; pathlength: 1cm), and the steady-state fluorescence spectra on a Hitachi 4010 spectrofluorimeter with Sigma fluorimeter cuvette (volume: 2 ml; pathlength: 1cm).

2.2. Protein purification

SBA was purified from the crude extract of soybean meal [19] by affinity chromatography on cross-linked guar gum matrix [16]. Since aggregation of SBA occurs on storage in the lyophilized state [20], affinity-purified SBA was precipitated by ammonium sulfate (80% saturation) and dialyzed against PBS (0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl) before use in denaturation experiments. The protein was eluted as a single peak in gel filtration on Sephadex G-100 column and was free from the aggregated form of protein. The purity of the preparation was checked by polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions [21], and the assay of activity was done by hemagglutination assay [22] using 3% suspension of trypsin treated rabbit erythrocytes. Protein concentration was determined spectrophotometrically using $A^{1\%,\ 1\ cm} = 12.8$ at 280 nm and expressed in terms of subunit ($M_r = 30\,000$) [23]. The neutral sugar content of SBA was estimated by the phenol-sulfuric acid method [24] using D-mannose as standard.

2.3. Chemical modification

Reductive methylation of SBA was carried out in 0.1 M phosphate buffer, pH 7.0, in presence of sodium cyanoborohydride and formaldehyde according to the procedure described [25] to prepare the *N*-dimethyl derivative. Acetyl derivative was prepared by acetylation of SBA with acetic anhydride in saturated sodium acetate, pH 8.3, as described [26]. In the final step, each derivative was subjected to affinity chromatography on cross-linked guar gum column. The extent of amino group modification was determined by TNBS method [27].

2.4. Protein denaturation

For each denaturation experiment, a known amount of PBS was mixed with a fixed amount of the protein stock solution and varying amounts of the concentrated denaturant (both in PBS) in a final volume of 2 ml, and incubated at 37 °C for 18 h to ensure that the equilibrium was achieved. The protein concentrations were in the range of 0.5–1.5 μM. The steady-state fluorescence measurements were made at 25 °C with excitation at 280 nm, and emission scanned from 300 to 400 nm. The excitation and emission slits were 5 nm and the scan rate was 60 nm min^{-1}. To test the reversibility of denaturation, the protein solution was first completely denatured in 8 M urea or 6 M GdnHCl, and then diluted with PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+} and incubated at 37 °C for up to 6 h before the fluorescence spectra were recorded as described above.

2.5. Size-exclusion chromatography

To verify the size corresponding to tetrameric structure of lectin, SBA was loaded onto a Sephadex G-100 column (0.9×70 cm) which was equilibrated with PBS at room temperature. Fractions of 2 ml were collected at a flow rate of 12 ml h^{-1} and monitored for protein at 280 nm. Gel filtrations of *N*-dimethyl SBA and acetyl-SBA on Sephadex G-100 were done similarly. As the Sephadex G-100 column could not be used to determine the size of the species in presence of denatu-

rant, size-exclusion chromatography on Sephacryl HR 100 was performed. SBA denatured in 3 M urea was loaded onto a Sephacryl HR 100 column (1.5 × 70 cm) which was equilibrated with PBS containing 3 M urea. The flow rate was 15 ml h^{-1} and the fraction size was 2 ml. The protein content in the fractions was assayed by the method of Lowry et al. [28], and the absorbances were read at 750 nm. Gel filtration experiments of *N*-dimethyl SBA denatured in 2.5 M urea, and acetyl-SBA denatured in 2 M urea were performed similarly. The chromatography of SBA and its derivatives in absence of urea and that of fully denatured protein in 8 M urea on Sephacryl HR 100 column were also performed. The Sephacryl column was precalibrated in presence of urea with the following marker proteins: bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), and soybean trypsin inhibitor (20.1 kDa).

3. Results and discussion

3.1. Properties of SBA and its derivatives

SBA is a tetrameric GalNAc/Gal-specific glycoprotein lectin composed of slightly different subunits ($M_r \approx 30\,000$) [29,30]. Chemical modifications of SBA by reductive methylation and acetylation to obtain *N*-dimethyl SBA and acetyl-SBA, respectively, showed that ~80% of the amino groups were modified (as estimated by TNBS method) even by repeating the procedures twice. However, each derivative possessed the similar structural and functional properties as unmodified SBA. Both *N*-dimethyl SBA and acetyl-SBA retained their capacity to bind to affinity column (cross-linked guar gum), and each derivative was affinity purified before using in the folding studies. The hemagglutinating activity of each derivative was also similar to that of native lectin (data not shown). The fluorescence properties of the derivatives were almost identical to those of the unmodified protein, suggesting that they have similar conformations. Size-exclusion chromatography of SBA and its derivatives on Sephadex G-100 (Fig. 5A) shows that they were eluted at the same elution volume corresponding to molecular mass of 120 kDa. This demonstrates that the quaternary structures of these derivatives remained same (tetrameric) after modification. The neutral sugar content of unmodified and modified proteins as determined by the phenol-sulfuric acid method [24] was 4.8% which is consistent with one Man9 oligomannose type chain per subunit.

3.2. Denaturation of SBA, N-dimethyl SBA and acetyl-SBA

Steady-state fluorescence is a useful technique for studying the structure and dynamics of proteins [31,32]. The intrinsic fluorescence of proteins from aromatic amino acid residues is an excellent built-in reporter [33]. For example, the microenvironment surrounding Trp residues can be probed since these exhibit red-shift of emission maximum as their environment changes from nonpolar to polar [34]. A fluorescence red-shift also occurs when Trp side chains buried in the interior of the protein become solvent exposed as a result of denaturation. The fluorescence spectra of SBA in varying concentrations of urea at pH 7.2 are shown in Fig. 1A, and the urea denaturation curve is shown in Fig. 2A. At 0 M urea the protein exhibits emission maximum at 329 ± 1 nm, which gradually red-shift to 351 ± 1 nm in ≥ 6.8 M urea, indicative of protein denaturation and Trp exposure to the aqueous environ-

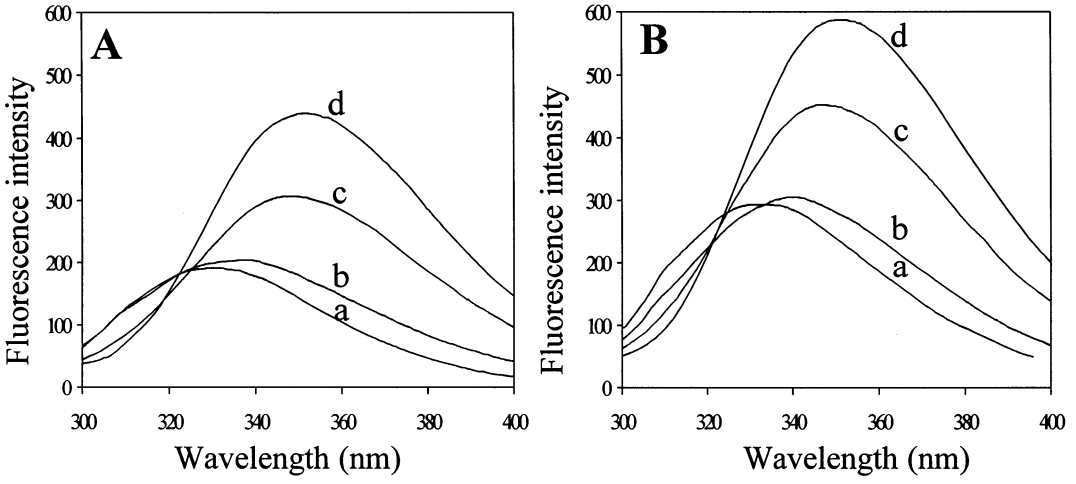

Fig. 1. Fluorescence spectra at 25 °C of (A) SBA (1.1 μM) in 0 M (a), 3 M (b), 5 M (c) and 8 M (d) urea in PBS; and (B) *N*-dimethyl SBA (1.5 μM) in 0 M (a), 2.5 M (b), 4.5 M (c) and 8 M (d) urea in PBS. The spectra were corrected for the buffers containing requisite concentrations of urea. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm min^{-1}.

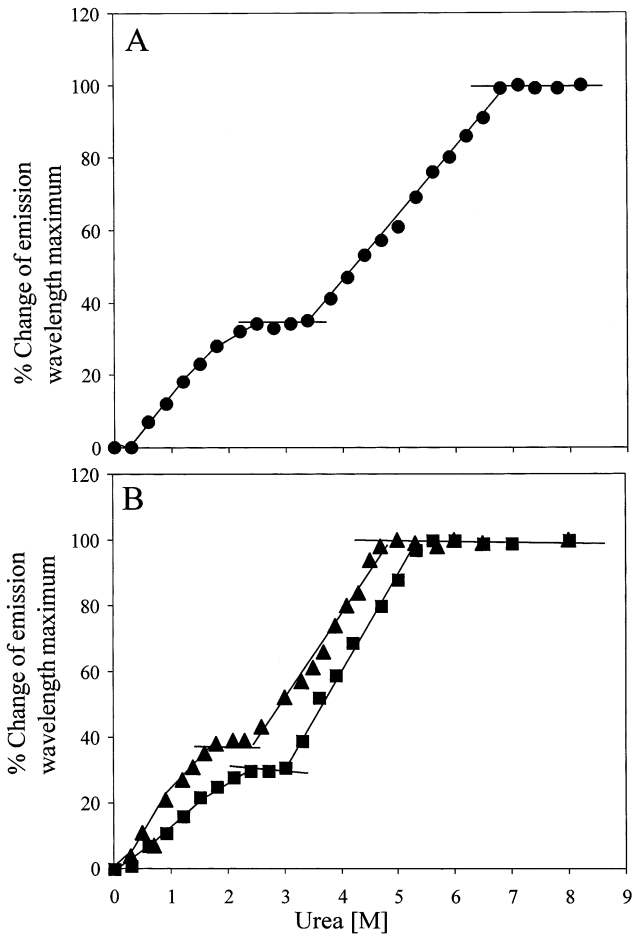

Fig. 2. (A) Urea denaturation curve of SBA (●). (B) Urea denaturation curves of *N*-dimethyl SBA (■) and acetyl-SBA (▲). Curves were calculated from the results of change in emission wavelength maximum as a function of urea concentration in PBS. Each data point represents average of three determinations.

ment. The denaturation curve (Fig. 2A) reveals two distinct transitions. A stable intermediate appears in 2.5–3.4 M urea with an emission maximum around 337 nm corresponding to ~35% of emission maximum red-shift obtained for complete denaturation. The intermediate exhibits the relative fluorescence intensity similar to that of native lectin (curves *a* and *b* in Fig. 1A), followed by increased fluorescence with further rise in urea concentration and concomitant unfolding, which levels off at ~2-fold in ≥6.8 M urea (curves c and d in Fig. 1A). As SBA is a noncovalently associated tetramer, the formation of intermediate structure in urea due to subunit dissociation was examined by size-exclusion chromatography. The integrity of tetrameric structure of native SBA was confirmed from gel filtration analysis on Sephadex G-100 (Fig. 5A). Gel filtration size analysis in presence of urea was performed on Sephacryl HR 100. It is interesting to note that the native tetrameric lectin in absence of urea and the completely unfolded monomer in 8 M urea were eluted in the void volume in separate experiments on Sephacryl column (data not shown). This may be due to the extensive randomly coiled conformations of the denatured subunits leading to an appreciable decrease in elution volume compared with that of a compact globular conformation [35]. When SBA denatured in 3 M urea (corresponding to the intermediate structure in the denaturation curve (Fig. 2A)) was subjected to size-exclusion chromatography on Sephacryl column, a protein peak appeared at a position of molecular mass 31 kDa (Fig. 5B) that corresponds to the lectin monomer. It may be mentioned that increasing the concentration of protein loaded to the column facilitated the monitoring of the protein peak but resulted in a fraction of protein being eluted in the void volume (not shown), probably as a result of partial aggregation. The above results demonstrate that the stable intermediate as seen in the denaturation curve (Fig. 2A) arises from the dissociation of tetramer into monomers that retain largely the tertiary structure of the protein. These results agree with a previous report [36] of formation of lectin monomer as intermediate in GdnHCl denaturation of SBA.

Denaturation curves are useful to probe the relationship between chemical structure and the protein stability [31]. These can also provide information as to whether a small change in the chemical structure can affect the mechanism of denaturation of the protein. Fig. 1B shows the fluorescence spectra of *N*-dimethyl SBA in varying urea concentrations at pH 7.2 and the urea denaturation curve is shown in Fig. 2B. As with SBA, the urea denaturation curve of *N*-dimethyl SBA shows two distinct transitions, with a stable intermediate in 2.2–3.0 M urea. In absence of urea, the derivative exhibits an emission maximum at 330 ± 1 nm, and when the intermediate forms in urea, ~30% change in emission wavelength maximum occurs; however, there was no appreciable difference in fluorescence intensity between the intermediate and the derivative in 0 M urea (curves a and b in Fig. 1B), as was observed for SBA. Gel filtration size analysis on Sephacryl HR 100 shows that the molecular mass of the intermediate corresponds to that of protein monomer (Fig. 5C). The denaturation curve of acetyl-SBA (Fig. 2B) also shows two transitions with an intermediate corresponding to protein monomer (Fig. 5D).

3.3. Denaturation curve analysis

The nature of the denaturation curves of SBA and its derivatives reveals that the denaturation process can be described as two distinct and separable transitions:

$$N_4 \leftrightarrow 4N \quad \text{(dissociation)} \tag{1a}$$

$$N \leftrightarrow U \quad \text{(unfolding)} \tag{1b}$$

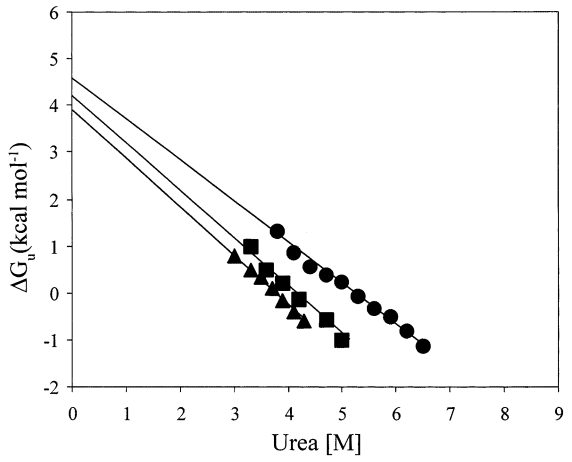

Fig. 3. ΔG_u as a function of urea molarity for unfolding transitions for SBA (●), *N*-dimethyl SBA (■) and acetyl-SBA (▲). ΔG_u values were calculated from the data in Fig. 2 using Eq. (2) with $T = 298$ K. Lines for unfolding transitions were drawn according to Eq. (5) using the least squares analysis of the data. See the text for more details.

where N_4, N and U denote native tetramer (quaternary state), 'native' monomer (tertiary state) and unfolded monomer (unfolded state) of the protein. These transitions were found to be completely reversible.

3.3.1. Analysis of unfolding transition

The unfolding transition $N \leftrightarrow U$ depicting a two-state mechanism is analyzed according to the method of Pace [31]. The equilibrium constant (K_u) is related to the fraction of the unfolded protein (f_U), and the free energy of unfolding (ΔG_u) by:

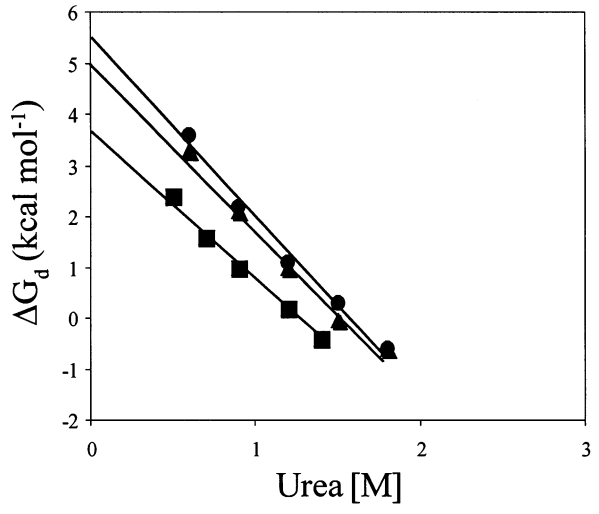

Fig. 4. ΔG_d as a function of urea molarity for dissociation transitions for SBA (●), N-dimethyl SBA (■) and acetyl-SBA (▲). ΔG_d values were calculated from the data in Fig. 2 using Eq. (8) with $T = 298$ K. Lines for dissociation transitions were drawn according to Eq. (9) using the least squares analysis of the data. See the text for more details.

$$\Delta G_u = -RT \ln K_u = -RT \ln\left(\frac{f_U}{1 - f_U}\right) \quad (2)$$

f_U and hence K_u can be calculated from the fluorescence data as follows:

$$f_U = \left(\frac{\Delta\lambda_{obs} - \Delta\lambda_N}{\Delta\lambda_U - \Delta\lambda_N}\right) \quad (3)$$

$$K_u = \left(\frac{\Delta\lambda_{obs} - \Delta\lambda_N}{\Delta\lambda_U - \Delta\lambda_{obs}}\right) \quad (4)$$

where $\Delta\lambda_{obs}$ is the observed change in emission wavelength maximum in the transition region and $\Delta\lambda_N$ and $\Delta\lambda_U$ are the values characteristic of the 'native' (structured) monomer and unfolded monomer of the protein, respectively.

ΔG_u depends on the denaturant concentration according to the linear extrapolation method (Fig. 3).

$$\Delta G_u = \Delta G_{u,aq} - m_u[M] \quad (5)$$

where $[M]$ is the molar concentration of the denaturant, and m_u is a measure of the dependence of ΔG_u on denaturant concentration. $\Delta G_{u,aq}$ represents the free energy of stabilization of tertiary monomeric structure relative to the unfolded state under normal conditions (aqueous solutions in the absence of denaturant at room temperature).

The $\Delta G_{u,aq}$ values and the midpoints of transitions, $[D]_{1/2}$ are shown in Table 1. In urea, $\Delta G_{u,aq}$ for SBA is 4.6 kcal mol^{-1} (monomer mass). Similar free energy value was obtained for SBA in GdnHCl (Table 1) estimated from the GdnHCl denaturation curve (not shown). The consistency of the results in both denaturants support the use of a linear extrapolation method (Eq. (5)) which has been justified on thermodynamic grounds [37] and which has the advantage that no assumption about the binding of denaturants to the native and unfolded forms of the protein are needed [31]. The free energy of stabilization of the tertiary structure of SBA is thus obtained as 4.5–4.6 kcal mol^{-1} which is in the lower range of values reported for globular monomeric proteins where the native state appears to be stabilized by 2–15 kcal mol^{-1} [31,38]. The $\Delta G_{u,aq}$ values for unfolding in urea for *N*-dimethyl SBA and acetyl-SBA (Table 1) are 4.2 and 3.9 kcal mol^{-1} compared with a value of 4.6 kcal mol^{-1} for SBA, which lead to a decrease in free energy of stabilization of tertiary structure by ~ 9 and $\sim 15\%$, respectively. An estimate [38] of decrease in stability of tertiary structure from the difference in $[D]_{1/2}$ values multiplied by m_u value for SBA (0.9 kcal mol^{-1} M^{-1}) (Table 1) also gives the similar result, being ~ 13 and $\sim 15\%$ for *N*-dimethyl SBA and acetyl-SBA, respectively. (Difference in $[D]_{1/2}$ values was calculated after subtracting the respective urea concentrations required for intermediate monomer formation in each case). These results show that reductive methylation and

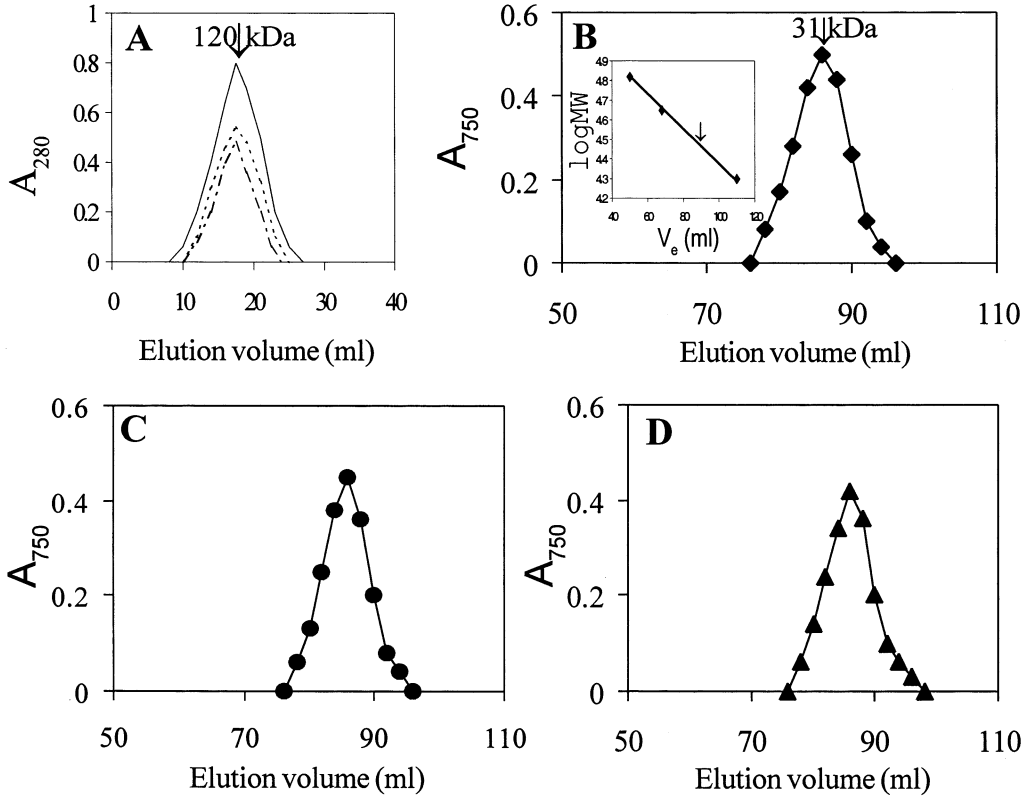

Fig. 5. (A) Elution profiles of SBA (4 mg) (––), *N*-dimethyl SBA (2 mg) (…) and acetyl-SBA (2 mg) (---) from Sephadex G 100 column (0.9 × 70 cm). Gel filtrations on Sephacryl HR 100 column (1.5 × 70 cm) of (B) SBA (0.5 mg) denatured in 3 M urea, (C) *N*-dimethyl SBA (0.5 mg) denatured in 2.5 M urea and (D) acetyl-SBA (0.5 mg) denatured in 2 M urea. Experimental conditions have been described in the text. Inset: Molecular weight calibration curve. The columns were precalibrated with standard marker proteins (from left to right): bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and soybean trypsin inhibitor (20.1 kDa). The elution position of the dissociated SBA (or its derivative) is marked by arrow in the calibration curve and the calculated molecular mass is shown by arrow in the elution profile.

acetylation lead to the destabilization of tertiary structure of SBA by ~10–15% and their effects do not differ markedly.

Table 1 shows that the m_u values for SBA and its derivatives are very similar for unfolding processes. For a two-state mechanism, the m_u value will depend mainly on the size and composition of the part of the polypeptide chain which is freshly exposed to denaturant during denaturation pathways [39]. These results thus suggest similar mechanism of unfolding of SBA and its two derivatives.

3.3.2. Analysis of dissociation transition

For the $N_4 \leftrightarrow 4N$ dissociation, we consider a two-state transition between native tetramer and 'native' (structured) monomer under the conditions of the experiment. Unlike unfolding, 1 M tetramer dissociates into 4 M monomers, and we express equilibrium constant for dissociation (K_d) in terms of mole fraction of monomer (f_N) as:

$$K_d = \frac{(f_N)^4}{(1-f_N)} \tag{6}$$

where f_N can be obtained from the fluorescence data as:

$$f_N = \left(\frac{\Delta\lambda_{obs} - \Delta\lambda_{N_4}}{\Delta\lambda_N - \Delta\lambda_{N_4}}\right) \tag{7}$$

where $\Delta\lambda_{obs}$ is the observed change in emission wavelength maximum in the transition region and $\Delta\lambda_{N_4}$ and $\Delta\lambda_N$ are the values characteristic of the tetramer and monomer, respectively.

The free energy of dissociation (ΔG_d) is then given by:

$$\Delta G_d = -RT \ln K_d = -RT \ln\left[\left(\frac{(\Delta\lambda_{obs} - \Delta\lambda_{N_4})^4}{(\Delta\lambda_N - \Delta\lambda_{obs})(\Delta\lambda_N - \Delta\lambda_{N_4})^3}\right)\right] \tag{8}$$

Assuming a linear dependence of ΔG_d on denaturant concentration [*M*], we get:

$$\Delta G_d = \Delta G_{d,aq} - m_d[M] \tag{9}$$

m_d is the slope of the plot of free energy of dissociation versus denaturant concentration, and $\Delta G_{d,aq}$ represents the free energy term for the quaternary structure resulting from the association of monomers in aqueous solution.

Table 1
Thermodynamic parameters for unfolding of soybean agglutinin and its two derivatives

Protein in denaturant	Unfolding (N↔U)[a]		
	$\Delta G_{u,aq}$ (kcal mol^{-1})[b]	m_u (kcal M^{-1})[b]	$[D]_{1/2}$ (M)[c]
Urea			
SBA	4.6 ± 0.1	0.9	5.2
N-Dimethyl SBA	4.2 ± 0.1	1.0	4.2
Acetyl-SBA	3.9 ± 0.2	1.1	3.7
GdnHCl			
SBA	4.5 ± 0.1	1.8	2.5

[a] Two-state transition from tertiary to unfolded state.
[b] Calculated using the least-squares analysis with $T = 298$ K; $\Delta G_{u,aq}$ represents the free energy at zero denaturant concentration for unfolding process (tertiary monomer↔unfolded monomer), and m_u denotes the slope of the linear dependence of ΔG_u on denaturant concentration.
[c] Denaturant concentration at the midpoint of the unfolding curve, where $\Delta G_u = 0$.

The linear extrapolation analyses for SBA and its derivatives, viz, *N*-dimethyl SBA and acetyl-SBA are shown in Fig. 4. In urea, the $\Delta G_{d,aq}$ for SBA is 5.5 kcal mol^{-1} (tetramer mass), which is in good agreement with the value of 6.5 kcal mol^{-1} for SBA in GdnHCl (Table 2) estimated from the GdnHCl denaturation curve (not shown). The $\Delta G_{d,aq}$ values obtained for dissociation in urea of *N*-dimethyl SBA and acetyl-SBA are 5.0 and 3.6 kcal mol^{-1}, respectively, compared with a value of 5.5 kcal mol^{-1} for SBA. Thus the decrease in free energy parameter for the quaternary structure amounts to ~9% for *N*-dimethyl SBA and ~35% for acetyl-SBA. The decrease in stability calculated [38] by taking the difference between $[D]_{1/2}$ values for SBA and each of the derivatives and multiplying these by the m_d value for the unmodified protein (3.4 kcal mol^{-1} M^{-1}) (Table 2) gives ~6 and ~25% for *N*-dimethyl SBA and acetyl-SBA, respectively, which agrees fairly well with the estimate obtained from the difference in $\Delta G_{d,aq}$ values. These results clearly show that acetylation has a more pronounced destabilizing effect than reductive methylation on the stability of the quaternary structure of SBA. Since reductive methylation does not alter significantly the net charge of the protein [25], and the added methyl groups are small compared with other protein modifying reagents, the effect of reductive methylation on protein quaternary structure is small, and is less than that of acetylation which brings about changes in overall charge distribution on the protein in the neutral range and thus may contribute to a substantial decrease in stability of the quaternary structure, i.e. in the assembly of subunits. In this context, mention may be made of acetylation of the Glc/Man-specific lectin concanavalin A when the tetrameric protein is converted to dimeric derivative [40]. As with unfolding, the m_d values for SBA and its derivatives (Table 2) are very similar, suggesting similar dissociating pathways for these proteins.

Table 2
Parameters for dissociation of soybean agglutinin and its two derivatives

Protein in denaturant	Dissociation (N_4↔4N)[a]		
	$\Delta G_{d,aq}$ (kcal mol^{-1})[b]	m_d (kcal M^{-1})[b]	$[D]_{1/2}$ (M)[c]
Urea			
SBA	5.5 ± 0.2	3.4	1.6
N-Dimethyl SBA	5.0 ± 0.2	3.3	1.5
Acetyl-SBA	3.6 ± 0.3	3.0	1.2
GdnHCl			
SBA	6.5 ± 0.2	5.9	1.1

[a] Dissociation from tetrameric quaternary to monomeric tertiary state.
[b] Expressed in terms of mol of tetramer; calculated by linear extrapolation of ΔG_d values wherein K_d is expressed in terms of mole fractions (see text for details).
[c] Denaturant concentration at the midpoint of the dissociation curve.

It may be mentioned that the free energy parameter ($\Delta G_{d,aq}$) for dissociation involves K_d in terms of mole fractions, which is not the same as equilibrium constant if the components are expressed in molar concentrations. For unfolding the K_u is the same, being independent of concentration units. However, though the absolute value of $\Delta G_{d,aq}$ would vary on the concentration terms used, the parameter would give the same relative stability order under a given set of experimental conditions.

4. Conclusions

The present study provides an estimate and a comparison of the protein stability of a tetrameric plant lectin SBA and its *N*-dimethyl and acetyl derivatives at both tertiary and quaternary levels of structure formation. The enhancement of stability in the multisubunit lectin in an ordered state when compared with monomeric forms may arise, at least in part, due to the energetic tendency to exclude water molecules from hydrophobic intermolecular surfaces for achieving the complementarity of the subunit interfaces [41]. The functional property of lectin depends, apart from its unique carbohydrate specificity, on this attainment of specific quaternary structure which provides multiple binding sites (multivalency) for its interaction with car-

bohydrate receptors in biological systems. The involvement of dimers as equilibrium intermediates does not appear in the present scheme of analysis. Further work on the kinetic investigation of the pathway of association of SBA following the pathway of folding is in progress. The present study also shows that a small change in chemical structure by chemical modification through reductive methylation and acetylation promotes differential effects on the stability of quaternary and tertiary structure of the lectin. These modification reactions are widely used as general protein radiolabeling methods. The present results showing decrease in protein stability effected through these modifications thus call for careful consideration while using these derivatives as probes in several studies including lectin-carbohydrate cross-linking interactions.

Acknowledgements

This work was supported by a research grant from the Department of Science and Technology, Government of India. We thank Professor S. Ghosh, Head of the Department of Chemistry, for providing the facilities and helpful discussions.

References

[1] Drickamer K, Taylor ME. Annu Rev Cell Biol 1993;9:237.
[2] Lotan R, Raz A. Ann New York Acad Sci 1988;551:385.
[3] Konstantinov KN, Robbins BA, Liu F-T. Am J Pathol 1996;148:25.
[4] Perillo NL, Pace KE, Seilhamer JJ, Baum LG. Nature 1995;378:736.
[5] Sharon N, Lis H. Science 1989;246:227.
[6] Sharon N, Lis H. Scientific Am 1993;268(1):82.
[7] Novogrodski A, Katchalski E. Proc Natl Acad Sci USA 1973;70:2515.
[8] Sharon N. Adv Immunol 1983;34:213.
[9] Dessen A, Gupta D, Sabesan S, Brewer CF, Sacchettini JC. Biochemistry 1995;34:4933.
[10] Olsen LR, Dessen A, Gupta D, Sabesan S, Sacchettini JC, Brewer CF. Biochemistry 1997;36:15073.
[11] Loris R, Hamelryck T, Bouckaert J, Wyns L. Biochim Biophys Acta 1998;1383:9.
[12] Nicolson GL. Biochim Biophys Acta 1976;457:57.
[13] Chung K-N, Walter P, Aponte GW, Moore H-P. Science 1989;243:192.
[14] Brewer CF. Chemtracts Biochem Mol Biol 1996;6:165.
[15] Mandal DK, Brewer CF. Biochemistry 1992;31:8465.
[16] Mandal DK, Brewer CF. Biochemistry 1992;31:12602.
[17] Appukuttan PS, Surolia A, Bachhawat BK. Indian J Biochem Biophys 1977;14:382.
[18] Frohlich O, Jones SC. J Membr Biol 1987;98:33.
[19] Bhattacharyya L, Haraldsson M, Brewer CF. Biochemistry 1988;27:1034.
[20] Lotan R, Lis H, Sharon N. Biochem Biophys Res Commun 1975;62:144.
[21] Laemmli UK. Nature 1970;227:680.
[22] Osawa T, Matsumoto I. Methods Enzymol 1972;28:323.
[23] Lotan R, Siegelman HW, Lis H, Sharon N. J Biol Chem 1974;246:1219.
[24] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Anal Chem 1956;28:350.
[25] Jentoft N, Dearborn DG. Methods Enzymol 1983;91:570.
[26] Khan MI, Mandal DK, Brewer CF. Carbohydr Res 1991;213:69.
[27] Habeeb AFSA. Arch Biochem Biophys 1967;119:264.
[28] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. J Biol Chem 1951;193:265.
[29] Lotan R, Cacan R, Cacan M, Debray H, Carter WG, Sharon N. FEBS Lett 1975;57:100.
[30] Mandal DK, Nieves E, Bhattacharyya L, Orr GA, Roboz J, Yu Q-T, Brewer CF. Eur J Biochem 1994;221:547.
[31] Pace CN. Methods Enzymol 1986;131:266.
[32] Eftink MR. In: Lakowicz JR, editor. Topics in Fluorescence Spectroscopy, vol. 2. New York: Plenum, 1991:53.
[33] Creighton TE. Proteins: Structure and Molecular Properties. New York: Freeman, 1993.
[34] Burnstein EA, Vedenkina NS, Ivkova MN. Photochem Photobiol 1973;18:263.
[35] Mann KG, Fish WW. Methods Enzymol 1972;26:28.
[36] Nagai K, Shibata K, Yamaguchi H. J Biochem 1993;114:830.
[37] Schellman JA, Hawkes RB. In: Jaenike R, editor. Protein Folding. Amsterdam: Elsevier/NorthHolland Biomedical Press, 1980:331.
[38] Cupo JF, Pace CN. Biochemistry 1983;22:2654.
[39] Greene RF Jr, Pace CN. J Biol Chem 1974;249:5388.
[40] Gunther GR, Wang JL, Yahara I, Cunningham BA, Edelman GM. Proc Natl Acad Sci USA 1973;70:1012.
[41] Richards FM. Annu Rev Biophys Bioeng 1977;6:151.