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The Minimal Structural Requirement of Concanavalin A that Retains Its Functional Aspects

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A systematic investigation of the effects of several commonly used detergents on the conformation and function of concanavalin A at pH 7 in solution form was made by using circular dichroism (CD), intrinsic fluorescence, 1-anilino 8-sulphonic acid (ANS) binding, dynamic light scattering (DLS) and sugar inhibition assay. In the presence of 6.0 mM sodium dodecyl sulphate (SDS), an anionic detergent, and 0.8 mM cetyl tri methyl ammonium bromide (CTAB), a cationic detergent, intermediate states of concanavalin A were obtained having a negative CD peaks at 222 and 208 nm respectively, a characteristic of α-helix. These states also retained tertiary contacts with altered tryptophan environment and high ANS binding (exposed hydrophobic area) which can be characterized as molten globule states. Concanavalin A in the presence of 5.0 mM 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate (CHAPS), a zwitterionic detergent, and 0.07 mM brij-35, a non-ionic detergent, also exists in intermediate states. These intermediates (molten globules) had high ANS binding with native-like secondary (inherent β-sheet) and tertiary structure. The intermediate states were characterized further by means of dynamic light-scattering measurements and kinetic data. To study the possible functional requirement of the minimum structure, the intermediate states characterized in the presence of detergents were shown to retain the activity with polysaccharide (dextran). The pattern of activity observed was brij-35 > CHAPS > CTAB > SDS. The specific binding and activity of concanavalin A with ovalbumin was investigated as a function of time by turbidity measurements. Cationic and anionic detergents showed significant effects on the structure of concanavalin A as compared with zwitterionic and non-ionic detergents.

Key words: circular dichroism, concanavalin A, detergents, molten globule, percent residual activity.

Abbreviations: ANS, 1-anilino-8-naphthalenesulphonate; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate; CTAB, cetyl tri methyl ammonium bromide; DLS, dynamic light scattering; MG, molten globule; R_h, hydrodynamic radius; SDS, sodium dodecyl sulphate; TMB, Tris buffer containing metal ions; Tris, [2-Amino-2-(hydroxy methyl) propane-1,3-diol].

To be biologically active a protein has its particular three-dimensional conformation, which is governed by the amino acid sequence of that protein and acquired by the process of protein folding (*1*, *2*). On protein folding pathway there are many partially folded intermediate (PFI) states. One of these intermediate states can be defined as a molten globule (MG) state in which the protein molecule is almost as compact as in the native state (*3*) and has a loosely packed non-polar core (*4*). The specific structure of the protein is altered by some denaturants like surfactants, alkali, urea, guanidine chloride, formamide, formaldehyde, trifluoroacetate, ethylene glycol, dimethylsulphoxide, *etc*. due to which the protein loses its proper function (*5–9*)].

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Surfactants have a polar head and a non-polar tail; the later causes a hydrophobic effect, which arises primarily from the strong attractive forces between water molecules themselves (*10*). The forces involved between protein and surfactants are both electrostatic and hydrophobic (*11*, *12*). Earlier scattering and hydrodynamic data were mainly used to probe the structural changes in protein–detergent complexes. However, spectroscopic approaches have gained more attention (*13*). Detergents like brij-35, cetyl tri methyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) find their major application for solubilization and recovery of recombinant proteins (*14*).

Detergent extraction of integral proteins from the membrane often results in the disruption of the protein subunits or even converts β form into helices. Once membrane glycoproteins are solubilized and stabilized in buffered detergent solutions, they can be purified by immobilized lectin affinity column chromatography (*15*, *16*).

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Lotan *et al*. (*17*) have determined saccharide-binding activities in lectin affinity chromatography of membrane glycoproteins on their immobilized derivatives. Their study was confined to the haemagglutination assay and the effect of detergents on the solution form of lectins as well as their immobilized derivatives (*17*). No study up to now reports the spectroscopic studies on the effect of detergents on the conformational and functional changes of concanavalin A, a lectin from jack bean, which is homotetrameric with a molecular weight of 104 kDa (*18*) and is classified as all-β protein. Although effect of detergents on the haemagglutination activity is well known, here for the first time, we report the effect of detergents on structure of concanavalin A and its interaction with glycoproteins. Lectin–carbohydrate interaction is a well-studied phenomenon (*19*). Here concanavalin A was used to study this phenomenon with dextran and ovalbumin.

Immobilized lectins are used for purification of membrane glycoproteins in the presence of detergents, thus disrupting the native structure and carbohydrate binding of the lectin. The present study includes the effect of detergents: SDS (anionic), CTAB (cationic), 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate (CHAPS) (zwitterionic) and brij-35 (non-ionic) on concanavalin A (as a model of lectin used for the purification of glycomembrane proteins).

MATERIALS AND METHODS

Materials—Concanavalin A was purchased from Himedia (Mumbai, India). Ovalbumin from Sigma Chemical Co. (St Louis, MO, USA), [2-Amino-2-(hydroxy methyl) propane-1,3-diol] (Tris) from Qualigens Fine Chemicals (Mumbai, India), SDS from Sisco Research Laboratories (Mumbai, India), CTAB and brij-35 from Merck (Germany), CHAPS from PIERCE and Dextran from BDH Chemicals (Poole, England) were used without further purification. All other chemicals used in this study were of analytical grade.

Methods—The purity of concanavalin A was checked by polyacrylamide gel electrophoresis where more than one band revealed probably the presence of nick fragments which were removed by gel filtration. Samples containing concanavalin A (5 μM) in freshly prepared 20 mM phosphate buffer pH 7 with different concentrations of SDS (0–9.0 mM), CTAB (0–3.2 mM), CHAPS (0–10 mM) and brij-35 (0–0.12 mM), were filtered through 0.1 polyvinylidene filters (millipore) and equilibrated at room temperature for 1 h before recording. The concentration of native concanavalin A was determined using an extinction coefficient of 13.7 at 280 nm for a 0.1% solution.

Circular Dichroism Measurements—Circular dichroism (CD) measurements were performed with a JASCO J-720 spectropolarimeter (Tokyo, Japan) calibrated with ammonium D-10-camphorsulphonate. A cell of path length 0.1 and 1 cm was used for scanning between 200 and 250 nm and between 250 and 300 nm, respectively. Protein concentration of the samples was typically 5 μM in 20 mM phosphate buffer, pH 7 for the far-UV and near-UV CD studies. The results were expressed as θ in mdeg (*20*).

Fluorescence Measurements—Fluorescence spectra were recorded using a Shimadzu RF 1501 spectrofluorophotometer (Japan) in a 10 mm path length quartz cell. The excitation wavelength was 280 nm and emission wavelength was recorded from 300 to 400 nm, respectively (*21*). The final protein concentration was 5 μM. For each sample, proper blank was taken into consideration.

ANS Fluorescence Measurements—ANS (1-anilino-8-naphthalenesulphonate) binding was measured by fluorescence emission with excitation at 380 nm and emission was recorded from 400 to 600 nm, respectively (*22*). Typically, ANS concentration was 100 M excess of protein concentration. The final protein concentration was ~5 μM in 20 mM phosphate buffer, pH 7. Proper blank was made for every point.

Dynamic Light Scattering—For dynamic light scattering (DLS) experiments, concanavalin A (20 μM) in 20 mM freshly prepared phosphate buffer pH 7 was incubated for 1 h with 6.0 mM SDS, 0.8 mM CTAB, 5.0 mM CHAPS and 0.07 mM brij-35. The filtered samples were manually injected into flow cell (30 μl) and illuminated by 100 mW, 660 nm laser diode. Corresponding to the hydrodynamic radii (R_h) and molecular weight a particular R_h was calculated by software PMgr v3.01p17 supplied along with the instrument RiNA Laser spectroscatter 201.

Percent Residual Activity—The interaction of concanavalin A (5 μM) with dextran (1.5 mg/ml) in the presence of varying concentration of detergents was studied in 20 mM Tris–HCl pH 7 buffer containing Ca^{2+} and Mn^{2+} as metal ions (TMB) by turbidity method at 360 nm using spectrophotometer model Hitachi U 1500 (*23*). For each sample, proper blanks of native concanavalin A and dextran were taken into account. The percent residual activity was calculated relative to native concanavalin A-dextran complex as 100% from the turbidity measurements at 360 nm at pH 7 and 37°C.

Kinetic Study—The interaction of concanavalin A (5 μM) with ovalbumin (20 μM) in the presence of 6.0 mM SDS, 0.8 mM CTAB, 5.0 mM CHAPS and 0.07 mM brij-35 was studied at different time intervals in 20 mM TMB of pH 7 by turbidity method at 360 nm on spectrophotometer model Hitachi U-1500 (*23*). Proper blank having respective detergent with concanavalin A was taken in to consideration.

Data Analysis—Data were expressed in term of fraction unfolded (F_D) calculated from the equation:

$$F_D = [(Y - Y_N)/(Y_D - Y_N)] \quad (1)$$

where Y is the observed variable parameter and Y_N and Y_D are the values of the variable characteristics of the folded and unfolded conformations. The difference in free energy between the folded and unfolded states, ΔG was calculated by the following equation:

$$\Delta G = -RT \ln K = -RT \ln[F_D/(1 - F_D)] \quad (2)$$

where K is the equilibrium constant, R is the gas constant (1.987 cal/deg/mol) and T is 298 K (*24*).

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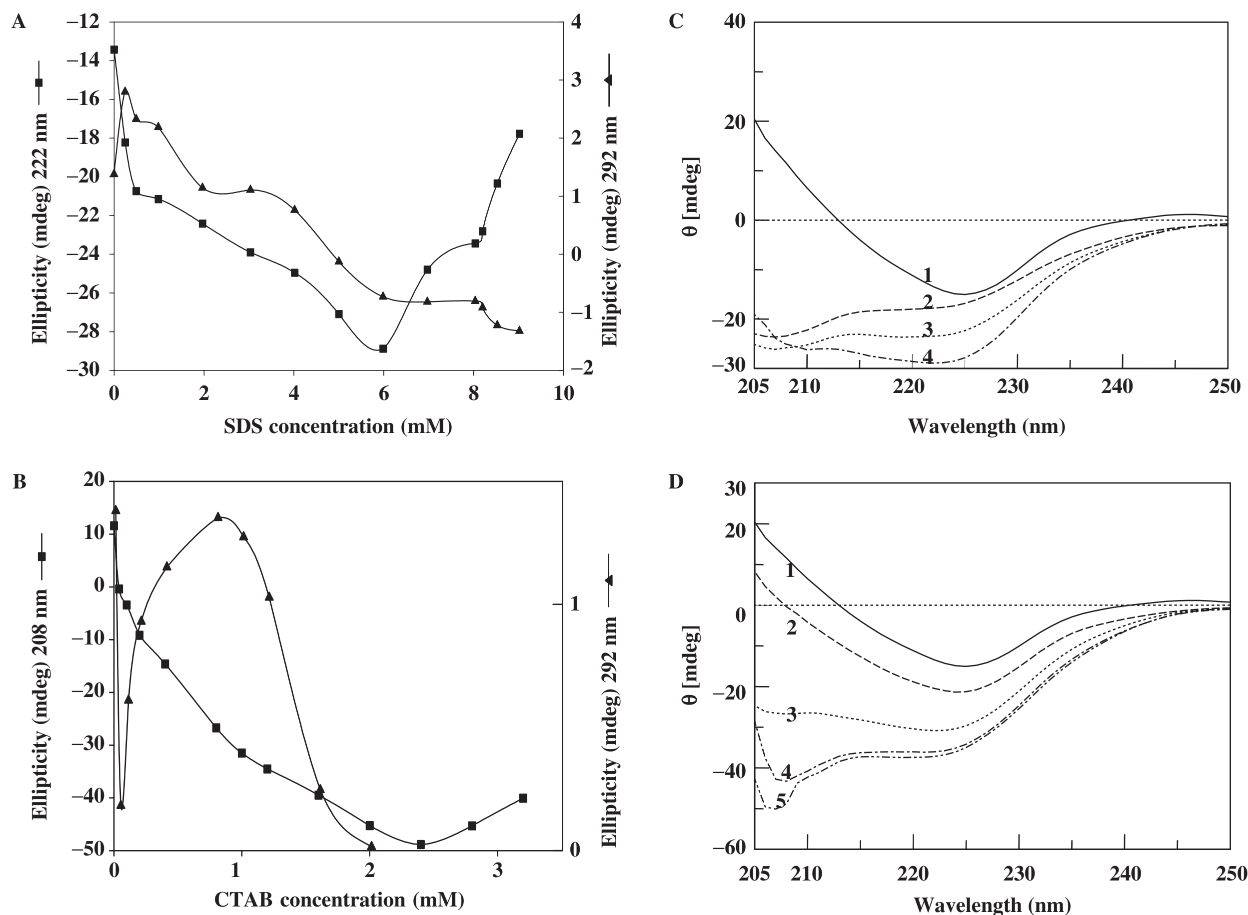

Fig.1. **(A) Effect of increasing concentration of SDS (0–9.0 mM) on concanavalin A (5 μM) at pH 7 as followed by ellipticity measurement at 222 nm (filled square) and 292 nm (filled triangle).** (B) Effect of increasing concentration of CTAB (0–3.2 mM) on concanavalin A at pH 7 as followed by ellipticity measurement at 208 nm (filled square) and 292 nm (filled triangle).(C) Far-UV CD spectra of concanavalin A as a function of SDS concentration (0–9.0 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the native concanavalin A, curve 2 with 9 mM, curve 3 with 8 mM and curve 4 with 6.0 mM of SDS. The protein concentration was 5 μM and the path length was 0.1 cm. (D) Far-UV CD spectra of concanavalin A as a function of CTAB concentration (0–3.2 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the native concanavalin A, curve 2 with 0.04 mM, curve 3 with 0.8 mM, curve 4 with 2.0 mM and curve 5 with 2.4 mM CTAB. The protein concentration was 5 μM and the path length was 0.1 cm.

RESULTS AND DISCUSSION

Effect of Detergents on the Structure of Concanavalin A—SDS and CTAB

Anionic detergent SDS induced conformational transitions on concanavalin A, and was studied by far-UV and near-UV CD. Figure 1A shows the ellipticity at 222 and 292 nm in the presence of varying concentrations of SDS (0–9 mM) and CTAB (0–3.2 mM) at pH 7. CD signal measured at 222 nm is a more selective probe for the helicity because interference caused by other secondary structure elements is relatively weak at this wavelength (*25*). Up to 6.0 mM SDS there was induction of secondary structure; beyond this concentration, loss of signal in far-UV CD spectrum was observed. An increase in ellipticity up to 6.0 mM can be suggestive of an increase in secondary structure content. Figure 1B shows the ellipticity at 208 and 292 nm in the presence of varying concentration of CTAB (0–3.2 mM) at pH 7. Ellipticity values at 208 nm showed a gradual denaturation of concanavalin A up to 1.2 mM. Beyond this concentration, there was a sharp change in the ellipticity values and the maximum loss in secondary structure was observed at 2.4 mM. As the concentration of SDS and CTAB increases further, the polarity of the solvent decreases creating a hydrophobic environment, which results in the disruption of secondary structure. SDS and CTAB being hydrophobic in nature may interact with hydrophobic side chains of the lectins exposed upon unfolding. So most probably, SDS perturbs the structure of protein surface partially modifying the layer of water and the microenvironment of amino acid residues, which is in agreement with the observed alterations in CD spectra. Six millimolar SDS retained partial amount of tertiary structure of concanavalin A as evident from the value of θ mdeg at 292 nm. Changes in ellipticity were followed at 292 nm with respect to CTAB molar concentration in near-UV CD spectra. A decrease

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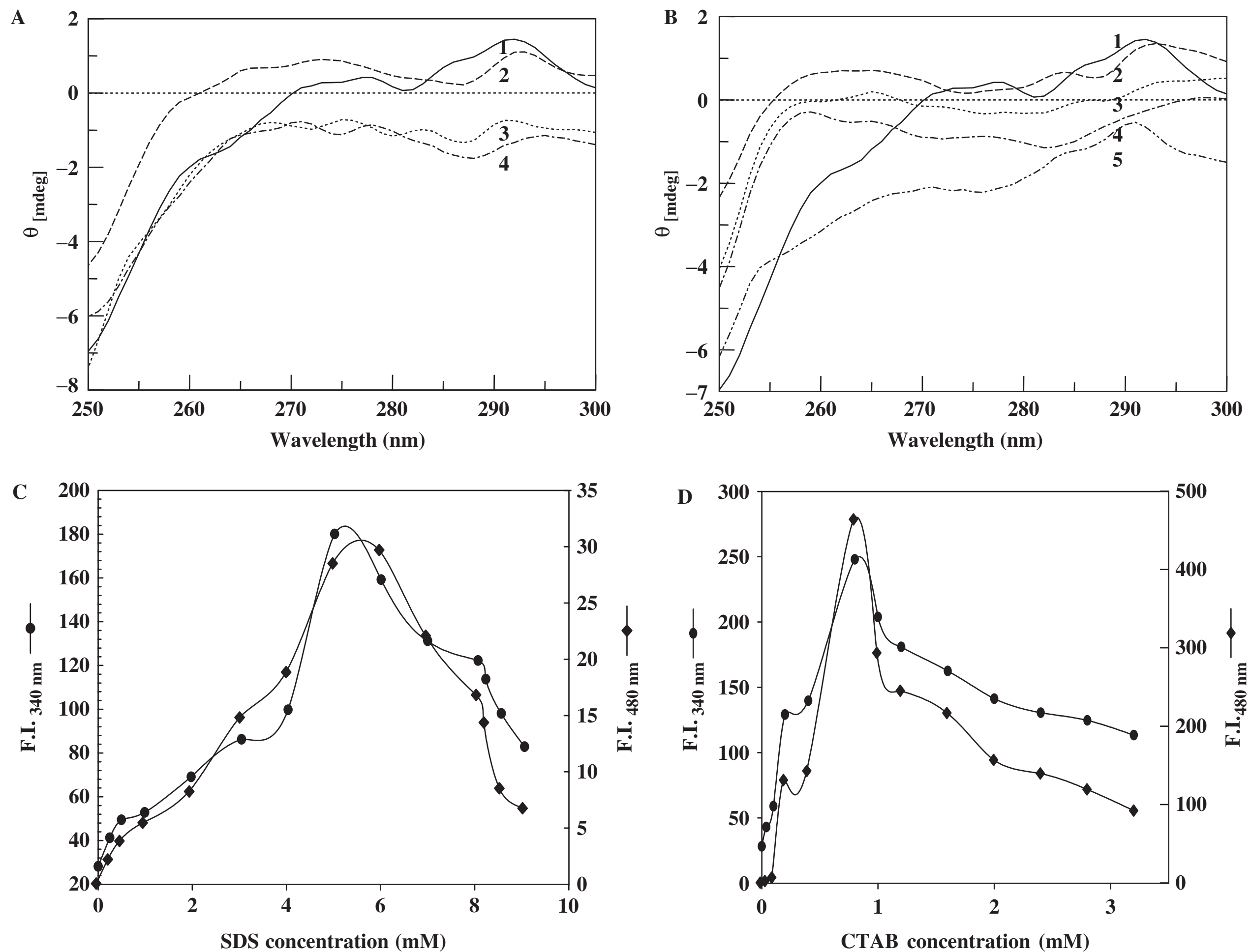

Fig. 2. **(A) Near-UV CD spectra of concanavalin A as a function of SDS concentration (0–9.0 mM) in 20 mM sodium phosphate buffer, pH 7.** Curve 1 represents the native concanavalin, curve 2 with 6.0 mM, curve 3 with 8 mM and curve 4 with 9 mM SDS. The protein concentration was 5 μM and the path length was 1 cm. (B) Near-UV CD spectra of concanavalin A as a function of CTAB concentration (0–3.2 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the native concanavalin A, curve 2 with 0.8 mM, curve 3 with 2.0 mM, curve 4 with 2.4 mM and curve 5 with 0.04 mM CTAB. The protein concentration was 5 μM and the path length was 1 cm. (C) Effect of increasing concentration of SDS (0–9.0 mM) on concanavalin A at pH 7 as followed by fluorescence intensity measurement at 340 nm (filled circle) and ANS fluorescence measurement at 480 nm (filled diamond). (D) Effect of increasing concentration of CTAB (0–3.2 mM) on concanavalin A at pH 7 as followed by fluorescence intensity measurement at 340 nm (filled circle) and ANS fluorescence measurement at 480 nm (filled diamond).

in ellipticity was observed in the presence of 0.04 mM CTAB. With the increase in concentration of CTAB (0.1–0.8 mM), there was increase in ellipticity that becomes constant beyond the mentioned concentration. At 0.8 mM concentration of CTAB, the ellipticity observed was near to that of the native concanavalin A.

As can be seen from Fig. 1C and D, 6.0 mM SDS (curve 4 of Fig. 1C) and 0.8 mM CTAB (curve 3 of Fig. 1D) induce the negative peaks at 208 and 222 nm in native concanavalin A, a characteristic of α-helix. Thus, SDS and CTAB induce non-native α-helix in native β-sheeted concanavalin A.

Figure 2A and B show the near-UV CD spectra in the range of 250–300 nm in the presence of different concentration of SDS and CTAB. Curve 1 of Fig. 2A represents the native preparation, curves 2, 3, 4 and 5 of Fig. 2A are in the presence of 0.25, 6.0, 8.0 and 9.0 mM SDS, respectively. Two prominent positive peaks at 292 and 277 nm characterize the native concanavalin A (curve 1 of Fig. 2A and curve 1 of Fig. 2B). On addition of 0.25 mM SDS there was a change in ellipticity of the native preparation. On further addition of SDS up to 6.0 mM the ellipticity of lectin (curve 2 of Fig. 2A) approaches to the native preparation (curve 1 of Fig. 2A) with the appearance of above-mentioned peaks. However, further addition of SDS up to 8 mM and 9 mM leads to loss of the native peaks as well as the CD signal (curves 3 and 4 of Fig. 2A). Upon addition of 0.04 mM CTAB (curve 5 of Fig. 2B) to native concanavalin A solution (curve 1), there was loss in ellipticity with disappearance of the peak at 277 and 292 nm. However, the spectrum of concanavalin A in the presence of 0.8 mM CTAB (curve 2 of Fig. 2B) resembled more to

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that of the native protein as compared with those at 2.4 mM (curve 4 of Fig. 2B) and 0.04 mM (curve 5 of Fig. 2B), where significant structure was lost. Thus, concanavalin A in the presence of 6.0 mM SDS and 0.8 mM CTAB retained the non-native secondary and tertiary structures.

The intrinsic fluorescence maximum is an excellent parameter to monitor the polarity of tryptophan environment in the protein (*21*). Figure 2C and D depicts the fluorescence intensity at 340 nm as a function of SDS and CTAB concentration, respectively. On increasing the SDS concentration there was an increase in fluorescence intensity up to 6.0 mM, indicating that the tryptophan environment of concanavalin A in 6.0 mM SDS was different from its native and denatured form in the presence of 9 mM SDS. The fluorescence intensity at 340 nm gradually increased with increase in concentration of CTAB up to 0.8 mM indicating that the lectin is acquiring a different environment from native form. The fluorescence intensity of tryptophan beyond 0.8–3.2 mM was found to decrease, suggesting that the tryptophan residues were again relocating to the same environment.

Binding of ANS to the hydrophobic regions of the protein results in an increase in fluorescence intensity that has been widely used to detect the MG state of different proteins (*26*). Titration of concanavalin A at pH 7 by SDS and CTAB was also monitored by ANS fluorescence at 480 nm (Fig. 2C). ANS shows negligible binding at pH 7. The notch in Fig. 2C and D at about 6.0 mM (Fig. 2C) and 0.8 mM CTAB (Fig. 2D), respectively, indicate the intermediate compact states appearing during structural transition from the native to SDS denatured state. The compactness of this intermediate state was lost on further increasing the concentration of SDS up to 9 mM or CTAB concentration up to 3.2 mM.

Thus, it appears that concanavalin A in presence of 6.0 mM SDS and 0.80 mM CTAB has the exposure of sizeable amounts of hydrophobic residues and retains a significant amount of 'non-native'-like secondary and fluctuating tertiary structure. This suggests the accumulation of a compact MG-like intermediate possessing 'non-native'-like tertiary as well as secondary structure.

CHAPS and brij-35

Figure 3A and B shows the far-UV CD spectra of the effect of CHAPS and brij-35 on native concanavalin A, respectively. As can be seen from the figures the native concanavalin A exhibits a trough (negative peak) at 225 nm (curve 1 of Fig. 3A and B). The position of the trough is a characteristic feature of β-conformation in lectins (*27*). Addition of CHAPS with increasing concentration resulted in increase in negative ellipticity with the position of trough remaining the same. The maximum increase was observed at 5.0 mM of CHAPS (curve 4 of Fig. 3A). On the other hand, with addition of increasing concentration of brij-35 in the solution of native concanavalin A, an intermediate state retaining the substantial amount of secondary structure was obtained between 0.05 mM and 0.12 mM (Fig. 3B). There was no major change in the far-UV CD spectra at different concentrations of brij-35 (in the Fig. 3B only the spectrum corresponding to 0.07 mM concentration of the detergent is shown for the sake of clarity). Thus, in the presence of brij-35 equilibrium is reached at 0.05 mM, which persists the entire concentration range studied.

The CD spectrum in the near-UV region was used to probe the asymmetry of the protein aromatic amino acid environment (*28*). Figure 3C and D shows the near-UV CD spectra in 250–300 nm range of native concanavalin A and in the presence of varying concentration of CHAPS and brij-35, respectively. At 0.16 mM of CHAPS (curve 1 of Fig. 3C), the protein showed the loss of tertiary structure as evident by the increase in CD signal. On further increasing the concentration up to 5.0 mM (curve 3 of Fig. 3C) the spectra retains the native-like characteristic with the prominent peaks at 292 nm and 277 nm (curve 4 of Fig. 3C). On addition of brij-35 up to 0.12 mM there was gradual loss of tertiary structure in concanavalin A (Fig. 3D). However, up to 0.07 mM of brij-35 (curve 3 of Fig. 3D), concanavalin A retains the native-like spectral features (curve 4 of Fig. 3D).

Figure 4A and B shows the fluorescence intensity at 340 nm as the function of increasing concentration of CHAPS and brij-35. In the presence of 5.0 mM CHAPS there was drastic increase in the tryptophan fluorescence intensity indicating the appearance of an intermediate state. In the presence of brij-35, the fluorescence intensity increases on increasing the concentration of detergent from 0 to 0.07 mM whereas it decreases beyond 0.07 mM to 0.12 mM.

In the native state of concanavalin A, there are two exposed tryptophan residues. One tryptophan is involved in monomer–monomer contact (residue No. 88) (*29*). The tetramer concanavalin A most probably dissociates into separate monomers on addition of CHAPS up to 5.0 mM and brij-35 up to 0.07 mM, thus exposing this tryptophan residue; hence, leads to increase in fluorescence intensity.

As can be seen from Fig. 4A, in the presence of CHAPS ANS shows minimal binding in the range of 0–4.0 mM. A drastic increase in ANS fluorescence was observed at 5.0 mM, suggesting the exposure of hydrophobic regions of the interior protein. On increasing the concentration of brij-35 up to 0.12 mM the native subunit acquired a random polypeptide conformation that led to decrease in fluorescence intensity (Fig. 4B). X-ray crystallographic analyses have delineated the exposed nature of two of the four tryptophan of concanavalin A (*30*). One tryptophan is situated near the lip of the large apolar-binding cavity (residue No.182). The alternate explanation can most probably be that addition of brij-35 creates a hydrophobic environment leading to an increase in fluorescence intensity. The decrease in fluorescence intensity can be explained that the buried tryptophan also contributes to the measured fluorescence and thus, the remaining signal is contributed by the internal tryptophan residues. Hence, at 0.07 mM concentration of brij-35 an intermediate state exists which has a different environment of tryptophan than the native state. This state also has exposed hydrophobic

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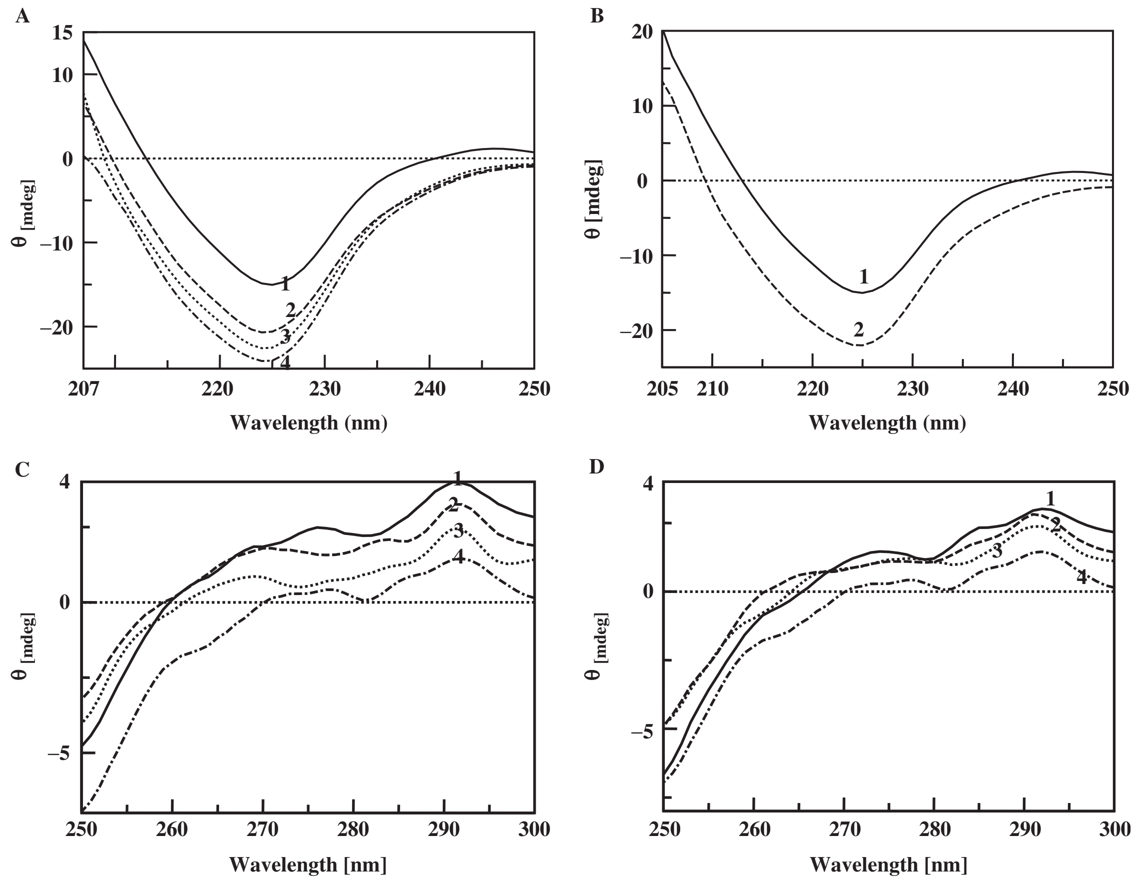

Fig. 3. **(A) Far-UV CD spectra of concanavalin A at different concentration of CHAPS (0–10 mM) in 20 mM sodium phosphate buffer, pH 7.** Curve 1 represents the native concanavalin A, curve 2 with 0.16 mM, curve 3 with 3 mM and curve 4 with 5.0 mM CHAPS. The protein concentration was 5 μM and the path length was 0.1 cm. (B) Far-UV CD spectra of concanavalin A as a function of brij-35 concentration (0–0.12 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the native concanavalin A, curve 2 with 0.07 mM brij-35. All curves in presence of brij-35 were overlapping on one another so only a single curve is shown here. The protein concentration was 5 μM and the path length was 0.1 cm. (C) Near-UV CD spectra of concanavalin A at different concentration of CHAPS (0–10 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the concanavalin A with 0.16 mM, curve 2 with 3 mM, curve 3 with 5.0 mM CHAPS and curve 4 is of native concanavalin A. The protein concentration was 5 μM and the path length was 1 cm. (D) Near-UV CD spectra of concanavalin A at different concentration of brij-35 (0–0.12 mM) in 20 mM sodium phosphate buffer, pH 7. Curve 1 represents the concanavalin A with 0.0018 mM, curve 2 with 0.0504 mM, curve 3 with 0.07 mM brij-35 and curve 4 is of native concanavalin A. The protein concentration was 5 μM and the path length was 1 cm.

residues; hence, maximum ANS binding was also observed in this intermediate state (Fig. 4B).

Thus, it appears that the CHAPS (5.0 mM) and brij-35 (0.07 mM) induced states retained a significant amount of 'native'-like secondary and tertiary structure as well as the exposed hydrophobic region. These states can be characterized as MG states that retain tertiary structure. A comparison of different structural and functional properties of detergent-induced PFIs of concanavalin A in the presence of SDS, CTAB, CHAPS and brij-35 at pH 7 is given in Table 1.

The fraction of concanavalin A-unfolded (F_D) at pH 7 in the presence of different detergents was calculated by taking concanavalin A at pH 7 in the absence of detergents as the native value. The calculated free energy of unfolding, ΔG, in the presence of detergents is shown in Table 2.

Dynamic light scattering

To investigate the quaternary structure of lectin in the presence of different detergents the DLS studies were carried out. Since concanavalin A is a non-covalently associated tetramer at pH 7 without intermolecular disulphide bonds. One possibility for these subunits is that they may be dissociated into monomers or dimers in the presence of any denaturants such as detergents. The DLS analyses of native concanavalin A confirms the tetrameric nature of the lectin as it showed the R_h of 4.3 nm which corresponds to ~109 kDa. When the lectin was incubated with 6.0 mM SDS for 1 h, the R_h was 2.45 nm that is approximately equal to 25 kDa indicating for the monomeric nature of lectin in solution. In the presence of 0.8 mM CTAB the R_h was 3.3 nm corresponding to 50 kDa as for the dimer but in the presence of 5.0 mM CHAPS and 0.07 mM brij-35 the R_h were 4.18 and 4.25 nm, respectively same as the native tetramer (Fig. 5).

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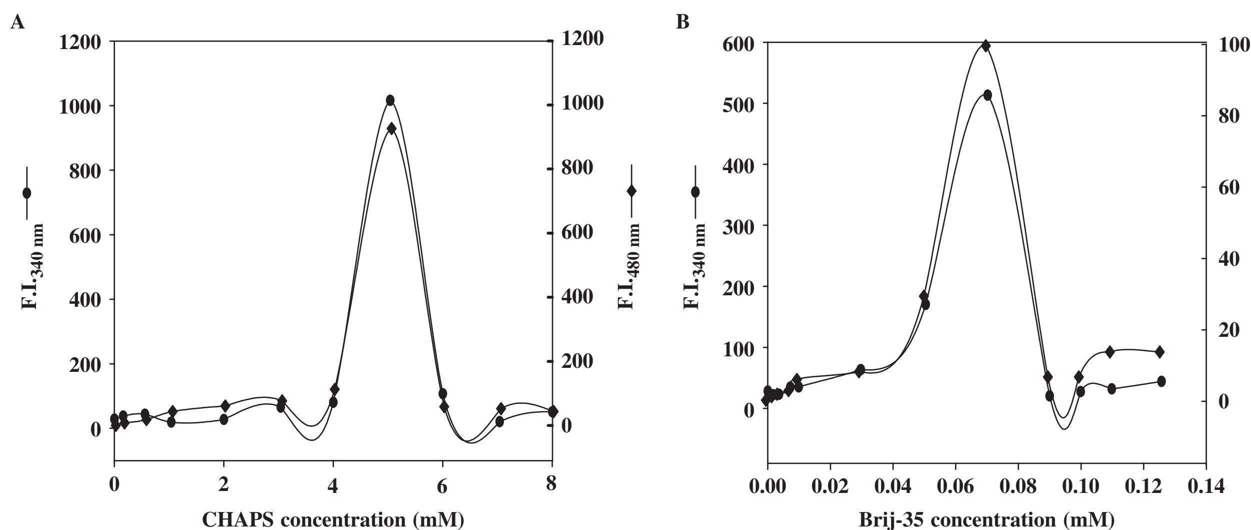

Fig. 4. **(A) Effect of increasing concentration of CHAPS (0–10 mM) on concanavalin A at pH 7 as followed by fluorescence intensity measurement at 340 nm (filled circle) and ANS fluorescence measurement at 480 nm (filled diamond).** (B) Effect of increasing concentration of brij-35 (0–0.12 mM) on concanavalin A at pH 7 as followed by fluorescence intensity measurement at 340 nm (filled circle) and ANS fluorescence measurement at 480 nm (filled diamond).

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Table 1. **Comparison of different structural and functional properties of detergent induced partially folded intermediates (PFI) of concanavalin A in the presence of SDS, CTAB, CHAPS and brij-35 at pH 7.**

Variable	SDS	CTAB	CHAPS	brij-35
Concentration (mM)	6.0	0.8	5.0	0.07
Trough position[a] (at λ_{nm})	222	208	225	225
θ_{mdeg}[b] (at 292 nm)	−0.73	1.35	2.31	2.37
FI[c] max (at 340 nm)	159	248	1017	513
FI[d] max (at 480nm)	30	464	920	100
R_h[e] (nm)	2.45	4.25	3.3	4.18
Residual activity (%)	51	57	69	78

[a]In far-UV CD. [b]In near-UV CD. [c]Intrinsic fluorescence intensity. [d]ANS fluorescence intensity. [e]Hydrodynamic radius from DLS measurement.

Table 2. **ΔG values for the detergent-induced transition of concanavalin A at pH 7 studied by far-UV CD measurements at 225 nm and fluorescence intensity measurements at 340 nm.**

Probe	ΔG_{H_2O} (Kcal/mol) SDS	ΔG_{H_2O} (Kcal/mol) CTAB	ΔG_{H_2O} (Kcal/mol) CHAPS	ΔG_{H_2O} (Kcal/mol) brij-35
$\theta_{mdeg\ (225\,nm)}$	−4.66	−0.518	−0.63	−3.99
$FI_{340\,nm}$	−5.45	−2.51	ND[a]	−0.518

[a]ND, not determined.

EFFECT OF DETERGENTS ON THE FUNCTION OF CONCANAVALIN A

Percent Residual Activity—Effect of the detergents on the precipitin reaction of concanavalin A with dextran was investigated by turbidity measurement at 360 nm by spectrophotometer. The absorbance obtained after the incubation of concanavalin A with dextran was taken as control. Since the MG states of concanavalin A were obtained in the presence of SDS, CTAB, CHAPS and brij-35 at 6.0, 0.8, 5.0 and 0.07 mM, respectively, so further experiments on activity were performed on the aforementioned concentrations. Addition of 6.0 mM SDS resulted in 49% activity loss of the native protein. Thus, the residual activity of the intermediate characterized in the presence of SDS was 51% of the native. In the presence of CTAB, the residual activity was 57%, in the presence of CHAPS and brij-35 it was 69% and 78%, respectively (Table 1).

The maximum loss in activity of concanavalin A was observed in the presence of SDS. We conclude that the intermediate state of concanavalin A obtained in the presence of SDS and CTAB consists of α-helix *i.e.* the transition of the protein inherent β-sheeted structure to α-helix. These states also lose 49% and 43% of the activity. The partial retention in activity is possibly due to the presence of some tertiary structure. In the presence of CHAPS and brij-35, there was retention of the high activity (∼70%) as consistent with the retention of native-like secondary and tertiary

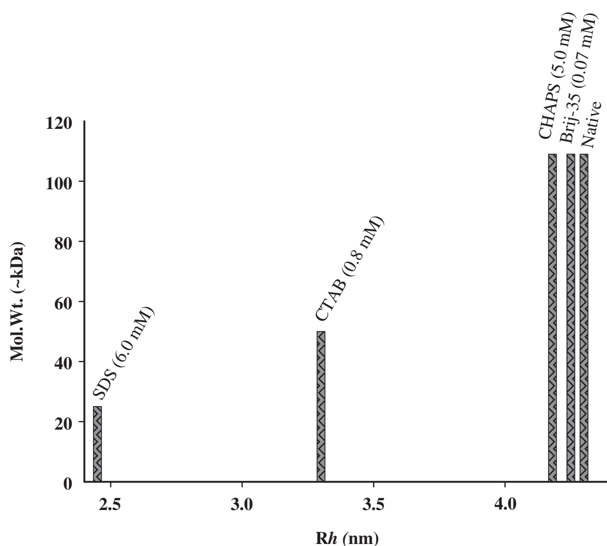

Fig. 5. **Relationship between molecular weight and hydrodynamic radii of native concanavalin A and in the presence of 6.0 mM SDS, 0.8 mM CTAB, 5.0 mM CHAPS and 0.07 mM brij-35.**

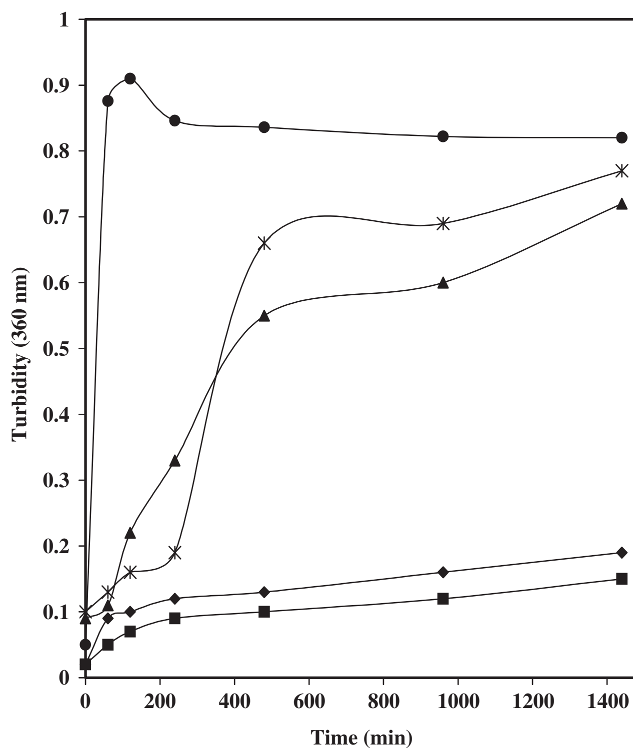

Fig. 6. **Curves for kinetic study of partially folded intermediates of concanavalin A (5 µM) obtained in the presence of different detergents *i.e.* 6.0 mM SDS (filled square), 0.8 mM CTAB (filled diamond), 5.0 mM CHAPS (filled triangle) and 0.07 mM brij-35 (asterisk) on concanavalin A-ovalbumin (filled circle) as a control, taking lectin with the respective detergents as blanks and quantitative precipitin reaction at 360 nm on spectrophotometer against time in TMB, pH 7.**

structure. As the spectroscopic studies show the loss of secondary and tertiary structure and DLS data show the loss of oligomeric structure, these results are consistent with our activity loss.

Activity with Respect to Time (Kinetics)—To monitor the activity of concanavalin A with time, the ligand ovalbumin was incubated with this lectin in the presence of different detergents and the turbidity was monitored after different time intervals (Fig. 6). CHAPS and brij-35 did not affect the activity of lectin up to concentrations approaching PFI for concanavalin A. The activity of this lectin was reduced in the presence of CTAB and even more dramatic reduction in activity was observed in the presence of SDS. The turbidity (precipitate) was lowest in SDS followed by CTAB, CHAPS and brij-35. Thus, it can be concluded that SDS has the property to denature the concanavalin A affinity column when used in membrane protein purification. The decrease in activity at low detergent concentration was less significant. High concentration resulted in dramatic decrease in activity of concanavalin A.

At neutral pH the charge on amino acid residues of concanavalin A is zero, as the pI of this lectin is 7 (*31*). SDS and CTAB being ionic detergents were found to be more effective denaturant as compared with CHAPS and brij-35 which are zwitterionic and non-ionic, respectively (SDS > CTAB > CHAPS > brij-35). SDS and CTAB were found to induce structural transitions in concanavalin A from β-sheet to α-helix while in the case of CHAPS and brij-35, there was change in CD signal but the conformation remains the same. All the detergents showed an altered tryptophan environment and high ANS binding.

The solubilization, fractionation, isolation and subsequent purification of most membrane glycoproteins by lectin affinity chromatography require the use of buffered detergent solutions. Lotan *et al.* (*17*) had reported the effect of temperature on glycoproteins binding efficiencies of lectins. They have also studied the effect of detergents on the binding capacity of immobilized lectins with glycoproteins. Till now, no study reports on the structural transition from β-sheet to α-helix on concanavalin A in the presence of SDS and CTAB. Here we have characterized the MG state of concanavalin A in the presence of different detergents. The data reported here represent our investigations on the effects of several detergents on the concanavalin A in solution by spectroscopic studies and its binding activity with ovalbumin and dextran. We chose detergents that are commonly used for membrane solubilization at concentrations below their critical micellar concentrations.

Thus, we can conclude that in purification of membrane glycoproteins from concanavalin A immobilized column, low concentration of detergent can be used. High concentration of detergents resulted in the loss in structure as well as function of the lectins. The changes were major in the presence of SDS and CTAB than CHAPS and brij-35. This study can be presented as a model for the purification of membrane glycoproteins where the detergents are commonly used for the purification combined with lectin affinity chromatography. For solubilization of membrane glycoproteins

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CHAPS and brij-35 can be proved to be more appropriate than SDS and CTAB.

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REFERENCES

1. Jacnicke, R. (1991) Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry* **30**, 3147–3161
2. Ptitsyn, O.B. (1987) How the molten globule became? *J. Protein Chem.* **6**, 273–293
3. Dolgikh, D.A., Gilmanshin, R.I., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminiv, S.Y., and Ptitsyn, O.B. (1981) Alpha-Lactalbumin: compact state with fluctuating tertiary structure? *FEBS Lett.* **136**, 311–315
4. Damaschun, G., Gernat, C., Damaschun, H., Bychkova, V.E., and Ptitsyn, O.B. (1986) Solvent dependence of dimensions of unfolded protein chains. *Int. J. Biol. Macromol.* **8**, 226–230
5. Goto, Y., Takahashi, N., and Fink, A.L. (1990) Mechanism of acid-induced unfolding of proteins. *Biochemistry* **29**, 3480–3488
6. Goto, Y. and Nishikiori, S. (1991) Role of electrostatic repulsion in the acidic molten globule of cytochrome c. *J. Mol. Biol.* **222**, 679–686
7. Naeem, A., Khan, K.A., and Khan, R.H. (2004) Characterization of a partially folded intermediate of papain induced by fluorinated alcohols at low pH. *Arch. Biochem. Biophys.* **432**, 79–87
8. Goto, Y., Calciano, L.J., and Fink, A.L. (1990) Acid-induced folding of proteins. *Proc. Natl Acad. Sci. USA* **87**, 573–577
9. Wuthrich, K. (1994) NMR, alcohols, protein solvation and protein denaturation. *EXS* **71**, 261–268
10. Barrick, D., Hughson, F.M., and Baldwin, R.L. (1994) Molecular mechanisms of acid denaturation. The role of histidine residues in the partial unfolding of apomyoglobin. *J. Mol. Biol.* **237**, 588–601
11. Hiramatsu, J. and Yang, T. (1983) Cooperative binding of hexadecyltrimethyl ammonium chloride and sodium dodecyl sulphate to cytochrome c and resultant change in protein conformation. *Biochim. Biophys. Acta* **743**, 106–114
12. Das, P., Mazumdar, S., and Mitra, S. (1998) Characterization of a partially unfolded structure of cytochrome c induced by sodium dodecyl sulphate and kinetics of its refolding. *Eur. J. Biochem.* **254**, 662–670
13. Naeem, A. and Khan, R.H. (2004) Characterization of molten globule state of cytochrome c at alkaline, native and acidic pH induced by butanol and SDS. *Int. J. Biochem. Cell. Biol.* **36**, 2281–2292
14. Cardamone, M., Puri, N.K., Sawyer, W.H., Capon, R.J., and Brandon, M.R. (1994) A spectroscopic and equilibrium binding analysis of cationic detergent-protein interactions using soluble and insoluble recombinant porcine growth hormone. *Biochim. Biophys. Acta* **1206**, 71–82
15. Lotan, R. and Nicolson, G.L. (1979) Purification of cell membrane glycoproteins by lectin affinity chromatography. *Biochim. Biophys. Acta* **559**, 329–376
16. Silman, I. and Katchalski, E. (1966) Water-insoluble derivatives of enzymes, antigens, and antibodies. *Annu. Rev. Biochem.* **35**, 873–908
17. Lotan, R., Beattie, G., Hubbell, W., and Nicolson, G.L. (1977) Activities of lectins and their immobilized derivatives in detergent solutions. Implications on the use of lectin affinity chromatography for the purification of membrane glycoproteins. *Biochemistry* **16**, 1787–1794
18. Naeem, A., Khan, A., and Khan, R.H. (2005) Partially folded intermediate state of concanavalin A retains its carbohydrate specificity, *Biochem. Biophys. Res. Comm.* **331**, 1284–1294
19. Sharon, N. and Lis, H. (1995) Lectin-carbohydrate complexes of plants and animals: an atomic view, *Essays Biochem.* **30**, 59–75
20. Chen, Y.H., Yang, J.T., and Martinez, H.M. (1972) Determination of the secondary structure of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11**, 4120–4131
21. Stryer, L. (1968) Fluorescence spectroscopy of proteins. *Science* **162**, 526–540
22. Matulis, D., Baumann, C.G., Bloomfield, U.A., and Lovrien, R.E. (1999) 1-anilino-8-napthalenesulfonate as a protein conformational tightening agents, *Biopolymers* **49**, 451–458
23. Waseem, A. and Salahuddin, A. (1983) Relevance of hydrophobic interactions in specific binding of mutivalent ligands to concanavalin A-effect of organic solvents. *Indian J. Biochem. Biophys.* **31**, 253–258
24. Naeem, A., Khan, K.A., and Khan, R.H. (2006) Characterization of partially folded intermediates of papain in presence of cationic, anion and non-ionic detergents at low pH. *Biopolymers* **1**, 1–10
25. Fink, A.L., Calciano, C.T., Goto, Y., Kurotsv, T., and Palleros, D. R. (1994) Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* **33**, 12504–12511
26. Matulis, D. and Lovrien, R. (1998) 1-Anilino-8-naphthalene sulfonate anion–protein binding depends primarily on ion pair formation. *Biophys. J.* **74**, 422–429
27. Pere, M., Bourrillon, R., and Jirgensons, B. (1975) Circular dichroism and conformational transition of *Dolichos Biflorus* and *Robinia Pseudoacacia* lectins. *Biochim. Biophys. Acta* **393**, 31–36
28. Dryden, D. and Weir, M.P. (1991) Evidence for an acid-induced molten-globule state in interleukin-2: a fluorescence and circular dichroism study. *Biochim. Biophys. Acta* **1078**, 94–100
29. Pelley, R. and Horowitz, P. (1976) Fluorimetric studies of tryptophyl exposure in concanavalin A. *Biochim. Biophys. Acta* **427**, 359–363
30. Reeke, G.N., Becker, J.W., and Edelman, G.M. (1975) The covalent and three-dimensional structure of concanavalin A IV. Atomic co ordinates, hydrogen quaternary structure. *J. Biol. Chem.* **250**, 1525–1547
31. Khan, R.H., Naeem, A., and Baig, M.A. (2005) Spectroscopic studies on the protective effect of a specific sugar on concanavalin A at acidic, neutral and alkaline pH. *Cell. Mol. Biol. Lett.* **10**, 61–72

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