MATERIALS

AND

METHODS
2. Materials and Methods

2.1. Materials

Jack bean seeds were purchased from Sigma. Sephadex G-100 and Superdex 75 were obtained from Pharmacia. Sephadex G-100 was suspended in distilled water and allowed to swell at 90ºC for five hours. Bio-Gel P-100 was obtained from Bio-Rad Laboratories. Guanidine hydrochloride (GdnHCl) (> 99 %), sodium cyanoborohydride, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 8-anilino-1-naphthalenesulfonate (ANS) and o-phthalaldehyde (OPA) were purchased from Sigma. Urea (AR, E. Merck, India) was further crystallized from hot ethanol to remove possible contamination by cyanate ions (Frohlich & Jones, 1987). Bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), and soybean trypsin inhibitor (20.1 kDa) used as marker proteins were obtained from Sigma. All other reagents used were of analytical grade.

The centrifugation was done in a super speed centrifuge (Sorvall Super T-21). Protein lyophilization was carried out in the lyophilizer from Savant Instruments, USA (SNL 108B). The pH of various solutions was measured with pH-meter from Systronic (India). Fraction collector (Redifrac) and peristaltic pump (P-1), used for chromatographic experiments were from Pharmacia. Exact dry weights of several compounds were measured by Metler AE 240 analytical balance. Constant and variable temperature bath used was from Techne.
2.2. Methods

2.2.1. Protein purification

Native ConA was purified from the seeds of jack bean (Canavalia ensiformis) by slight modification of the published procedure (Agrawal & Goldstein, 1967) using Sephadex G-100 as an affinity matrix.

The procedure is as follows:

Jack bean seeds (50 g) was soaked in 250 ml of PBS (10 mM sodium phosphate containing 0.15 M NaCl, pH 7.2). After soaking overnight, the seeds were crushed in a blender. The resulting slurry was placed for constant stirring on the magnetic stirrer for 3 hours and then filtered through cheese-cloth. The supernatant obtained by centrifugation was brought to pH 4.5 by 1 N HCl, and the precipitates were discarded. The pH of the supernatant was then adjusted to 7.2 by 2 N NaOH and centrifuged. Thereafter the supernatant was 80 % saturated with ammonium sulfate and kept at 4°C overnight. The precipitate collected by centrifugation was dissolved in minimum volume of PBS and dialyzed extensively against PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2. The lectin was purified by affinity chromatography at room temperature on Sephadex G-100 column (3.5 x 24 cm) preequilibrated with PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$. After the unabsorbed proteins were removed by extensive washing with the equilibrating buffer, the lectin was eluted from the column with 0.2 M glucose in the same buffer at a flow rate of 20 mL / h. Fractions of 4 mL were collected in tubes in the automatic fraction collector and examined for protein by measuring absorbance at 280 nm. The lectin fractions were pooled, made 80 % saturated with ammonium sulfate and kept at 4°C overnight. After centrifugation, the precipitate was dissolved and dialyzed extensively against...
distilled water. Finally, the purified protein was lyophilized and stored at 4°C. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970).

2.2.2. Chemical modification

Preparation of N-dimethyl ConA

Reductive methylation of ConA was carried out to prepare its N-dimethyl derivative according to the procedure described (Khan et al., 1991). ConA (100 mg) was dissolved in 7 mL of 0.05 M HEPES buffer containing 1 mM Mn²⁺ and 1 mM Ca²⁺, pH 7.2. To this solution, sodium cyanoborohydride (10 mg) was added, followed by 15 μL aliquots of 3.7% (v/v) formaldehyde at 10 min interval over a period of 1 h. The reaction mixture was slowly stirred at 25°C for 1 h. The protein solution was then thoroughly dialyzed against water, lyophilized and subjected to a second cycle of the above described steps. Finally, the N-dimethyl derivative was affinity purified using Sephadex G-100 as affinity matrix.

Preparation of acetyl-ConA

Acetyl-ConA was prepared as described (Mandal & Brewer, 1993). ConA (50 mg) was dissolved in saturated sodium acetate (pH 8.3; 5 mL) and cooled in an ice bath. To this protein solution, 40 μL aliquots of acetic anhydride was added with stirring at 10 min interval over a period of 40 minutes. The mixture was stirred for further 40 min in the ice bath. The resulting solution was then extensively dialyzed against water, lyophilized and subjected to a second derivatization. The crude acetyl-ConA was purified by affinity chromatography on Sephadex G-
100 column. The dimeric derivative was finally obtained by gel filtration on Bio Gel P-100 in PBS containing 0.1 mM Mn²⁺ and 0.1 mM Ca²⁺, pH 7.2.

Preparation of succinyl-ConA

Succinyl-ConA was prepared by modification of the method of Gunther et al., 1973. ConA (50 mg) was dissolved in saturated sodium acetate (pH 8.3; 5 mL) and cooled in an ice bath. To this solution, 30 mg of succinic anhydride was added and the mixture was stirred for 40 min in the ice bath. The resulting solution was then extensively dialyzed against water, lyophilized and subjected to a second cycle of the above steps. The crude succinyl-ConA was subjected to affinity chromatography on Sephadex G-100 column and the dimeric succinyl derivative was obtained by gel filtration on Bio Gel P-100 in PBS containing 0.1 mM Mn²⁺, and 0.1 mM Ca²⁺ pH 7.2.

2.2.3. Determination of extent of modification

The extent of chemical modification for each derivative was determined by TNBS (2,4,6-trinitrobenzenesulfonic acid) method (Habeeb, 1966). To 0.5 mL of protein solution (0.5 - 1 mg / mL) in 0.2 M NaCl was added 0.5 mL of 4 % sodium bicarbonate, pH 8.5, and 0.5 ml of 0.1% TNBS in water. The mixture was incubated at 40°C for 2 hours. Then 0.5 ml 10 % SDS was added to solubilize the protein and prevent its precipitation on addition of 0.25 mL of 1 N HCl. The absorbance of the solution was measured at 335 nm against a blank treated as above but with 0.5 mL of 0.2 M NaCl instead of the protein solution.
2.2.4. Determination of protein concentration

The concentration of native ConA was determined spectrophotometrically at 280 nm using $A^{1\%1\text{ cm}} = 13.7$ at pH 7.2 and $A^{1\%1\text{ cm}} = 12.4$ at pH 5.2, and expressed in terms of monomer ($M_r = 26\ 000$) (Goldstein & Poretz, 1986).

The concentrations of N-dimethyl ConA, acetyl-ConA and succinyl-ConA were measured spectrophotometrically at 280 nm using $A^{1\%1\text{ cm}} = 13.7$ at pH 7.2 (Gunther et al., 1973) or with Folin-Ciocalteu reagent according to the procedure described by Lowry et al., 1951 using bovine serum albumin as standard. The concentrations are expressed in terms of monomer ($M_r = 28\ 000$) (Gunther et al., 1973).

Absorption spectroscopy was performed on a Hitachi U 3210 UV-VIS spectrophotometer using Sigma cuvette (volume: 2 mL; pathlength: 1cm).

2.2.5. Determination of denaturant concentration

The concentrations of stock solutions of denaturants - urea and GdnHCl, were determined by dry weight or by refractive index measurements as described (Pace, 1986).

Based on dry weight, 8 M urea solution required 75.5 g of urea per 100 mL of water, and 6 M GdnHCl solution required 100.9 g of GdnHCl per 100 mL of water.

Alternatively, the concentration (molarity) of urea solution was calculated from the expression: $117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3$ and that of GdnHCl solution from $57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3$ where $(\Delta N)$ is the difference between the refractive index of the denaturant solution and water at the sodium D line.
2.2.6. Denaturation experiments

The denaturation experiments for native ConA tetramer in urea and GdnHCl were carried out in PBS (pH 7.2) at 25°C. For urea denaturation experiment, a known amount of PBS was mixed with a fixed amount of the protein stock solution and varying amounts of 8 M urea solution (both in PBS) in a final volume of 2 mL. A series of tubes containing the mixtures, in duplicate, were incubated at 25°C for 18 h to ensure that the equilibrium was achieved. The spectroscopic measurements to follow unfolding were made on these solutions having different urea concentrations. The protein concentrations were in the range of 0.4 - 2.0 μM. For GdnHCl denaturation, the experimental solutions were prepared in tubes by mixing a known amount of PBS, a fixed amount of protein stock solution and varying amounts of 6 M GdnHCl solution in a final volume of 2 mL. The solutions were incubated at 25°C for 18 h to ensure equilibrium and spectroscopic measurements were made on each solution.

The denaturation experiments for ConA dimer in urea and GdnHCl were carried out in 20 mM sodium acetate buffer containing 0.15 M NaCl, pH 5.2 at 25°C. The experimental solutions were prepared as described above in acetate buffer, pH 5.2. The denaturation experiments for N-dimethyl ConA, acetyl-ConA and succinyl-ConA in urea were carried out similarly in PBS (pH 7.2) at 25°C.

2.2.7. Renaturation experiments

Renaturation of the tetrameric ConA, after complete denaturation of 40 μM protein in 8 M urea or 6 M GdnHCl, was initiated by 20 to 100-fold dilution with reconstitution buffer (PBS containing 0.1 mM Mn²⁺ and 0.1 mM Ca²⁺, pH 7.2) to a residual denaturant concentration of
0.08-0.4 M urea or 0.06-0.3 M GdnHCl. The final protein concentration during renaturation was 0.4-2.0 \mu M. Immediately after dilution, the mixtures were vigorously stirred in a vortex mixer, and a series of tubes containing the mixtures, in duplicate, were incubated at 25°C for different periods of time up to a total time of 24 h. The process of reconstitution was examined with solutions at defined times of renaturation by intrinsic fluorescence, ANS binding, far-UV CD and activity assay.

The renaturation experiments for dimeric ConA, after denaturation in 8 M urea or 6 M GdnHCl in acetate buffer, pH 5.2 were carried out as described above by dilution with acetate buffer containing 1 mM Mn$^{2+}$ and 1 mM Ca$^{2+}$ at pH 5.2. The reconstitution experiments for N-dimethyl ConA, acetyl-ConA and succinyl-ConA at 25°C, after denaturation in urea, were designed similarly using PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2 as the renaturation buffer.

2.2.8. Fluorescence spectroscopy: Intrinsic fluorescence measurements

Fluorescence spectroscopy was performed on a Hitachi 4010 spectrofluorimeter equipped with a constant temperature cell holder. The spectra were measured at 25°C using Sigma fluorimeter cuvette (volume: 2 mL; pathlength: 1 cm). 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 band pass was 5 nm each and the scan rate was 60 nm / min. The intrinsic fluorescence measurements for the urea and GdnHCl-induced denaturation of tetrameric ConA and dimeric ConA were made in PBS (pH 7.2) and acetate buffer (pH 5.2), respectively. The spectra were corrected for the buffer containing requisite concentrations of
denaturant. The fluorescence measurements for the urea-induced denaturation of chemically modified derivatives (N-dimethyl ConA, acetyl-ConA and succinyl-ConA) were made similarly in PBS (pH 7.2). Relative change (\%) of emission wavelength maximum was calculated on the basis of the change of wavelength maximum between the native and the unfolded state taken as 100 \%. Relative change of fluorescence intensity at 336 nm was determined as percent change relative to the fluorescence intensity of the native protein in absence of denaturant.

The fluorescence measurements for reconstitution of tetrameric ConA were carried out in PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2 and those for dimeric ConA in acetate buffer containing 1 mM Mn$^{2+}$ and 1 mM Ca$^{2+}$, pH 5.2. The spectra were corrected for the buffer containing residual concentrations of denaturant present during renaturation. The measurement for the renaturation of chemically modified derivatives of ConA were done in a similar manner at pH 7.2. Relative change of fluorescence intensity at 336 nm during renaturation was determined as described above.

2.2.9. Fluorescence spectroscopy: Use of ANS as an external probe

For the urea and GdnHCl-induced denaturation of ConA and its derivatives, ANS-binding experiments were performed in the presence of 2 μM protein, 50 μM ANS, and various concentrations of denaturant at 25°C. The fluorescence spectra of the samples were measured with an excitation wavelength of 370 nm, and the emission scanned from 450 - 500 nm. The excitation and emission band pass was 5 nm each and the scan rate was 60 nm / min. The fluorescence intensities measured at various concentrations of denaturant at 470 nm were plotted as a function of the denaturant concentration.
For the renaturation studies, the ANS fluorescence spectra were measured in the presence of 0.8 μM protein and 50 μM ANS at various times during reconstitution. The ANS fluorescence intensity at 470 nm as a function of time during reconstitution of ConA and its derivatives was determined.

### 2.2.10. Circular dichroism (CD) spectroscopy

The far-UV CD spectra were measured on a JASCO J-720 spectropolarimeter purged with N₂, and equipped with a constant temperature cell holder. The buffer used in the denaturation experiments was PBS (pH 7.2) for ConA tetramer and acetate buffer (pH 5.2) for ConA dimer. The spectra were measured at 25°C in 1 mm pathlength cell using a scan speed of 20 nm/min with a response time of 2 s, and averaged over five scans to eliminate signal noise. The data are represented as the mean residue ellipticity \([\theta]\), which is defined as \([\theta] = 100\theta_{\text{obs}}/l(c)\), where \(\theta_{\text{obs}}\) is the observed ellipticity in degrees, \(l\) is the length of the light path in centimeters, and \(c\) is the concentration in moles of residue per liter. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions.

The far-UV CD spectra during renaturation of tetrameric and dimeric ConA were measured at defined times in appropriate renaturation buffer at 25°C as described above.

### 2.2.11. Size-exclusion chromatography

The size-exclusion chromatography of native tetrameric ConA was performed on Bio-Gel P-100 at pH 7.2. ConA was loaded onto a Bio-Gel P-100 column (1.1 × 100 cm) which was
equilibrated with PBS containing 0.1 mM Mn²⁺ and 0.1 mM Ca²⁺ at room temperature.

Fractions of 2 ml were collected at a flow rate of 12 mL / h and monitored for protein at 280 nm. The column was precalibrated with the following marker proteins: bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), and soybean trypsin inhibitor (20.1 kDa). To determine the size of the intermediate species in presence of denaturant, size-exclusion chromatography on Superdex 75 was performed. Tetrameric ConA denatured in 2.7 M urea was loaded onto a Superdex 75 column (1.1 x 19 cm) which was equilibrated with PBS containing 2.7 M urea. The flow rate was 6 mL / h and the fraction size was 0.8 mL. The protein content in the fractions, after dilution with buffer, was monitored at 280 nm. The Superdex column was precalibrated in presence of urea with the marker proteins mentioned above. The chromatography of fully denatured protein in 8 M urea was also performed. As the native ConA binds to the Superdex column, the elution behavior of native ConA on Superdex 75 was examined in presence of 0.2 M glucose in PBS after equilibration of the column with the same buffer.

The dimeric structure of Con A at pH 5.2 was verified from gel filtration on Bio-Gel P-100 column (1 x 75 cm) which was pre-equilibrated with 20 mM sodium acetate buffer containing 0.15 M NaCl, 1 mM Mn²⁺ and 1 mM Ca²⁺, pH 5.2. Fractions of 2 mL were collected at a flow rate of 10 mL / h and monitored for protein at 280 nm. The size of the intermediate species in presence of 2.1 M GdnHCl was determined by size-exclusion chromatography also on Bio-gel P-100 column (1 x 50 cm). The fraction size was 1.2 mL and the flow rate 6 mL / h. The protein content in the fractions, after dilution with buffer, was monitored at 280 nm.

The size-exclusion chromatography of chemically modified derivatives (N-dimethyl ConA, acetyl-ConA and succinyl-ConA) was performed on Bio-Gel P-100 at pH 7.2, and the
size of the intermediate species in urea for N-dimethyl ConA and acetyl-ConA was determined by gel filtration on Superdex 75 at pH 7.2 as described before.

2.2.12. Hemagglutination assay

The activity of ConA and its derivatives in native and renatured states was assayed by measuring their ability to agglutinate trypsinized rabbit erythrocytes at pH 7.2.

Preparation of standard trypsinized erythrocyte suspension

Stock blood suspension was made by mixing equal volumes of venous whole rabbit blood and Alsever's solution (2.05 g of glucose, 0.8 g of sodium citrate and 0.42 g of NaCl dissolved in 100 mL of water and brought to pH 6.1 by the addition of solid citric acid) containing 1/30 volume of the anticoagulant (8 g of sodium citrate, 54 mL of 37% formaldehyde and 100 mL of 0.9% NaCl solution), and stored at 4°C. Erythrocytes were collected from stock blood suspension on the day of the assay by centrifugation at room temperature (2000 rpm, 5 min) and washed 3-4 times with PBS, pH 7.2. The washed erythrocytes were suspended in PBS (3% v/v). To this suspension was added 1/10 volume of 1% trypsin solution and the mixture was incubated at 37°C for 1 h. The trypsinized erythrocytes were washed 3-4 times with PBS, and finally suspended in PBS to give a 3% (v/v) suspension of trypsinized erythrocytes.

Hemagglutinating activity

The hemagglutinating activity of the lectin in native and renatured states was assayed by the 2-fold serial dilution technique (Osawa & Matsumoto, 1972) in PBS containing 0.1 mM Mn$^{2+}$ and 0.1 mM Ca$^{2+}$, pH 7.2. The assay was done in microtiter plates using 25 μL of lectin
solution and 25 µL of 3 % suspension of trypsin-treated rabbit erythrocytes in each well. The 2-fold serial dilution was done as follows: 25µl buffer was added to 25 µl protein solution in a well, mixed and then 25 µl of this diluted solution was transferred to the next well and the process was repeated. The protein concentrations for lectin and its derivatives range from 16 to 26 µg / mL in the first well. The mixture was shaken and allowed to stand overnight at room temperature. The agglutination in each well was estimated visually and the minimal hemagglutinating concentration of lectin was determined.

2.2.13. Quantitative analysis of lectin activity during reconstitution using a combination of affinity technique and OPA method of protein estimation

The activity of the reconstituting protein at various times was determined by affinity binding. The fraction of bound as well as unbound protein present in the renaturing solution was estimated relative to the sample of native protein which gave the percent recovery of activity during reconstitution.

**Affinity binding**

A series of syringe columns with Sephadex G-100 as affinity matrix was prepared. For the reactivation experiments with tetrameric ConA at pH 7.2, each column was equilibrated with PBS containing 0.1 mM Mn²⁺ and 0.1 mM Ca²⁺, and 1 mL aliquot of the reconstituting protein solution (20 µg / mL) was loaded onto it after different time intervals during reconstitution. The unbound protein was eluted within 2 min and quickly subjected to protein quantitation by OPA fluorimetric method. The protein which was bound to the affinity matrix was also eluted by 0.2 M glucose in PBS and quantitated. A control experiment using native tetrameric ConA was
performed which showed that the native protein was completely bound to the affinity column under the same conditions.

The reactivation studies with the dimeric ConA in 20 mM acetate buffer containing 0.15 M NaCl, 1 mM Mn$^{2+}$ and 1 mM Ca$^{2+}$, pH 5.2 were carried out similarly.

*o*-Phthalaldehyde (OPA) fluorescent method for protein quantitation

The quantitation of the unbound and bound protein present in the reconstituting solution was done by OPA fluorimetric method as described (Peterson, 1983), which can be used to quantitate as little as 100 ng of intact protein. Briefly, the procedure is as follows.

Stock OPA solution was first prepared by dissolving 120 mg of OPA in 1.5 mL of ethanol which was then added to 100 mL of 1 M borate buffer, pH 10.4 followed by addition of 0.6 mL of 30 % Brij 35. The solution was stored at room temperature up to 3 weeks. The OPA reagent was prepared at least 30 min prior to use by adding 3 µL of 2-mercaptoethanol per 1 mL aliquot of the stock OPA solution.

The protein solution (100 µL) was mixed with the OPA reagent (100 µL). After 15 min, 2 mL of 0.5 M NaOH was added and fluorescence was measured at 430 nm relative to that of a reagent blank with an excitation wavelength of 340 nm. The quantitation of protein was made from a linear calibration curve for native protein.