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Stability Study on Lectin from *Dolichos purpureus* Seeds by Means of Fluorescence and Circular Dichroism

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The effect of temperature and urea on the stability of *Dolichos purpureus* lectin is presented here. *Dolichos purpureus* lectin completely lost its hemagglutinating activity after being treated at 95 ºC for 0.5 h or with 4 M urea for 2 h. SDS-PAGE showed that heat treatment induced the aggregation of the protein above 75 ºC. Intrinsic tryptophan fluorescence studies and far-UV circular dichroism spectroscopy showed that the molecular backbone of *Dolichos purpureus* lectin began to unfold upon incubation at 75 ºC or with 6 M urea and that *Dolichos purpureus* lectin unfolding with heat and denaturant urea was a two-state mechanism. Furthermore, the secondary structure of *Dolichos purpureus* lectin is predominantly β-sheet (39.1 %) with relatively less α-helical structure (18.4 %). 1,8-Anilinonaphthalene sulfonic acid binding experiments indicated that the probe 1,8-anilinonaphthalene sulfonic acid bound to *Dolichos purpureus* lectin at relatively high temperatures. The present results indicated that *Dolichos purpureus* lectin was greatly resistant to temperature and denaturant like urea and the hemagglutinating activity of *Dolichos purpureus* lectin was greatly associated with the α-helix domain.

Key Words: Lectin, Hemagglutinating activity, Fluorescence, Circular dichroism, 1,8-Anilinonaphthalene sulfonic acid, Stable.

INTRODUCTION

Lectins, the carbohydrate binding proteins of non-immune origin, can specifically and reversibly bind to carbohydrates[1]. They are widespread in nature and have been found in almost all living organisms, ranging from viruses and bacteria to plants and animals where as recognizing molecules they have important applications in a number of biological processes such as cell-cell interactions, signal transduction and cell growth and differentiation[1-3] With more efforts that have been put into the purification and characterization of lectins. It is better known that lectins are of great value in many fields including biochemistry, agriculture and biomedicine, *e.g.*, recently, lectins are also found to be useful in the fractionation of cells for their use in bone marrow transplantation[3-5].

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Dolichos purpureus lectin (DPL) is a glucose/mannose specific glycoprotein isolated from the seeds of legume plant *Dolichos purpureus*, with the carbohydrate content of 1.6 % (w/w) and the isoelectric point at 6.18. It is a heterotetramer with a molecular mass of 155 kD and consists of two types of subunits with apparent masses of 36.8 and 39.8 kDa, respectively[6]. However, DPL has not been studied in detail with respect to the structural aspects. The structural studies here on DPL provide much insight into the relationship between physiological functions and structures of lectins. In fact, the true physiological roles of legume lectins and the corresponding mechanisms are still not well understood, such as the mechanism of lectin inhibition of membrane repair as in lectin-based food poisoning[7].

Fluorescence spectroscopy is an effective method to study the conformational change of proteins in aqueous solution[8]. Another efficient method, the measurement of circular dichroism (CD) is one of the most frequently used methods to study the conformational changes of proteins[9] and estimate the types and amounts of secondary structures[10]. 1,8-Anilinonaphthalene sulfonic acid (ANS) is a hydrophobic probe which can bind to macromolecules through hydrophobic interaction accompanied by the notable increase of fluorescence quantum yield of ANS[11]. In the present work, SDS-PAGE, intrinsic fluorescence and binding of ANS as well as circular dichroism were used to investigate the conformational change of DPL under different conditions. Here, it is reported that DPL is a remarkably stable lectin. The high stability of DPL can be favorable to the study of its characteristics and fulfillment of its potential functions, for example, as candidates for targeted drug delivery or affinity ligands to purify other glycoproteins.

EXPERIMENTAL

Tris(hydroxymethyl)methylamine, urea and bovine serum albumin (BSA) were obtained from Amresco. BCA Protein Assay Kit was from Pierce. Sodium dodecyl sulfate (SDS), glycine, acrylamide and N,N'-methylene-*bis*-acrylamide were from Bio-Rad. 1,8-Anilinonaphthalene sulfonic acid (ANS) was purchased from sigma. All the other chemicals used in this research were of analytical grade and acquired from Chengdu Kelong Chemical Reagents Co. All solutions were prepared in high quality de-ionized water from a Milli-Q water purification system (Millipore Corporation, USA).

Preparation of *Dolichos purpureus* lectin: *Dolichos purpureus* lectin was purified as described previously[6]. The concentration of purified DPL was measured with a BCA kit using BSA as a standard protein.

Assay of hemagglutinating activity: A serial 2-fold dilution of the lectin solution in microtiter plates (25 μL) was mixed with 25 μL of a 2 % (v/v) suspension of rabbit red blood cells in phosphate buffered saline (pH 7.2) at room temperature. The hemagglutinating activity of DPL incubated at different temperatures or with different urea concentrations was assessed after 1 h when the red blood cells in the control well had fully sedimented. Agglutination activity was expressed as the reciprocal of the highest dilution that gave a positive result and was reckoned as one hemagglutination unit[12].

Examination of DPL thermostability by SDS-PAGE: *Dolichos purpureus* lectin of aliquots of 20 µL were treated at 35, 45, 55, 65, 75, 85 and 95 ºC for 0.5 h followed by centrifugation at 10000 rpm for 10 min. The supernatants were transferred to other Eppendorf tubes and the initial Eppendorf tubes were added with 20 µL buffer. All the samples were subjected to SDS-PAGE with the DPL sample at room temperature used as a control.

Fluorescence measurement: Fluorescence measurements were performed on a Hitachi F-4500 spectrophotometer. The spectra of all the solutions were obtained with a 10 mm path length quartz cuvette. For emission spectra the following parameters were used in all experiments: excitation slit width, 5 nm; emission slit width, 5 nm; scan speed, 240 nm/min; response time, 0.5 s. In thermodenaturation, DPL was incubated in the temperature range 25-95 ºC at an increment of 10 ºC for 0.5 h. In urea denaturation, DPL was incubated with urea at 1, 2, 3, 4, 6 or 8 M for 2 h. Intrinsic spectra were recorded between 300 and 400 nm with an excitation wavelength of 295 nm. The fluorescence spectra of ANS were investigated upon incubation with DPL at an appropriate temperature for 0.5 h. The excitation wavelength was at 380 nm and the emission spectra were taken in the range of 400-600 nm.

Circular dichroism measurement: Far-ultraviolet CD spectra were recorded on a model 400 spectropolarimeter from AVIV corporation (USA), using a quartz cell path length of 2 mm and a spectral bandwidth of 1 nm. Each data point of the spectra was an average of three accumulations. In thermal denaturation, the sample was kept for 0.5 h at a specific temperature (25, 35, 45, 55, 65, 75, 85 or 95 ºC) before CD measurement. In urea denaturation, DPL was incubated with urea at various concentrations for 2 h before being measured.

Calculation of secondary structure contents: CD data from samples that were subjected to thermal treatment were analyzed by the method of CONTINLL[13], the protein set being 43 proteins for the temperatures from 25-65 ºC and 43 plus 5 denatured proteins for 85 and 95 ºC. The calculation results of DPL denatured by urea were of no referential value, perhaps because the CD data were not compatible with the analyzing methods.

RESULTS AND DISCUSSION

Effect of heat and urea treatment on the hemagglutinating activity of DPL: The results of thermal denaturation of DPL showed that the hemagglutinating activity was stable upto 55 ºC for 0.5 h and lost its activity by 70 % at 65 ºC and beyond 95 ºC no activity was observed [Fig. 1(A)]. The transition temperature for half lost activity of DPL was at 60 ºC. By comparison, *P. sylvestris* lectin loses all of its hemagglutinating activity at 55 ºC[14] and *Pseudostellaria heterophylla* lectin retains only 50 % of its hemagglutinating activity at 40 ºC[15].

The hemagglutinating activity of DPL decreased as the concentration of urea increased and thoroughly lost at 4 M urea [Fig. 1(B)]. The denaturant urea is known to disturb the three-dimensional conformation and binding sites of lectins by affecting the hydrophobic interactions that play a crucial role[14].

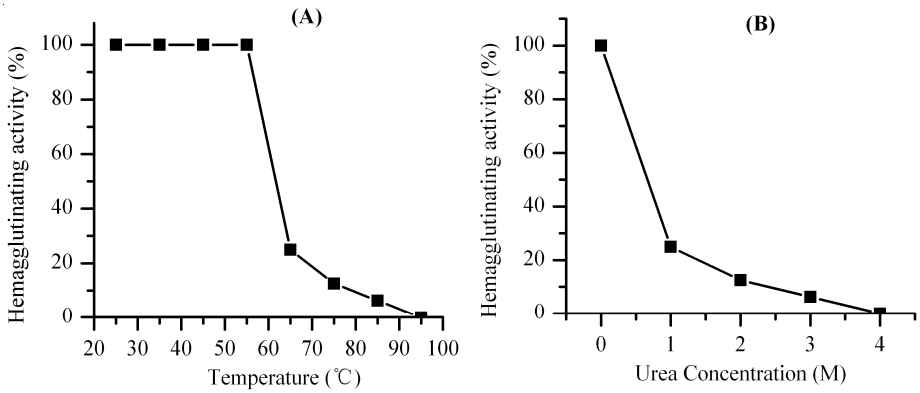

Fig. 1. Effect of temperature or urea on the activity of DPL. (**A**) The hemagglutinating activity of DPL was examined after the sample was incubated at a specific temperature for 0.5 h; (**B**) The hemagglutinating activity of DPL was examined after the sample was incubated with urea of a specific concentration for 2 h

Heat treatment induced the aggregation of DPL molecules: *Dolichos purpureus* lectin is a heterotetramer legume lectin, gave two bands with apparent masses of 36.8 and 39.8 kDa in SDS-PAGE[6]. Heat treatments were done at various temperatures for 0.5 h, then centrifuged and subjected to SDS-PAGE for analysis the supernatants and the pellets. The results showed clearly that DPL was still remained in the supernatants after being treated at temperatures from 35-65 ºC; however, part of it was present in the pellet after being heated at 75 ºC. In contrast, most of it was present in the pellets when DPL was denatured by heating at 85 and 95 ºC [Fig. 2(A)]. These results suggest that under 75 ºC heat treatment, DPL initially denatures and the unfolded protein associates to form soluble aggregates. Eventually, these aggregates associate to form a precipitate on centrifugation. The amounts of soluble and aggregate proteins were also determined [Fig. 2(B)].

Effect of heat and urea treatment on the fluorescence spectra of DPL: As shown in Fig. 3(A), at 25 ºC, native DPL had an intrinsic fluorescence emission maximum of about 330 nm when excited at 295 nm, which indicated that the tryptophan residues were in a predominantly hydrophobic environment[16]. The tryptophan emission maximum was not affected in the temperature range of 35-75 ºC, however, the fluorescence intensity decreased with increasing the temperature. With further increasing the temperature, the tryptophan emission maximum of the protein was red-shifted, reaching 344 nm and the fluorescence intensity decreased further at 95 ºC. This result indicates that the tryptophan residues are gradually exposed to a more hydrophilic environment and DPL is gradually unfolded. Previous study indicated that DPL contains four tryptophan residues with only one located in the surface hydrophobic pocket, which was proved to be not involved in the active center.

It had been proved that the emission peak positions of tryptophan residue could be red shifted and the fluorescence intensity decreased when the microenvironment

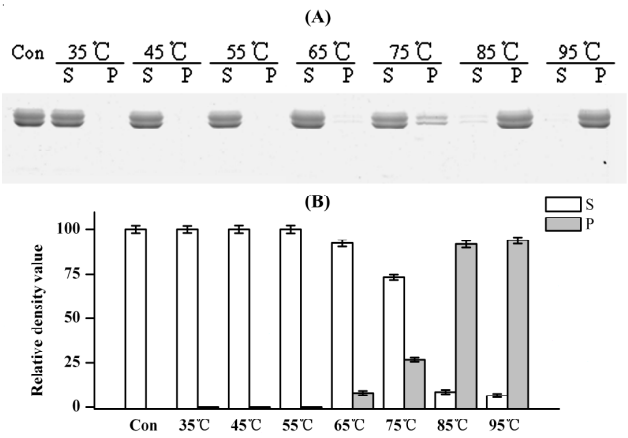

Fig. 2. SDS-PAGE of DPL incubated at various temperatures. DPL of aliquots of 20 μL was incubated at a specific temperature for 30 min and centrifuged at 10000 rpm for 10 min. Con, S and P indicate control, supernatant and precipitation, respectively. The supernatants were transferred to other Eppendorf tubes and the initial Eppendorf tubes were added with 20 μL buffer. All the samples were subjected to SDS-PAGE with the DPL sample at room temperature used as a control

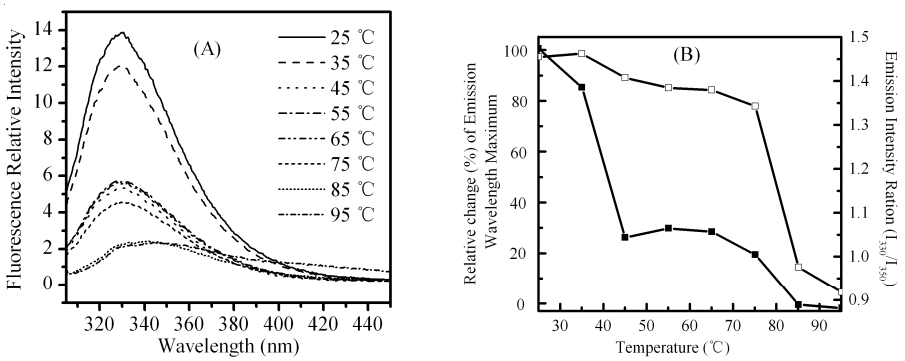

Fig. 3. Intrinsic fluorescence of DPL incubated at various temperatures. (A) The fluorescence spectra of DPL were measured after being incubated at different temperatures for 30 min. The pH 7.8, 20 mM tris-HCl buffer was used. The concentrations of DPL were 0.25 μM; the excitation wavelength was at 295 nm; (B) Heat denaturation curves of DPL measured in terms of relative change of emission wavelength maximum and emission intensity ratio at 330 and 350 nm as a function of increasing temperture

of tryptophan residue was increased in polarity[17,18]. The further decreasing of fluorescence intensity of DPL after treatment at 75 ºC and the decrease of hemagglutinating activity indicated that the active center of DPL altered. After incubation at 85 ºC, the peak position of DPL underwent a red shift of 11 nm accompanied by the loss of major hemagglutinating activity. Treatment at 95 ºC also resulted in a red shift of

peak position to 345 nm with a completely loss of hemagglutinating activity, indicating that heat treatment had effect on the structure and activity of the protein. It is assumed that the thermal denaturation of DPL involved a simple two-state transition [Fig. 3(B)].

The urea denaturation curves for DPL, as monitored by fluorescence, were shown in Fig. 4(A). The fluorescence intensity of DPL declined with the increase of urea concentration. Native DPL had an emission maximum at 331 nm, which shifted to 342 nm on denaturation with 8 M urea. The changes in fluorescence intensity and the red shift of emission maximum suggest that the unfolding of DPL took place and tryptophan residues in DPL were exposed to more polar environment. The denaturation profile [Fig. 4(B)], in terms of the relative change of the fluorescence intensity at 330 nm as a function of urea concentration, suggesting that DPL unfolding is a two-state process. The denaturation curve monitored by fluorescence intensity ratio at 330 and 350 nm supported the conclusion. It should be note that the protein denatured partially in 8 M urea at room temperature in present condition, a higher temperature was needed in order to denature DPL completely, which would had an emission maximum at 350 nm.

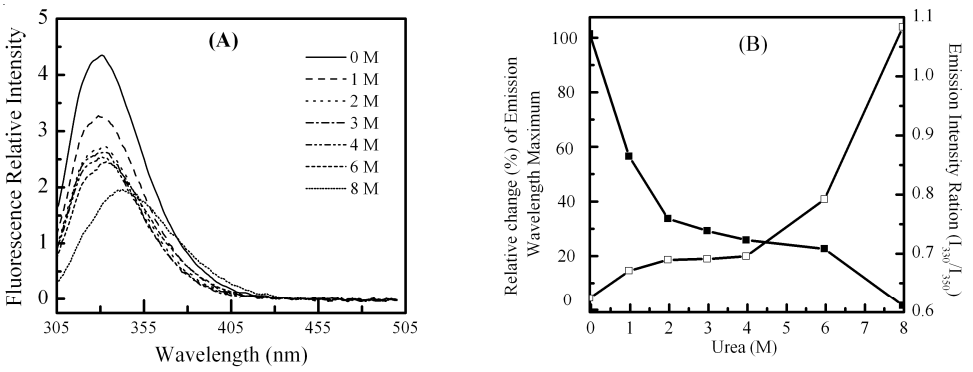

Fig. 4. Intrinsic fluorescence of DPL incubated at different concentration of urea. (A) The fluorescence spectra of DPL were measured after being incubated at different concentrations of urea for 2 h. The concentrations of DPL were 0.13 μM; the excitation wavelength was at 295 nm; (B) Urea denaturation curves of DPL measured in terms of relative change of emission wavelength maximum and emission intensity ratio at 330 and 350 nm as a function of urea concentration

Heat treatment induced the change of 1,8-anilinonaphthalene sulfonic acid fluorescence spectra: The structural changes induced by heat treatment in DPL were also monitored by ANS fluorescence. 1,8-Anilinonaphthalene sulfonic acid is an environment-sensitive fluorescent dye that normally binds to exposed hydrophobic surfaces in partially folded intermediates more tightly than to both the native and random coil states of proteins. Binding to exposed hydrophobic domains in proteins brings about a large increase in 1,8-anilinonaphthalene sulfonic acid fluore-

scence emission and a blue shift of the emission maximum[11]. Fig. 5 showed that 1,8-anilinonaphthalene sulfonic acid binds weakly to the native state of DPL and that there was no observed change both in the fluorescence intensity and the peak positions upon increasing the temperature from 35-75 ºC. However, a 2.0-fold increase in ANS fluorescence intensity together with a blue shift of ANS emission maximum (from 500-475 nm) took place at 85 ºC, indicating the exposure of hydrophobic patches on the protein surface. At 95 ºC, the fluorescence intensity of ANS decreased to a small extent, which may be due to the transformation of the exposed hydrophobic sites to polar environment.

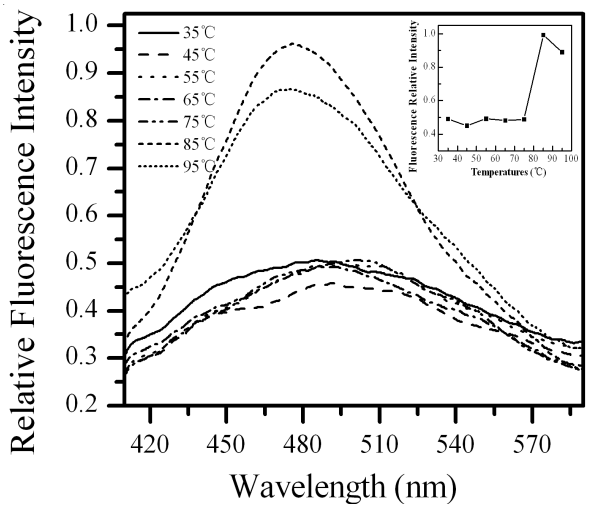

Fig. 5. 1,8-Anilinonaphthalene sulfonic acid fluorescence of DPL incubated at different temperatures. The fluorescence of ANS was measured after ANS and DPL was incubated at a temperature for 30 min. The excitation wavelength was at 380 nm. The concentration of DPL and ANS used were 1.4 and 30 μM, respectively. The peak positions for temperatures from 35-75 ºC are at 500 nm, for 85 and 95 ºC are at 475 nm. Inset, the fluorescence intensity values of ANS at peak positions were plotted against temperature

Effect of heat and urea treatment on the CD spectra of DPL: Far-UV CD spectroscopy was used to measure the changes in secondary structure upon thermal denaturation of DPL (Fig. 6). The native DPL exhibits a far-UV CD double minimum at 216 and 231 nm, which was typical of the protein conformation containing both α-helices and β-strands. Since the typical spectrum of α-helices shows double minima at 208 and 222 nm, the typical spectrum of β-strands shows a single negative band at around 216 nm while that of unordered coils shows a negative band at 200 nm[19]. Therefore, it was assumed that native DPL contains a high content of β-strands, which was confirmed by the calculation results of secondary structures (Table-1) with the content of β-strands of 39.1 % and that of α-helix to be 18.4 %. As a matter of fact, many legume lectins contain a high content of β-strands[20].

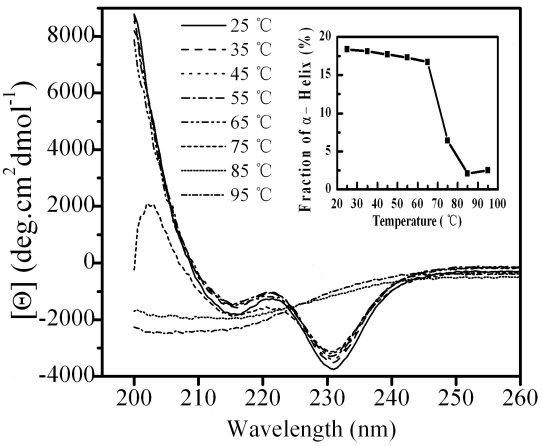

Fig. 6. Far-UV CD spectra of DPL incubated at various temperatures. Far-UV CD spectra of DPL were measured after DPL being incubated at temperatures from 25-95 ºC at an increment of 10 ºC for 0.5 h. The pH 7.8, 20 mM tris-HCl buffer was used. The concentration of DPL was 1.2 μM. Inset, the values of α-helix fraction of DPL were plotted against temperature

TABLE-1
CONTENTS OF THE SECONDARY STRUCTURES OF DPL AT DIFFERENT TEMPERATURES FROM 25-95 ºC, CALCULATED BY THE CONTINLL METHOD[10]

Temperature (ºC)	α-Helix (%)	β-Strand (%)	β-Turn (%)	Unordered coil (%)
25	18.4	39.1	18.9	23.5
35	18.1	39.9	18.6	23.5
45	17.7	40.0	18.4	23.8
55	17.3	39.0	19.0	24.8
65	16.7	38.3	20.1	24.9
75	6.4	40.7	20.0	32.9
85	2.1	43.5	16.7	37.7
95	2.5	42.9	16.5	38.1

DPL was incubated at a specific temperature for 30 min, followed by the measurement of CD spectra. The data then were subjected to the analysis program. The protein set was 43 proteins for the temperatures from 25-75 ºC and 43 plus 5 denatured proteins for 85 and 95 ºC.

As shown in Fig. 6, native DPL showed a notable negative band at 231 nm which was assumed to be attributed to tryptophan residues[21]. With the increase of temperature from 25-65 ºC, the intensity of CD signals and CD spectrum shape changed slightly, indicating that the secondary structures did not change much. However, the spectrum at 75 ºC showed an extra positive band at 202 nm apart from the minima at 216 and 231 nm, which suggested that the secondary structure of DPL had undergone some alteration. The calculation results showed that the contents of α-helices and β-strands were 6.4 and 40.7 %, respectively (Table-1). When denatured at 85 and 95 ºC, the CD spectra of DPL showed the loss of secondary structures for the completely unfolded state of the protein, the calculation results also showed further decrease of α-helix and the increase of unordered coils (Table-1).

As shown in the inset in Fig. 6, it was found that the decrease of α-helices was parallel to the loss of hemagglutinating activity of DPL [Fig. 1(A)]. When incubated at temperatures from 35-55 ºC, DPL maintained its hemagglutinating activity while the content of α-helices almost remained the same. At 75 ºC DPL lost 75 % activity, accompanied by the decrease of α-helices to a great degree. When incubated at 95 ºC, DPL completely lost the hemagglutinating activity with a large range of decrease of α-helices, suggesting that the active center of DPL was greatly associated with its α-helix domain. The profile of change of α-helix content also suggested that the heat induced unfolding of DPL is a two-state process and the transition temperature (Tm) was 70 ºC. The CD and intrinsic fluorescence data are in excellent agreement, although the Tm values from the latter were a few degrees lower.

The urea-induced denaturation curves for DPL were shown in Fig. 7(A). Compared to the native state, the CD shape and signal intensity of DPL treated with 1, 2 and 4 M urea only altered slightly, suggesting the maintenance of its secondary structures. However, denaturation in 6 or 8 M urea resulted in the notable decrease of signal intensity [Fig. 7(B)], indicating that some loss of secondary structures for the partly unfolded state of the protein.

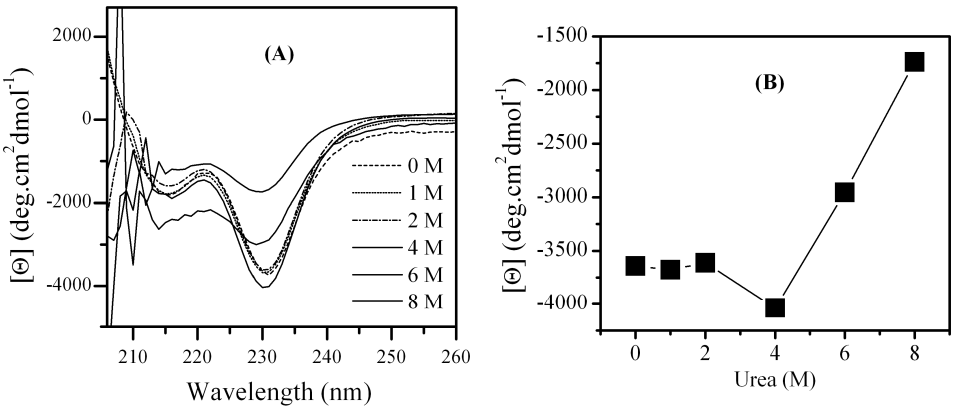

Fig. 7. Far-UV CD spectra of DPL incubated at different concentration of urea. (A) Far-UV CD spectra of DPL were measured after DPL being incubated with different concentrations of urea for 2 h. The pH 7.8, 20 mM tris-HCl buffer was used. The concentration of DPL was 1.2 μM. (B) Urea denaturation profile of EIL as monitored by CD ellipticity at 226 nm. Each data point represents average of two determinations

These results, in combination with the results of fluorescence measurement, indicated that DPL was stable against high concentration of urea, which just like the lectin EIL[22]. The loss of hemagglutinating activity of DPL treated with urea of low concentrations could be explained that urea induced the lysis of rabbit blood erythrocytes and therefore inhibited the detection of hemagglutination. The high

stability of DPL can be favourable to the study of its characteristics and fulfillment of its potential functions, for example, as candidates for targeted drug delivery or affinity ligands to purify other glycoproteins.

In summary, the studies reported here indicate that DPL is a remarkably stable lectin. The results of fluorescence and CD spectral studies demonstrate that the molecular backbone of DPL begins to unfold upon incubation at around 75 ºC or with 6 M urea and that the DPL unfolding with heat and denaturant urea was a two-state mechanism. Furthermore, the secondary structure of DPL is predominantly β-sheet (39.1 %) with relatively less α-helical structure (18.4 %) and the hemagglutinating activity of DPL was greatly associated with the α-helix domain.

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