DENATURATION BEHAVIOR AND CONFORMATIONAL STATES OF CHAMPEDAK GALACTOSE-BINDING LECTIN UNDER VARIOUS DENATURING CONDITIONS

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DENATURATION BEHAVIOR AND CONFORMATIONAL STATES OF CHAMPEDAK GALACTOSE-BINDING LECTIN UNDER VARIOUS DENATURING CONDITIONS

ABSTRACT

Protein denaturation studies of champedak galactose-binding (CGB) lectin were carried out in order to understand its folding mechanism as it occurs *in situ*. Urea denaturation (0−9.0 M urea) of CGB lectin was studied using different spectral probes such as far-UV CD spectral signal, fluorescence intensity, emission maximum and ANS fluorescence intensity. CGB lectin showed a two-step, three-state transition in the presence of urea. The first transition (marked by the increase in the spectral signals) started at ~2.0 M urea and ended at ~4.5 M urea, while the second transition (characterized by the decrease in the spectral signals except emission maximum) showed the start- and the end-points at ~5.75 M and ~7.5 M urea, respectively, when analyzed by the emission maximum probe. Transformation of tetramer into monomer represented the first transition, whereas the second transition reflected the unfolding of monomer. Conformational analysis of the native, 5.0 M urea-denatured and 9.0 M urea-denatured states of CGB lectin was made using far-UV CD, Trp fluorescence and ANS fluorescence spectra. The hemagglutinating activity of the lectin gradually decreased with increasing urea concentrations and was completely lost at ~4.0 M urea. Acid denaturation (pH 7.0−1.0) of CGB lectin was studied using intrinsic fluorescence and ANS fluorescence measurements. The lectin remained stable up to pH 5.0 and showed local disordering in the vicinity of the protein fluorophores within the pH range, 5.0−3.5. Decrease in the pH from pH 3.5 to pH 2.5 led to structural transition, which can be ascribed to the dissociation of the tetrameric lectin into monomeric form. Further

decrease in the pH up to pH 1.5 produced another transition, which specified the unfolding of monomers. Characterization of the conformational states obtained at pH 7.0, pH 2.5 and pH 1.5 based on intrinsic and ANS fluorescence spectra, gel chromatographic behavior and thermal denaturation confirmed the existence of the folded monomeric form at pH 2.5 and the unfolded state at pH 1.5. However, the acid-denatured state of CGB lectin at pH 1.5 retained significant residual structure, as evident from the greater loss of both secondary and tertiary structures in the presence of 6.0 M guanidine hydrochloride at pH 1.5. Anion-induced refolding below pH 1.5 was also evident from the ANS fluorescence results. Thermal denaturation of the acid-denatured states of CGB lectin resulted in formation of non-native β-structures. The influence of different polyols (ethylene glycol, erythritol, xylitol and sorbitol) on the acid-denatured states of CGB lectin at pH 2.5 and pH 1.5 was studied using ANS fluorescence, Trp fluorescence and far-UV CD measurements. The acid-denatured states of CGB lectin were stabilized against thermal denaturation in the presence of erythritol, xylitol and sorbitol as reflected by the shift of the thermal transition curves towards higher temperatures. On the other hand, ethylene glycol destabilized these states by shifting the thermal transition curves towards lower temperatures. Presence of erythritol, xylitol and sorbitol in the incubation mixture was found to stabilize the CGB lectin at both pH 2.5 and pH 1.5, as evident from the burial of the hydrophobic clusters and decreased polarity around Trp residues. Based on the spectral data, both sorbitol and erythritol appeared to exude relatively better stabilizing effect towards the secondary and tertiary structures of the acid-denatured states of CGB lectin. On the other hand, ethylene glycol was shown to destabilize it.

Keywords: champedak galactose-binding lectin, protein denaturation, polyols, circular dichroism, fluorescence spectroscopy

TABIAT PENYAHASLIAN DAN KEADAAN KONFORMASI LEKTIN PENGIKAT GALAKTOSA CHAMPEDAK DALAM PELBAGAI KONDISI PENYAHASLIAN

ABSTRAK

Kajian penyahaslian protein bagi lektin pengikat galaktosa champedak (lektin CGB) telah dijalankan untuk memahami mekanisme perlipatan *in situ* lektin tersebut. Penyahaslian urea (0−9.0 M urea) lektin CGB telah dikaji dengan menggunakan pelbagai kuar berlainan seperti isyarat spektra dikroisme bulatan ultralembayung jauh, keamatan pendarfluor, kepancaran maksimum dan kependarfluoran ANS. Lektin CGB telah didapati menghasilkan peralihan dua-langkah dan tiga-keadaan dalam kehadiran urea. Peralihan yang pertama (bercirikan peningkatan isyarat-isyarat spektra) bermula pada ~2.0 M urea dan tamat pada ~4.5 M urea, manakala peralihan yang kedua (bercirikan penurunan isyarat-isyarat spektra kecuali kepancaran maksimum) bermula pada ~5.75 M urea dan tamat pada ~7.5 M urea apabila dianalisa menggunakan kuar kepancaran maksimum. Transformasi tetramer kepada monomer mencirikan peralihan yang pertama, manakala peralihan yang kedua menggambarkan pemaparan monomer. Analisis konformasi bagi lektin CGB asli, ternyahasli-5.0 M urea dan ternyahasli-9.0 M urea telah dilaksanakan menggunakan isyarat spektra dikroisme bulatan ultralembayung jauh, kependarfluoran intrinsik dan kependarfluoran ANS. Aktiviti hemaglutinan lektin tersebut berkurangan secara beransur-ansur dengan peningkatan kepekatan urea dan akhirnya hilang pada ~4.0 M urea. Penyahaslian asid (pH 7.0−1.0) terhadap lektin CGB telah dikaji dengan menggunakan isyarat spektra keamatan pendarfluor dan kependarfluoran ANS. Lektin tersebut kekal stabil sehingga pH 5.0 dan menunjukkan kecelaruan setempat dalam lingkungan fluorofor protein pada julat pH 5.0−3.5. Penurunan pH dari pH 3.5 ke pH 2.5 menyebabkan peralihan struktur, iaitu penceraian tetramer lektin kepada bentuk monomer. Pencirian keadaan konformasi pada pH 7.0,

pH 2.5 dan pH 1.5 berdasarkan kependarfluoran intrinsik, kependarfluoran ANS, tabiat kromatografi gel dan penyahaslian terma telah mengesahkan kewujudan bentuk monomer terlipat pada pH 2.5 dan bentuk termapar pada pH 1.5. Walau bagaimanapun, lektin CGB ternyahasli-asid pada pH 1.5 mengekalkan struktur sisa yang ketara, seperti yang terlihat pada kehilangan struktur sekunder dan tertier yang lebih besar dengan kehadiran 6.0 M guanidin hidroklorida pada pH 1.5. Perlipatan semula teraruh-anion bawah pH 1.5 juga telah dikesan daripada kependarfluoran ANS. Penyahaslian terma bagi lektin CGB ternyahasli-asid telah menghasilkan pembentukan struktur β yang tidak asli. Pengaruh poliol yang berbeza (etilena glikol, eritritol, xilitol dan sorbitol) kepada lektin CGB ternyahasli-asid pada pH 2.5 dan pH 1.5 telah dikaji pada pH 2.5 dan pH 1.5 menggunakan isyarat spektra kependarfluoran ANS, kependarfluoran Trp dan dikroisme bulatan ultralembayung jauh. Lektin CGB ternyahasli-asid lebih stabil ketika penyahaslian terma dengan kehadiran eritritol, xilitol dan sorbitol, seperti yang digambarkan oleh anjakan keluk peralihan terma ke arah suhu yang lebih tinggi. Namun begitu, etilena glikol telah mengurangkan kestabilan keadaan lektin CGB tersebut dengan menganjak keluk peralihan terma ke arah suhu yang lebih rendah. Kehadiran eritritol, xilitol dan sorbitol dalam campuran pengeraman telah didapati menstabilkan lektin CGB pada pH 2.5 dan pH 1.5, seperti yang terlihat pada penimbusan kluster hidrofobik dan pengurangan polariti dalam lingkungan residu Trp. Berdasarkan data spektra tersebut, sorbitol dan eritritol kedua-duanya tampak memberi kesan penstabilan lebih baik kepada struktur sekunder dan tertier lektin CGB ternyahasli-asid. Walau bagaimanapun, etilena glikol telah didapati mengurangkan kestabilan lektin ternyahasli-asid tersebut.

Kata kunci: lektin pengikat galaktosa champedak, penyahaslian protein, poliol, dikroisme bulatan, spektroskopi kependarfluoran

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ADL 1.5	Acid-denatured lectin at pH 1.5
ADL 2.5	Acid-denatured lectin at pH 2.5
Ala	Alanine
ANS	1-Anilinonaphthalene-8-sulfonate
AOT	Sodium bis(2-ethylhexyl) sulfosuccinate
Arg	Arginine
Asp	Aspartic acid
a.u.	Arbitrary units
BSA	Bovine serum albumin
CD	Circular dichroism
CGB	Champedak galactose-binding
C_m	Mid-point concentration
CvL	*Cliona varians* lectin
Cys	Cysteine
GdnHCl	Guanidine hydrochloride
GdnSCN	Guanidine thiocyanate
Glu	Glutamic acid
h	Hour
His	Histidine
IgA1	Immunoglobulin A subclass 1
IgA2	Immunoglobulin A subclass 2
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ile	Isoleucine

Leu	Leucine
Lys	Lysine
M	Molar
mAu	Milliabsorbance unit
mdeg	Millidegree
Met	Methionine
min	Minute
mM	Millimolar
M_r	Molar mass
MWCO	Molecular weight cut-off
nm	Nanometer
Phe	Phenylalanine
pM	Picomolar
Pro	Proline
s	Second
sIgA	Secretory immunoglobulin A
Thr	Threonine
T_m	Mid-point temperature
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
w/v	Weight per volume
µM	Micromolar
°C	Degrees Celcius
%	Percentage

1. INTRODUCTION

1.1. Research background

Proteins produced in the cell acquire specific three-dimensional native conformation, which is essential for their biological functioning. This native structure is achieved by rapid folding of the polypeptide chains down the protein-folding funnel, towards a state of lowest free energy (Leopold et al., 1992; Dill & MacCallum, 2012). Protein folding is an intricate process, which must progress correctly for maintaining an organism's functional wellbeing. Given that proteins are only marginally stable and many events may affect the proper protein folding, several diseases such as Alzheimer's, Parkinson's and Creutzfeldt-Jakob disease, to name a few, are found associated with the misfolding of proteins (Chaudhuri & Paul, 2006; Valastyan & Lindquist, 2014). To ensure smooth occurrence of the correct protein folding, molecular chaperones work as guides to prevent aberrant protein folding in cells (Cortez & Sim, 2014). Furthermore, native protein conformation is also protected against stress by the presence of osmolytes in cells, which act to curb formation of misfolded proteins and aggregation, thus reducing the likelihood of disease (Kumar, 2009; Fonin et al., 2016). Treatment of proteins with denaturants may yield stable intermediates, which are believed to be important conformational states formed during the protein folding process occurring *in situ* (Baldwin, 1975; Kuwajima, 1989).

Protein denaturation process involves disruption of the native protein conformation induced by denaturants. These denaturants interfere with both main chain and side chain interactions of the polypeptide chain, which are supposed to maintain the native protein conformation (Tanford, 1968). For multimeric proteins, interchain interactions, in addition to the intrachain interactions, supplement their structural stability and also influence their folding process (Mei et al., 2005; Sakane et al., 2007; Fujii et al., 2013; 2017). Thus, denaturation of multimeric proteins is relatively more

complex, involving separation of the monomers as well as chain unfolding. Despite these challenges, several studies have advanced our knowledge towards understanding the folding and stability of multimeric proteins (Gloss, 2009; Aghera et al., 2011; Doyle et al., 2013; Rastegari et al., 2017).

Plant lectins are carbohydrate-binding proteins, present in all parts of plants and constitute around 5−10% of the total protein content in plants (Peumans & Van Damme, 2005; Van Damme, 2008). They play important roles in many biological functions such as cell-cell communication, pathogenic defence, plant development, organ growth, sugar transport, symbiotic plant-microbe interactions and are essential in regulating plant physiological processes (Sharon & Lis, 1989; Van Damme et al., 1998a; Brill et al., 2001; De Hoff et al., 2009; Yamaji et al., 2012). Plant lectins are grouped into seven families of structurally-related proteins (Peumans et al., 2001). One of the well-studied lectin families is the jacalin-related lectins, which include MornigaM, MornigaG, BanLec, PlanLec, Calsepa, Heltuba, Orysata, etc (Peumans et al., 1997; Van Damme et al., 1999; Peumans et al., 2000; Zhang et al., 2000; Van Damme et al., 2002). Lectins classified under this family possess one or more domains structurally similar to jacalin, a galactose-binding lectin, present in jackfruit seeds (Bunn-Moreno & Campos-Neto, 1981).

Champedak galactose-binding (CGB) lectin is a protein isolated from the seeds of *Artocarpus integer* (Hashim et al., 1991). CGB lectin binds reversibly to galactose and its derivatives (Hashim et al., 1991). In addition, it has also been shown to bind to *O*-glycosylated proteins present in the urine, which has been exploited for early detection of endometrial, ovarian and prostate cancers (Mu et al., 2012; 2013; Jayapalan et al., 2013). CGB lectin shared a high sequence homology to jacalin (Gabrielsen et al., 2014). The three-dimensional structure of CGB lectin has recently been elucidated using X-ray crystallography, which demonstrated its tetrameric structure with

noncovalently-bound monomers (Garbrielsen et al., 2014). Although several studies have been made on the denaturation behavior of different jacalin-related lectins (Campana et al., 2002; Khan et al., 2013; Datta & Swamy, 2017), there is a lack of denaturation data of CGB lectin under various denaturing conditions. Analysis of the denaturation data of CGB lectin would be helpful to understand its folding mechanism and conformational stability.

1.2. Problem statement and research objectives

Considering the above research background, several questions arise in mind:

1. What is the denaturation behavior and binding activity of the CGB lectin under different denaturing conditions?
2. Can the addition of cosolvents protect the conformational stability of the lectin against denaturing conditions?

Therefore, the aim of the present research was to study the denaturation behavior and conformational states of CGB lectin under various denaturing conditions. The following objectives were set in order to answer the above questions:

1. To study the effect of varying urea concentrations on the native conformation of CGB lectin.
2. To characterize the native and urea-denatured states of CGB lectin.
3. To investigate the effect of urea on the hemagglutinating activity of CGB lectin.
4. To study the acid denaturation behavior of CGB lectin and characterize the native and acid-denatured states of CGB lectin.
5. To study thermal denaturation behavior of the acid-denatured CGB lectin.
6. To evaluate the effect of different polyols on the acid-denatured states of CGB lectin.

2. LITERATURE REVIEW

2.1. Lectins

2.1.1. Background

Lectins are a group of ubiquitous carbohydrate-binding proteins with non-catalytic domain, possessing at least one specific sugar-binding site and of non-immune origin (Sharon & Lis, 1972; Goldstein et al., 1980; Peumans & Van Damme, 1995). Lectins have affinities toward specific sugars and bind them reversibly and noncovalently (Peumans & Van Damme, 1995). Due to their sugar-binding ability, lectins are involved in many biological functions such as defence, growth, cell communication and other physiological processes (Sharon & Lis, 1989; Van Damme et al., 1998a; Brill et al., 2001; De Hoff et al., 2009; Yamaji et al., 2012). In addition, plant lectins have also been utilized in various applications such as characterization of glycoconjugates, diagnostics, glycoprotein purification, blood banking, carriers in drug delivery systems, as well as anti-tumor applications (Mody et al., 1995; Gabor et al., 2002; Wu et al., 2009; Fu et al., 2011; Lam & Ng, 2011; Gorakshakar & Ghosh, 2016). Being highly related proteins, plant lectins have been grouped into relatively few lectin families (Van Damme et al., 1998b). Although lectins have been found in many parts of the plant, they constitute ~5−10% of the total protein content in plant seeds (Peumans & Van Damme, 2005; Van Damme, 2008). However, some seed lectins may dominate the total seed protein content, amounting up to 50% or more (Van Damme et al., 1998b).

2.1.2. Distribution and functions

Lectins are widely distributed among different types of organisms, which include bacteria, viruses, yeast, fungi, animals and plants (Gabius, 1997; Van Damme et al., 1998b; Kilpatrick, 2002; Varrot et al., 2013; Nizet et al., 2017). Table 2.1 shows the distribution of lectins across different groups of organisms. As evident from the table, lectins from plants have been widely explored compared to other organisms, possibly

Table 2.1: Distribution of lectins among various organisms.

Lectin	Source/Location	Reference
Bacterial lectins		
Pertussis toxin	*Bordetella pertussis*	Toomey & McClelland (1933)
Cholera toxin	*Vibrio cholera*	Holmgren et al. (1975)
PapG	*Escherichia coli*	Lund et al. (1985)
FimH	*Escherichia coli*	Krogfelt et al. (1990)
BabA	*Helicobacter pylori*	Ilver et al. (1998)
LecA (PA-II)	*Pseudomonas aeruginosa*	Winzer et al. (2000)
LecB (PA-III)		
SabA	*Helicobacter pylori*	Mahdavi et al. (2002)
Viral lectins		
Capsid protein	Foot-and-mouth disease virus	Loeffler & Frosch (1898)
	Polyoma virus	Stehle et al. (1994)
Envelope protein	Dengue virus	Ashburn & Caraig (1907)
Hemagglutinin-neuraminidase	Newcastle disease virus	Mountcastle et al. (1971)
Agglutinin	Influenza viruses	Rogers & Paulson (1983); Rogers et al. (1986)
Yeast lectins		
a-Factor	*Saccharomyces cerevisiae*	Terrance & Lipke (1981)
α-Factor		

Table 2.1, continued.

Lectin	Source/Location	Reference
α-Agglutinin	*Pichia amethionina*	Mendonça-Previato et al. (1982)
Adhesin	*Candida albicans*	Critchley & Douglas (1987)
Kb-CWL I Kb-CWL II	*Kluyveromyces bugaricus*	Al-Mahmood et al. (1988)
Cell wall proteinic receptor	*Kuyveromyces lactis*	El-Behhari et al. (1998)
Adhesin	*Candida glabarata*	Cormack et al. (1999)
Paracoccin	*Paracoccidioides brasiliensis*	Coltri et al. (2006)
Fungal lectins		
ABL	*Agaricus bisporus*	Ahmann & Sage (1974)
Pallidin	*Polysphondylium pallidum*	Simpson et al. (1975)
Bolesatine	*Boletus satanas*	Kretz et al. (1989)
SRL	*Sclerotium rolfsii*	Wu et al. (2001)
AAL	*Agrocybe aegerita*	Chenguang et al. (2003)
XCL	*Xerocomus chrysenteron*	Trigueros et al. (2003)
CGL2	*Coprinopsis cinerea*	Walser et al. (2004)
BEL	*Boletus edulis*	Bovi et al. (2011)
Animal lectins		
L-type lectins	Endoplasmic reticulum, ER-Golgi intermediate compartment, Golgi	Arar et al. (1995); Bottazzi et al. (2006)

Table 2.1, continued.

Lectin	Source/Location	Reference
Intelectins (X-type lectins)	Extracellular, cell membrane	Tsuji et al. (2001)
Siglecs (I-type lectins)	Cell membrane	Angata & Brinkman-Van ver Linden (2002)
P-type lectins	Secretory pathway	Dahms & Hancock (2002)
C-type lectins	Cell membrane, extracellular	Zelensky & Gready (2005)
Calnexins	Endoplasmic reticulum	Ireland et al. (2006)
R-type lectins	Golgi, cell membrane	Plüddemann et al. (2006)
Galectins (S-type lectins)	Cytoplasm, extracellular	Elola et al. (2007)
Plant lectins		
Abrin	Rosary pea	Martin (1887)
Ricin	Castor bean	Osborne et al. (1905)
Concanavalin A	Jack bean	Sumner (1919)
Wheat germ agglutinin	Wheat germ	Aub et al. (1965)
Pokeweed mitogen	*Phytolacca americana*	Reisfeld et al. (1967)
DBA	*Dolichos biflorus*	Etzler & Kabat (1970)
Soybean agglutinin	Soybean	Gordon et al. (1972)
Peanut agglutinin	Peanut	Trowbridge (1974)
Favin	Broad bean	Wang et al. (1974)

Table 2.1, continued.

Lectin	Source/Location	Reference
Frutalin	Breadfruit seed	Lotan et al. (1975)
Modeccin	*Adenia digitata*	Gasperi-Campeni et al. (1978)
Gelonin	*Gelonium multiflorum*	Stirpe et al. (1980)
Jacalin	Jackfruit seed	Bunn-Monero & Campos-Neto (1981)
Viscumin	Mistletoe	Olsnes et al. (1982)
GS4	*Griffonia simplicifolia*	Shibata et al. (1982)
Volkensin	*Adenia volkensii*	Barbieri et al. (1984)
PHA-L	Red kidney bean	Pineau et al. (1990)
Pea lectin	Pea	Koshte et al. (1990)
CGB lectin	*Artocarpus integer*	Hashim et al. (1991)
Artocarpin	Jackfruit seed	Misquith et al. (1994)
Calsepa	Hedge bindweed	Peumans et al. (1997)
CMB lectin	*Artocarpus integer*	Lim et al. (1997)
Heltuba	Jerusalem artichoke	Van Damme et al. (1999)
BanLec	Banana	Peumans et al. (2000)
Orysata1 Orysata2	Rice	Zhang et al. (2000)
PlanLec	Plantain	Rabijns et al. (2001)

Table 2.1, continued.

Lectin	Source/Location	Reference
MornigaM	Black mulberry tree bark	Van Damme et al. (2002)
MornigaG	Black mulberry tree bark	Van Damme et al. (2002)

due to the higher yield of plant lectins compared to other lectins (Lam & Ng, 2011). Lectins play different roles in different types of organisms. For example, microbial surface lectins function primarily to attach microorganisms to the surface of the host cells through binding to glycoproteins and glycolipids of the host cell membrane to initiate infection (Nizet et al., 2017). Animal lectins, on the other hand, are involved in the immune system, cell adhesion, cellular communication, regulation of protein levels, etc. (Spiess, 1990; Kilpatrick, 2002). Plant lectins help in germination, resistance against pathogens, rhizobial attachment in symbiotic plant-microbe interactions and sugar transport (Van Damme et al., 1998a; De Hoff et al., 2009).

2.1.3. Isolation and purification

Lectin purification procedures involve either a single step or multiple steps. Table 2.2 lists a few methods involved in the isolation and purification of various lectins from different sources. As seen from the table, single-step procedures involve affinity chromatography, whereas multiple-step purification might involve protein precipitation and various chromatographic techniques. The protein is often precipitated from the crude extract by salt (e.g. ammonium sulfate) fractionation or using polar / nonpolar solvents such as acetic acid, ethanol, acetone and polyethylene glycol. Alternatively, thermal and isoelectric precipitations have also been employed for protein precipitation (Burgess, 2009). Precipitation step is followed by various chromatographic procedures, such as affinity chromatography, ion exchange chromatography, hydrophobic chromatography and gel chromatography.

Among different isolation procedures listed in Table 2.2, affinity chromatography is widely employed as a method to purify lectins. Ligands used for immobilization to the stationary phase of the affinity column are often sugars such as mannose and galactose or glycoproteins such as mucin (Lis & Sharon, 1981; Pohleven et al., 2012). Additionally, lectins such as concanavalin A and *Cratylia mollis* seed

Table 2.2: Isolation and purification of various lectins.

Lectin	Method of purification	Protein recovery (%)	Reference
Fungal lectins			
Macrophomina phaseolina lectin	Affinity chromatography (Fetuin-Sepharose 4B) + Ion exchange chromatography (DEAE Sephadex A-50)	3.3	Bhowal et al. (2005)
Fusarium solani lectin	Hydrophobic chromatography × 2 (Phenyl-Sepharose) + Gel chromatography (Sephacryl S-200)	26.0	Khan et al. (2007)
Ganoderma lucidum lectin	Ammonium sulfate precipitation (0−30%) + Hydrophobic chromatography (Phenyl-Sepharose)	35.7	Thakur et al. (2007)
Inocybe umbrinella lectin	Ammonium sulfate precipitation (80%) + Ion exchange chromatography (DEAE-Cellulose) + Ion exchange chromatography (CM-Cellulose) + Gel chromatography (Superdex 75)	0.07	Zhao et al. (2009)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Aspergillus pamanensis lectin	Affinity chromatography (Mucin-Sepharose 4B)	8.4	Singh et al. (2015)
Yeast lectins			
Candida albicans cell wall adhesin	Affinity chromatography (Synsorb H-2) + Desalting (Sephadex G-25)	-	Tosh & Douglas (1992)
Candida albicans fimbrial adhesin	HPLC (Waters Protein-PAK 300 SW) + Reversed phase HPLC (C_4)	-	Yu et al. (1994)
Animal lectins			
Sarcophaga peregrine larvae hemolymph lectin	Affinity chromatography (Sepharose 4B)	-	Komano et al. (1980)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Grass carp lectin	Affinity chromatography (Rhamnose-Sepharose 6B) + Ion exchange chromatography (Mono S)	0.01	Lam & Ng (2002)
Serpula vermicularis (sea worm) lectin	Ion exchange chromatography (DEAE-Toyopearl) + Affinity chromatography (Cross-linked ovalbumin) + Gel chromatography (Sephadex G-150)	0.2	Molchanova et al. (2007)
Holothuria scabra (sea cucumber) lectin	Ultrafiltration (10 kDa membrane) + Hydrophobic chromatography × 2 (Phenyl-Sepharose)	1.9	Gowda et al. (2008)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Bighead carp lectin	Ion exchange chromatography (DEAE-Sepharose FF) + Gel chromatography (Sephacryl S-200 HR) + Gel chromatography (Superdex 200 10/300 GL)	3.6	Pan et al. (2010)
Cinachyrella apion (sponge) lectin	Acetone precipitation (0.5 / 1.0 / 2.0 vol) + Affinity chromatography (IgG anti-CvL-Sepharose[1]) + Gel chromatography (Superose 6 10/300 GL)	0.005	Medeiros et al. (2010)
Stonefish lectin	Affinity chromatography (Concanavalin A-Sepharose 4B) + Hydrophobic chromatography (Phenyl-Sepharose CL-4B)	3.0	Kato et al. (2016)
Portunus pelagicus (blue swimmer crab) lectin	Affinity chromatography (Mannose-Sepharose CL-4B)	-	Jayanthi et al. (2017)
Human serum pronase-inducible lectin	Affinity chromatography (Concanavalin A-Sepharose 4B)	24.1	Manikandan et al. (2017)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Plant lectins			
Peanut agglutinin	Defatting (Petroleum ether) + Ammonium sulfate precipitation (60%) + Affinity chromatography (Sepharose-ε-aminocaproyl-β-galactopyranosylamine)	1.4	Lotan et al. (1975)
CGB lectin	Ammonium sulfate precipitation (60%) + Affinity chromatography (Galactose-Sepharose 4B) + Gel chromatography (Sephacryl HR-100)	56.7	Hashim et al. (1991); Lim et al. (1997)
CMB lectin	Ammonium sulfate precipitation (60%) + Affinity chromatography (Galactose-Sepharose 4B) + Affinity chromatography (Mannose-Sepharose 4B) + Gel chromatography (Sephacryl HR-100)	30.1	Lim et al. (1997)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Pea lectin	Acetic acid precipitation (pH 5) + Affinity chromatography (Sephadex G-100)	0.4	Sitohy et al. (2007)
Dolichos lablab lectin	Ammonium sulfate precipitation (0−60%) + Affinity chromatography (Mannose-Sepharose) + Affinity chromatography (Galactose-Sepharose)	0.8	Rameshwaram & Nadimpalli (2008)
Macrotyloma axilare lectin	Thermal precipitation (85°C) + Ethanol precipitation (20−60%) + Ion exchange chromatography (HiLoad 16/10 Q-Sepharose)	12.4	de Santana et al. (2008)
Ophiopogon japonicas lectin	Affinity chromatography (DEAE-Sepharose) + Gel chromatography (Sephacryl S-100)	6.9	Tian et al. (2008)

Table 2.2, continued.

Lectin	Method of purification	Protein recovery (%)	Reference
Red kidney bean lectin	Defatting × 2 (Sherwood oil) + Reversed micelle (AOT[2] in isooctane) + Ion exchange chromatography (DEAE Sephadex A-50) + Gel chromatography (Sephadex G-150)	7.2	Hou et al. (2010)
Chinese pinto bean lectin (CPBL)	Affinity chromatography (Affi-Gel Blue Gel) + Ion exchange chromatography (Q Sepharose) + Gel chromatography (Superdex 75 10/300 GL) + Desalting (Centricon centrifugal filter)	0.6	Ang et al. (2014)

[1]CvL = *Cliona varians* lectin; [2]AOT = Sodium di-(2-ethylhexyl) sulfosuccinate.

lectin have also been used as ligands to purify glycosylated lectins (Coelho et al., 2012). Moreover, some polysaccharide matrices such as Sephadex (polymer of glucose) and agarose (polymer of galactose and 3,6-anhydro-L-galactopyranose) are also used without any ligands to purify lectins (Mohr, 1985; Coelho et al., 2012). Affinity chromatography might also be used in conjunction with other chromatographic procedures in order to separate undesired lectins, lectin isoforms and carbohydrate-binding non-lectin proteins.

Ion exchange chromatography is widely used in the multiple-step purification of lectins. Anionic exchangers (diethyl-aminoethyl (DEAE)-grouped resins and Q Sepharose) seem to be more frequently used compared to cationic exchangers (carboxymethyl (CM)-grouped resin and Mono S). While strong ion exchangers might be advantageous due to maintenance of charge almost independent of the pH, weak ion exchangers are preferred for purifying proteins, so as not to subject the protein to harsh elution conditions (Millner, 1999).

Gel chromatography is also a popular method in lectin purification. This technique is suitable for removing proteins both larger and smaller than the desired protein. Various stationary phases such as Sephacryl (HR-100, S-100, S-200), Sephadex (G-150, G-200), Sepharose 6 and Superdex (75, 200) have been used in gel chromatographic separation of the desired lectin. Hydrophobic chromatography (Phenyl-Sepharose), reversed phase HPLC (C_4) and ultrafiltration (10 kDa membrane) have also been applied as an additional step in some of the lectin purification procedures. Desalting has been placed as the last step to remove low molecular weight compounds.

As can be seen from Table 2.2, recovery of purified lectin varies considerably regardless of the source. The percentage recovery of lectins generally falls below 10% of the total protein content of the crude extract. Various factors such as low prevalence

of the lectin in the cells or protein loss in consecutive purification steps may contribute towards low percentage recovery of lectins. A significant number of lectins have a percentage recovery lower than 1%. However, some lectins are present in higher concentrations in the source organism, with percentage recovery effectively reaching ~30% or more (Van Damme et al., 1998b).

2.1.4. Jacalin-related lectins

Jacalin is a galactose-binding lectin, isolated from jackfruit seeds (Bunn-Moreno & Campos-Neto, 1981). Jacalin-related lectins (JRLs) are lectins belonging to a lectin family, where each protein member possesses one or more domains with structural and sequence similarity to jacalin (Peumans et al., 2001). Figure 2.1 shows the taxonomical view of the distribution of JRLs in plants. JRLs are found across different plant families including Moraceae, Fabaceae, Poaceae and Musaceae (Raval et al., 2004). The distribution of JRLs in both monocots and dicots indicates their origin from a common ancestor (Van Damme et al., 1998b). Lectins with jacalin-like domains play an important role in plant resistance against abiotic and biotic stresses (Esch & Schaffrath, 2017). This property was evident from the up-regulation of JRLs under stressed conditions and increased susceptibility of plants towards pathogen upon attenuation of JRL-like gene (Xiang et al., 2011; Song et al., 2014; He et al., 2017). JRLs have been divided into two subgroups, i.e., mannose-binding JRLs (mJRLs) and galactose-binding JRLs (gJRLs) subgroups (Van Damme et al., 1998b). The former has been found more widely distributed across the plant kingdom compared to the latter. These two subgroups show high degree of similarity in the amino acid sequences. However, gJRL protomers were each cleaved during evolution to form a long α- and a short β-chain, whereas mJRLs remained as a single long polypeptide chain (Van Damme et al., 1998b). The differences in the structures of gJRLs and mJRLs might be required for performing specific functions at different locations of the cell; gJRLs are located in the vacuolar

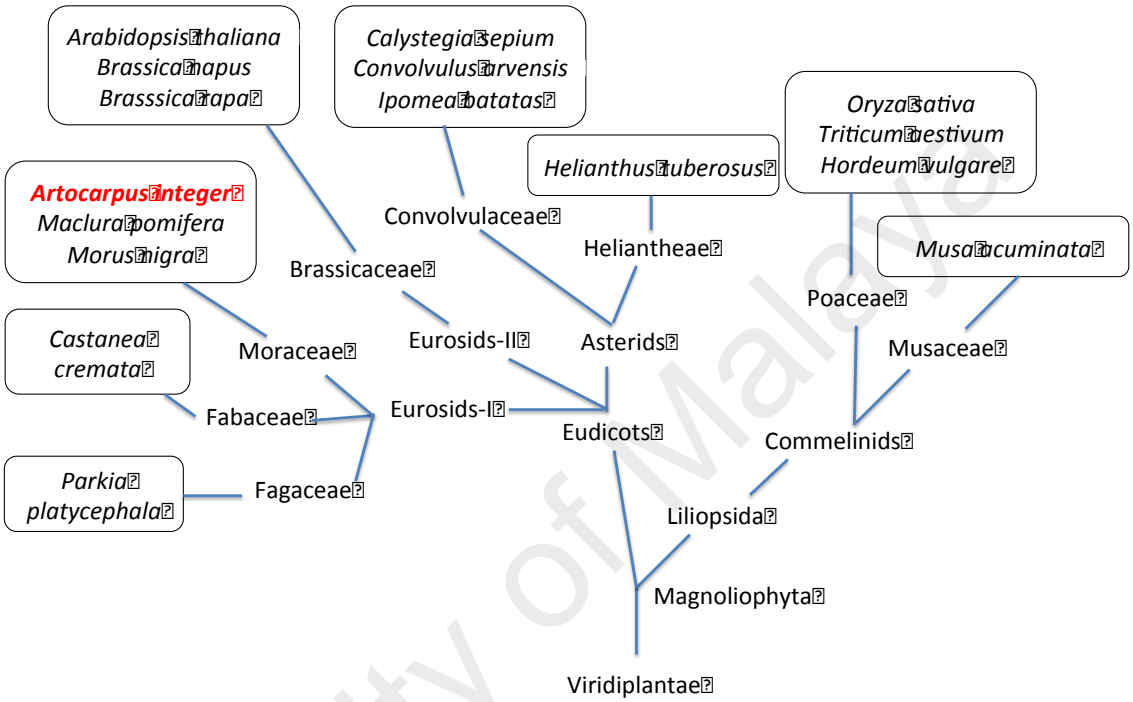

Figure 2.1: Taxonomical view of the distribution of jacalin-related lectins in different plant families. *Artocarpus integer*, the source plant for CGB lectin, is shown in red. Adapted from Raval et al. (2004).

compartment whereas mJRLs are restricted in the cytoplasmic or nucleoplasmic compartments of the cell (Nakamura-Tsuruta et al., 2008).

2.1.5. Champedak galactose-binding lectin

Lectins of the genus *Artocarpus* have been extensively studied in terms of physicochemical, structural and biological properties (Loris et al., 1998; Jagtap & Bapat, 2010; Hari et al., 2014). The plant of origin of champedak galactose-binding (CGB) lectin, *Artocarpus integer* (local name in Malay language: *cempedak*) (Figure 2.2) is local to Southeast Asia, particularly in the Malay Peninsula, Indonesia and New Guinea. It falls under the family Moraceae and shares the same genus as jackfruit (*Artocarpus heterophyllus)*. Two lectins which have been isolated from *A. integer* seeds include the CGB lectin (earlier termed as lectin-C) and the mannose-binding counterpart, champedak mannose-binding (CMB) lectin (earlier termed as lectin-M) (Hashim et al., 1991; Lim et al., 1997). Recovery of CGB lectin from the total protein content of the crude seed extract has been reported to be nearly twice compared to CMB lectin, being ~57% against ~30%, respectively (Table 2.2) (Lim et al., 1997). CGB lectin purified from six different clones of *A. integer* retained uniformity in structure and function (Hashim et al., 1993).

2.1.5.1. Molecular properties

The known molecular properties of CGB lectin are listed in Table 2.3. Four identical monomers form the parent molecule of CGB lectin as a homotetrameric lectin. Each monomer consists of an α-chain with a molecular mass of ~13 000 Da and a β-chain of ~2 100 Da (Gabrielsen et al., 2014). The total number of amino acid residues in CGB lectin tetramer is 616, with a distribution of 154 residues in each monomer. Furthermore, the α-chain in each monomer is comprised of 133 residues while 21 residues form the β-chain. CGB lectin has been characterized as a β-protein due to the prevalence of β structure. Eighty-nine out of 133 residues form the β-sheet structure

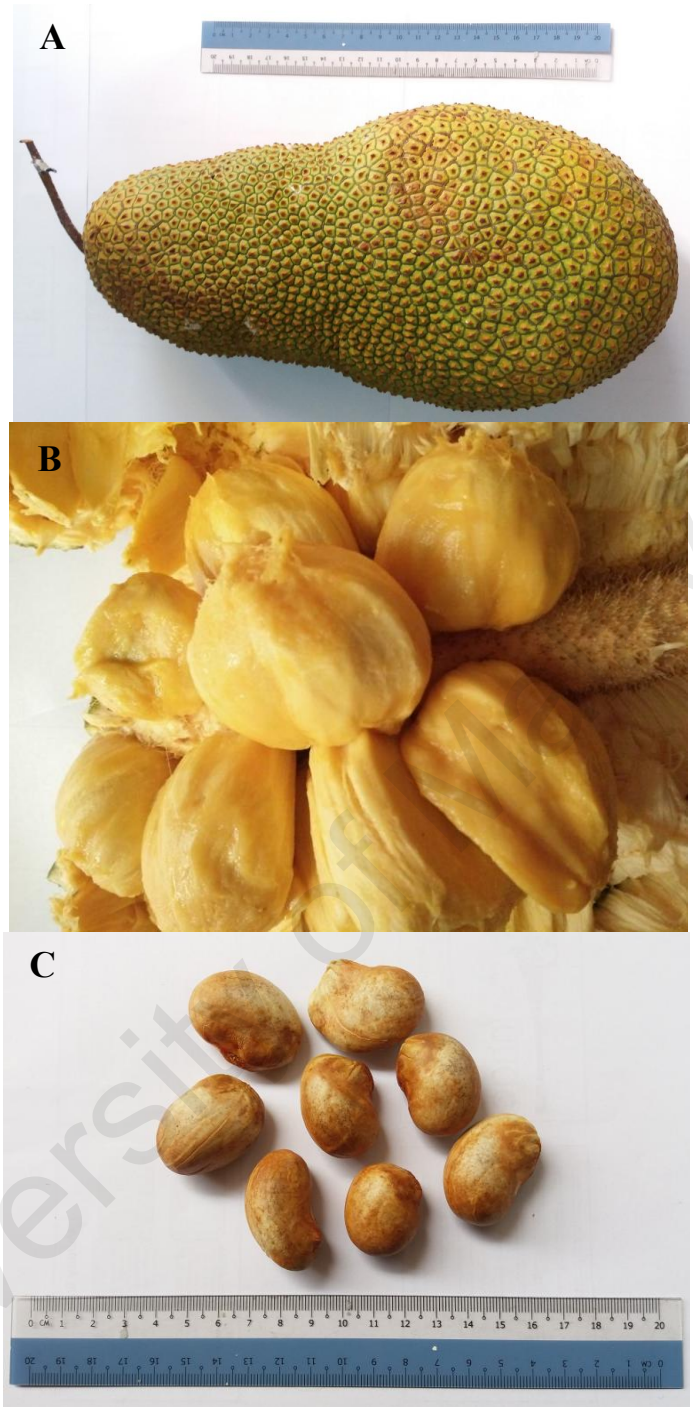

Figure 2.2: *Artocarpus integer* (local name: cempedak) fruit. (A) Whole fruit; (B) Arils; (C) Seeds.

Table 2.3: Molecular properties of CGB lectin.

Property	Value	Reference
Molecular weight • α-Chain • β-Chain	 ~13 000 Da ~2 100 Da	Gabrielsen et al. (2014)
Number of amino acid residues • Total (per tetramer) • α-Chain • β-Chain	 616 133 21	Gabrielsen et al. (2014)
β-sheet structure • α-Chain • β-Chain	 66% 33%	Gabrielsen et al. (2014)
Carbohydrate binding specificity* • *p*-Nitrophenyl-*β*-D-galactopyranoside • 1-O-Methyl-α-D-galactopyranoside • D-Melibiose • *N*-Acetyl-D-galactosamine • D-Galactose	 <0.02 0.40 1.83 1.90 6.15	Hashim et al. (1991)
Hemagglutinating activity (Human ABO blood types)	Positive	Hashim et al. (1991)
Immunoglobulin binding activity • Serum IgA1 • Colostral sIgA	 Positive	Hashim et al. (1991)
• IgA2 • IgD • IgG • IgM	Negative	

Table 2.3, continued.

Serum precipitating activity • Monkey • Sheep • Rabbit • Cat • Hamster • Rat • Guinea pig	Positive	Hashim et al. (1993)

*Concentration of sugar (mM) to produce 50% inhibition on lectin-IgA binding.

(66%) in the α-chain, whereas 7 out of 21 residues constitute the β-sheet structure (33%) in the β-chain (Gabrielsen et al., 2014). CGB lectin is known to bind reversibly to galactose and its derivatives. The sugar binding affinity of the lectin follows the order: *p*-nitrophenyl-*β*-D-galactopyranoside > 1-O-methyl-α-D-galactopyranoside > D-melibiose > *N*-acetyl-D-galactosamine > D-galactose (Hashim et al., 1991). In addition, CGB lectin has been found to agglutinate human erythrocytes of all ABO blood types without discrimination (Hashim et al., 1991). It has also exhibited specific binding to serum IgA1, colostral sIgA and C1 inhibitor molecules, while showing no binding activity towards IgA2, IgD, IgG and IgM (Hashim et al., 1991; 1993). Furthermore, CGB lectin has also displayed serum-precipitating activity with sera of different mammalian species (Table 2.3) (Hashim et al., 1993).

2.1.5.2. Amino acid composition

Table 2.4 shows the amino acid composition of CGB lectin as determined from the cDNA. CGB lectin contains a total of 154 amino acid residues for the lectin monomer, which makes a total of 616 residues per lectin tetramer (Table 2.4) (Gabrielsen et al, 2014). The aromatic residues of the lectin are distributed as 10 Phe, 11 Tyr and 2 Trp residues in each monomer, making a total of 40 Phe, 44 Tyr and 8 Trp residues per lectin tetramer. Due to the presence of Trp residues, the lectin has been characterized as a Class B protein (Teale, 1960). Nearly 40% of the total amino acid residues are contributed by hydrophobic amino acids (Phe, Ile, Trp, Leu, Val, Met, Ala and Pro) (Aftabuddin & Kundu, 2007; Gabrielsen et al, 2014). This was not unusual, as distribution of hydrophobic amino acids in globular proteins has been found to lie between 25% and 30%, or higher (Nakai & Modler, 1996). On the other hand, number of acidic (Asp and Glu) and basic (His, Lys and Arg) amino acid residues has been found the same, being 13 residues each per lectin monomer. Thus, one can expect the pI

Table 2.4: Amino acid composition of CGB lectin.*

Amino acid	Number of residues per monomer
Alanine	3
Arginine	2
Asparagine	7
Aspartic acid	6
Cysteine	0
Glutamic acid	7
Glutamine	4
Glycine	20
Histidine	1
Isoleucine	10
Leucine	9
Lysine	10
Methionine	2
Phenylalanine	10
Proline	7
Serine	16
Threonine	12
Tryptophan	2
Tyrosine	11
Valine	15
Total	**154**
Number of residues per tetramer	**616**

*Calculated from CGB lectin amino acid sequence (Gabrielsen et al., 2014).

of CGB lectin to be close to 7.0. It is interesting to note the absence of Cys residues, which translates to the lack of disulphide bonds in forming the lectin structure.

2.1.5.3. Primary structure

The amino acid sequence of CGB lectin is shown in Figure 2.3, which also includes the amino acid sequence of jacalin for comparison. As shown in the figure, CGB lectin shares a high degree (>95%) of sequence homology to jacalin (Gabrielsen et al., 2014). This justifies the classification of CGB lectin as a member of JRL family. The polypeptide chain undergoes post-translational modification by cleavage at the sequence shown in grey (Figure 2.3) to form the α- and β-chains. A single Trp residue is present in each of the α- and β-chains. The distribution of acidic, basic and hydrophobic amino acid residues in the α- and β-chains of CGB lectin is shown in Figure 2.4. Hydrophobic residues are evenly distributed in the α-chain, while clustered in the C-terminal half of the β-chain. The N-terminal half of the α-chain contains most (10 out of 13) of the acidic residues of the CGB lectin monomer, whereas the basic residues are more or less uniformly dispersed throughout the α-chain. The β-chain contains only one each of the acidic and basic amino acid residues within its N-terminal half.

2.1.5.4. Three-dimensional structure

Figure 2.5 shows the three-dimensional structure of CGB lectin (PDB accession code 4ak4), as obtained from X-ray crystallographic analysis (Gabrielsen et al., 2014). Most of the lectin structure is dominated by the presence of β-sheet structure (Figure 2.5A). However, segments of β-bend / β-turn are also present. There seems to be the complete absence of the α-helical structure in the protein (Gabrielsen et al., 2014). The three-dimensional structure of the lectin is a homotetramer of noncovalently-bound monomers (Figure 2.5B). Each monomer is further divided into a longer α-chain and a shorter β-chain (Figure 2.5C). The monomer is characterized by the presence of a β-prism structure, which is formed by the noncovalent association of three four-stranded

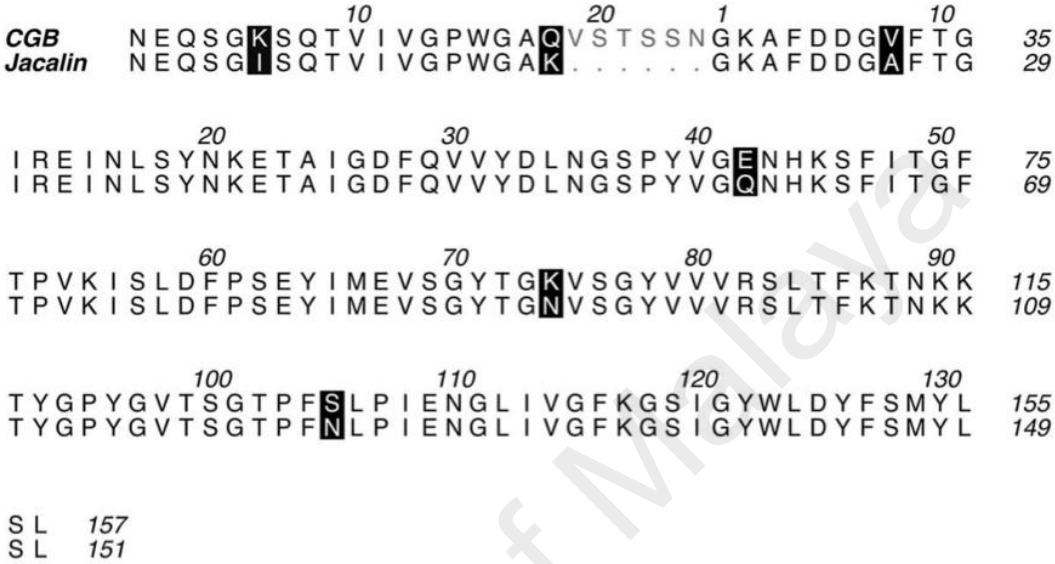

Figure 2.3: Amino acid sequence of CGB lectin monomer, aligned with the amino acid sequence of jacalin monomer. Amino acid residues shown in grey represent the cleaved fragment, separating the longer α-chain from the shorter β-chain (Gabrielsen et al., 2014).
(*Reproduced with permission of the International Union of Crystallography*)

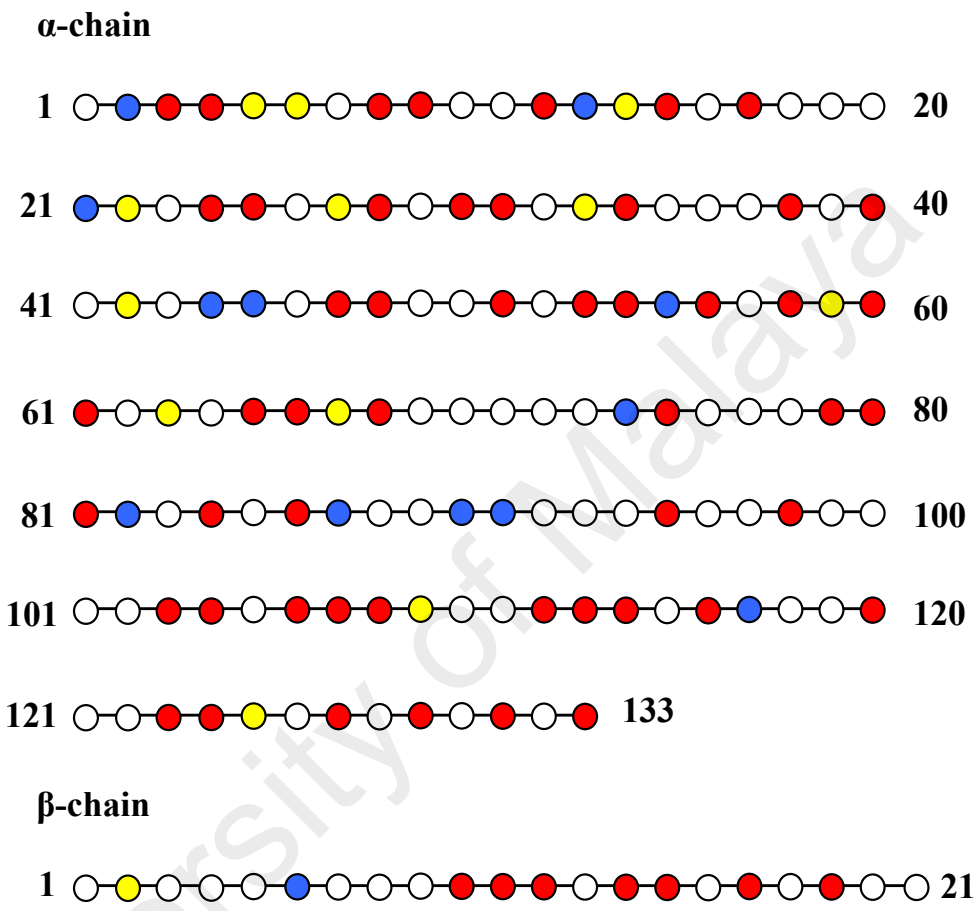

Figure 2.4: Diagram showing distribution of acidic, basic and hydrophobic amino acid residues in the α- and β-chains of CGB lectin monomer. Acidic, basic and hydrophobic residues are shown in yellow, blue and red, respectively.

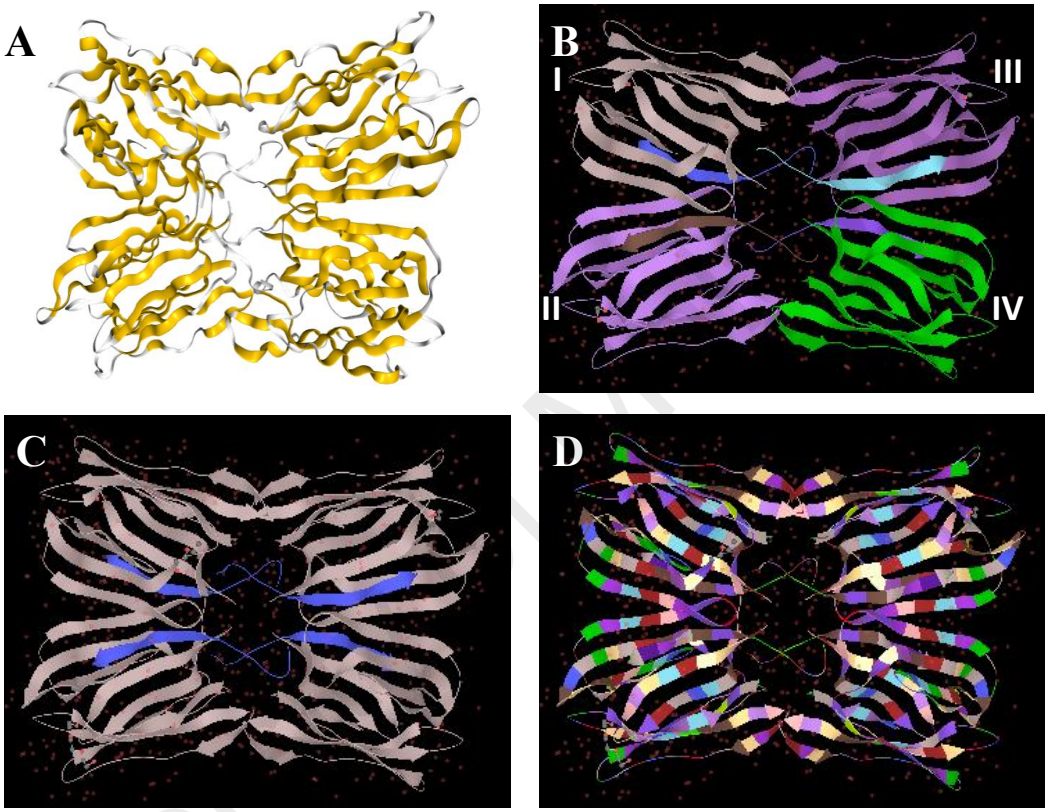

Figure 2.5: Three-dimensional structure of CGB lectin. The structure is color-coded based on (A) secondary structure (β-sheet, yellow; β-bend or turn, silver), (B) different monomers (shown in beige, dark purple, light purple and green), (C) chain type (α-chain, beige; β-chain, blue) and (D) different amino acid residues (PDB accession code 4ak4) (Berman et al., 2000; Gabrielsen et al., 2014).

antiparallel β-sheets (Figure 2.6) (Gabrielsen et al., 2014). This β-prism structure is similar to the three-dimensional structure described for jacalin-related lectins (Sankaranarayanan et al., 1996; Barre et al., 2001). Out of the twelve β-strands involved in the formation of β-prism structure of CGB lectin monomer, one β-strand, namely β1, forms the β-chain while the other 11 β-strands form the α-chain (Gabrielsen et al., 2014). The tetrameric structure of CGB lectin is stabilized by noncovalent interactions involving both the α- and β-chains, as evident by the presence of hydrogen bonds and hydrophobic interactions in two types of interfaces. The first interface (between monomers I and II as well as III and IV; Figure 2.5B) is formed by the α-α, β-β as well as α-β chain associations between the adjacent monomers. On the other hand, the second interface (between monomers I and III as well as II and IV; Figure 2.5B) involves interactions between the neighboring α-α and α-β chains. Each lectin monomer binds only one galactose molecule. The sugar-binding site is comprised of residues from the α-chain, involving Gly1, Tyr122, Trp123 and Asp125 through hydrogen bond formation to the galactose oxygen atoms, stacking of Tyr78 against *B* face of the galactose as well as hydrophobic interactions with Tyr122 (Gabrielsen et al., 2014).

2.1.5.5. Applications

Due to its sugar-binding ability, CGB lectin has been found useful to detect *O*-glycosylated proteins from serum samples of endometrial and cervical cancer patients (Abdul Rahman et al., 2007). Horseradish peroxide (HRP)-conjugated CGB lectin was used in this application to characterize the *O*-glycosylated proteins in the Western blots. Detection of alteration in the expression of *O*-glycosylated proteins, e.g. α_1-antitrypsin, α_1-B-glycoprotein, kininogen, antithrombin III, clusterin and zinc α-2-glycoprotein, in the serum of the diseased patients by the use of CGB lectin in Western blotting has offered additional procedure for cancer diagnosis.

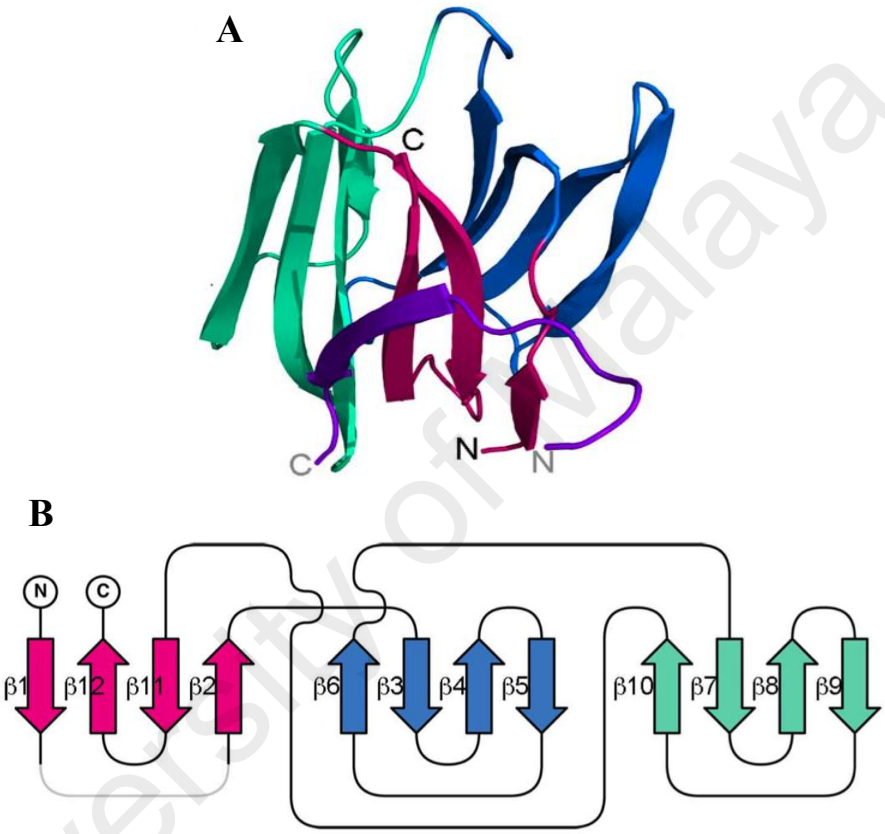

Figure 2.6: (A) Three-dimensional β-prism structure of CGB lectin monomer. The three sheets forming the prism structure (shown in pink, blue and green) are comprised of 12 β-strands as shown in (B). The region in gray is cleaved to separate strand β1 to form the β-chain, whereas the remaining 11 β-strands form the α-chain (Gabrielsen et al., 2014).
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CGB lectin has also been employed to detect *O*-glycosylated proteins from urine samples of early stage endometrial, ovarian and prostate cancer patients (Mu et al., 2012; 2013; Jayapalan et al., 2013). A decreased level of nebulin was detected in the urine samples of endometrial cancer patients using HRP-conjugated CGB lectin (Mu et al., 2012). Furthermore, this procedure also detected enhanced expression of clusterin and leucine-rich α-2-glycoprotein as well as reduced levels of kininogen in the urine samples of ovarian cancer patients (Mu et al., 2013). Diagnosis of prostate cancer can also be made with the help of CGB lectin by analyzing urine samples of the diseased patients to check enhanced expression of a truncated fragment of inter-α-trypsin inhibitor heavy chain 4 (Jayapalan et al., 2013).

2.2. Protein denaturation and stabilization

2.2.1. Protein denaturation

Protein denaturation studies have gained much interest in the pursuit of understanding the folding mechanism and structural stability of proteins (Herskovits et al., 1970; Privalov & Gill, 1988; Baldwin, 1989). Treatment of proteins with denaturants may yield stable intermediates, which are believed to be important conformational states, formed during the protein folding process occurring *in situ* (Baldwin, 1975; Kuwajima, 1989). Chemical denaturants such as urea and guanidine hydrochloride have been found popular in protein denaturation studies in being effective to disrupt all noncovalent interactions, which are known to stabilize the native protein conformation (Tanford, 1968; O'Brien, 2007). Other denaturants employed in protein denaturation studies include extreme conditions of pH, temperature and pressure.

To get complete insight about the folding/unfolding pathway, proteins may be subjected to more than one type of denaturants due to the involvement of different unfolding mechanisms (Camilloni et al., 2008; Kishore et al., 2012). Changes in the secondary, tertiary and quartenary structures of the protein as well as accumulation of

stable intermediates in the unfolding process are commonly monitored using probes such as near- and far-UV circular dichroism, fluorescence (both intrinsic and extrinsic), and absorption spectroscopy (Pace & Scholtz, 1997).

The unfolding process of monomeric or single-chain proteins is less complicated than multimeric proteins. The denaturation of multimeric proteins is more intricate due to the presence of intersubunit interactions between the protein subunits, in addition to the intrasubunit interactions within the protein subunit (Biswas & Kayastha, 2004; Sinha et al., 2005; Dev et al., 2006).

2.2.2. Denaturation of jacalin and JRLs

Recombinant jacalin (rJacalin) was found similar to native jacalin (nJacalin) in secondary and tertiary structures as well as GdnHCl denaturation behavior (Sahasrabuddhe et al., 2004). Although the sugar binding characteristics of these jacalins were found similar (Gal<MeαGal<MeαGalβ1-3GalNAc), reduced affinity of rJacalin towards methyl-α-galactose and Galβ1-3GalNAc was seen compared to nJacalin. On the other hand, refolding of the GdnHCl-denatured rJacalin showed complete recovery in its sugar binding affinity, unlike the partial recovery attained by the refolded nJacalin under similar conditions. However, nJacalin showed higher stability at extreme pH values compared to rJacalin (Sahasrabuddhe et al., 2004).

GdnHCl denaturation of jacalin-related lectin, frutalin showed monomerization coupled with unfolding above 4.0 M GdnHCl (Campana et al., 2002). Interestingly, the unfolded frutalin obtained at 4.0 M GdnHCl retained similar hemagglutinating activity as the native protein. The native frutalin exhibited a free energy of unfolding of 12.34 kJ mol^{-1}, which was increased to 17.12 kJ mol^{-1} when measured in the presence of D-galactose. During the refolding process of GdnHCl- and thermally-denatured frutalin, an additional misfolded species, representing the dead-end structure formed due to non-native refolding pathway was obtained along with the refolded species. The sugar-

binding site was identified as a site to initiate the refolding of frutalin (Campana et al., 2002).

Mulberry Latex Galactose-specific Lectin (MLGL) was found to be highly resistant against urea and GdnHCl denaturations (Datta & Swamy, 2017). However, it was completely denatured in 6.0 M guanidine thiocyanate (GdnSCN) with a C_m value of ~3.75 M. The emission maximum of MLGL was found to be red-shifted by 24 nm in the presence of 6.0 M GdnSCN compared to the native lectin. Furthermore, the lectin had also shown resistance to thermal denaturation, as the secondary structure and hemagglutinating activity remained unchanged up to 70°C. MLGL exhibited conformational stability within the pH range, 6.2−8.5, although a 50% decrease in the lectin activity was observed at pH 6.2 (Datta & Swamy, 2017).

GdnHCl denaturation study of the dimeric banana lectin (BanLec) showed a red shift of ~20 nm in its emission maximum at 4.0 M GdnHCl concentration (Ghosh & Mandal, 2014). Presence of sugar did not produce any effect on the GdnHCl denaturation behavior, as the denaturation curve was similar in the absence and presence of sugar (Gupta et al., 2008). Nonetheless, GdnHCl-induced unfolding of BanLec was temperature-dependent. Determination of the free energy change of unfolding (ΔG_o) showed higher ΔG_o value (17.8 kcal mol^{-1}) at 298 K compared to 15.3 kcal mol^{-1}, obtained at 318 K (Gupta et al., 2008). BanLec showed monomerization at 2.5 M GdnHCl, which was characterized by the increased ANS fluorescence intensity as well as delayed elution in gel chromatography (Ghosh & Mandal, 2014). Further increase in GdnHCl concentration beyond 2.5 M led to unfolding of monomers. However, an additional peak, similar in hydrodynamic radius to the protein at 2.5 M GdnHCl, appeared in gel chromatography at GdnHCl concentrations above 3.5 M. Thus, two distinct unfolded states (one randomly coiled and another globular) of BanLec were observed at different GdnHCl concentrations (Ghosh & Mandal, 2014).

Acid denaturation results of dimeric BanLec showed monomerization at pH 2.0 (Khan et al., 2013). However, two protein species with hydrodynamic radii of 1.7 and 3.7 nm were identified in gel chromatographic profile of BanLec at pH 2.0. On the other hand, the value of the hydrodynamic radius of native BanLec was found to be 2.9 nm. Furthermore, urea denaturation results of the dimeric and monomeric BanLecs revealed greater stability of the monomeric BanLec (C_m = 7.4 M) compared to the native dimeric lectin (C_m = 6.2 M). In addition, the monomeric lectin also exhibited greater resistance to high temperature, as revealed by the retention of significant secondary structures up to 95°C (Khan et al., 2013).

Denaturation of tetrameric *Artocarpus hirsuta* lectin with 5.0 M GdnHCl was characterized by a red shift of 21 nm in the emission maximum of the lectin, which remained unaffected in the pH range, 4.0−9.0 (Gaikwad et al., 2002). Similar GdnHCl denaturation behavior was observed when probed by fluorescence intensity in the pH range, 5.0−8.0. However, drastic changes were observed at pH 4.0 and pH 9.0. Fifty percent unfolding was achieved at lower GdnHCl concentrations (2.2 M) at pH 4.0 as well as pH 9.0, while it was delayed up to 3.2 M GdnHCl in the pH range, 6.0−8.0. Far-UV CD results also showed similar trend of GdnHCl denaturation in the pH range, 5.0−8.0, while higher unfolding rates were observed at pH 4.0 and pH 9.0. The tetrameric lectin showed initial dimerization followed by monomerization and unfolding with increasing GdnHCl concentrations, as revealed from gel chromatographic results. However, partial subunit dissociation and unfolding was also observed even at 6.0 M GdnHCl, as shown by the presence of multiple peaks in the gel chromatogram. Thermal denaturation of *A. hirsuta* lectin produced unfolded species, showing different fluorescence characteristics compared to the GdnHCl-denatured lectin. In addition, the sugar-binding activity of *A. hirsuta* lectin was lost at temperatures above 45°C, which was accompanied by lectin precipitation. Furthermore, renaturation from 5.0 M

GdnHCl-denatured lectin produced some recovery (23%) in the lectin sugar-binding activity compared to 0% recovery from thermally-denatured lectin at 70°C (Gaikwad et al., 2002).

2.2.3. Polyol-induced structural changes in proteins

Various strategies have been used to increase protein stability such as protein engineering involving both genetic and chemical approaches as well as solvent engineering (Arakawa & Timasheff, 1985; Arnold, 1993; Dombkowski et al., 2014). Whereas protein engineering yielded both more stable and less stable proteins compared to the native protein (Dani et al., 2003; Dombkowski et al., 2014), solvent engineering produced more promising results with better protein stability (Arakawa & Timasheff, 1985; Kamiyama et al., 1999; Wong et al., 2012). Polyols have been successfully used in solvent engineering to increase protein stability (Back et al., 1979; Gekko, 1981; Kaushik & Bhat, 1998; 2003; Sharma & Singh, 2017). Several reports have shown higher protein stability against stresses such as high pressure and temperature, acidic pH and denaturing chemicals in the presence of polyols (Kamiyama et al., 1999; Athès et al., 1999; Singh & Singh, 2003; Zaroog et al., 2013; Fonin et al., 2016). Furthermore, polyols have been found to induce significant refolding of denatured proteins (Rashid et al., 2006; Sharma & Singh, 2017).

Polyol-induced stabilization of proteins can be explained by the increase in the surface tension of water as well as preferential hydration of proteins (Timasheff & Arakawa, 1988; Kaushik & Bhat, 1998). The interactions between the added polyols and water molecules prevent these water molecules from permeating the protein structure, promoting hydrophobic interactions within the protein interior, thus leading to an increase in structural stability (Back et al., 1979; Gekko, 1981; Gekko & Morikawa, 1981; Gekko & Timasheff, 1981; Xie & Timasheff, 1997). Cyclic polyols, e.g. inositol, seem to strongly stabilize hydrophobic interactions of nonpolar side chains, but may

destabilize peptide-peptide hydrogen bonds (Gekko, 1981). Linear polyols, on the other hand, are found to stabilize the hydrophobic bonds more effectively with increasing hydroxymethyl chain length (Gekko & Morikawa, 1981; Kamiyama et al., 1999). However, Gekko (1981) postulated that peptide-peptide hydrogen bonds are in fact less stabilized with increased chain length. Hence, exceptions to this rule have also been documented in the literature (Athès et al., 1999; Rashid et al., 2006). In addition, the stabilizing effect of the polyols has also been shown to positively correlate with the polyol concentration (Kamiyama et al., 1999; Tiwari & Bhat, 2006).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Champedak galactose-binding lectin

Champedak galactose-binding (CGB) lectin was purified from *Artocarpus integer* seeds according to the method described by Hashim et al. (1991). The purified preparation was found to be homogeneous with respect to size and charge as it produced a single symmetrical peak on HiPrep 26/60 Sephacryl S-100 HR column chromatography and a single band on polyacrylamide gel electrophoresis (Figure 3.1). This preparation was used throughout this study.

3.1.2. Reagents used in protein estimation

Bio-Rad QuickStart™ Bradford 1× Dye Reagent (#500-0205) and Bio-Rad QuickStart™ Bovine Serum Albumin Standard Set (#500-0207) were procured from Bio-Rad Laboratories Inc., USA.

3.1.3. Reagents used in denaturation studies

Urea BioXtra (≥99%, type U0631), guanidine hydrochloride (GdnHCl) (≥99%, type G4505) and 1-anilinonaphthalene-8-sulfonate (ANS) (type A3125) were purchased from Sigma-Aldrich, Co., USA. Analytical grade samples of potassium chloride, glycine, sodium acetate, di-sodium hydrogen phosphate and sodium di-hydrogen phosphate were procured from Merck KGAa, Germany. Hydrochloric acid and acetic acid were obtained from R & M Chemicals, Malaysia.

3.1.4. Reagents used in structural transition studies with polyols

Sorbitol (99%, type 240850), xylitol (≥99%, type X3375), erythritol (≥99%, type E7500) and ethylene glycol (≥99%, type 102466) were supplied by Sigma-Aldrich, Co., USA. Chemical structures of these polyols are shown in Figure 3.2.

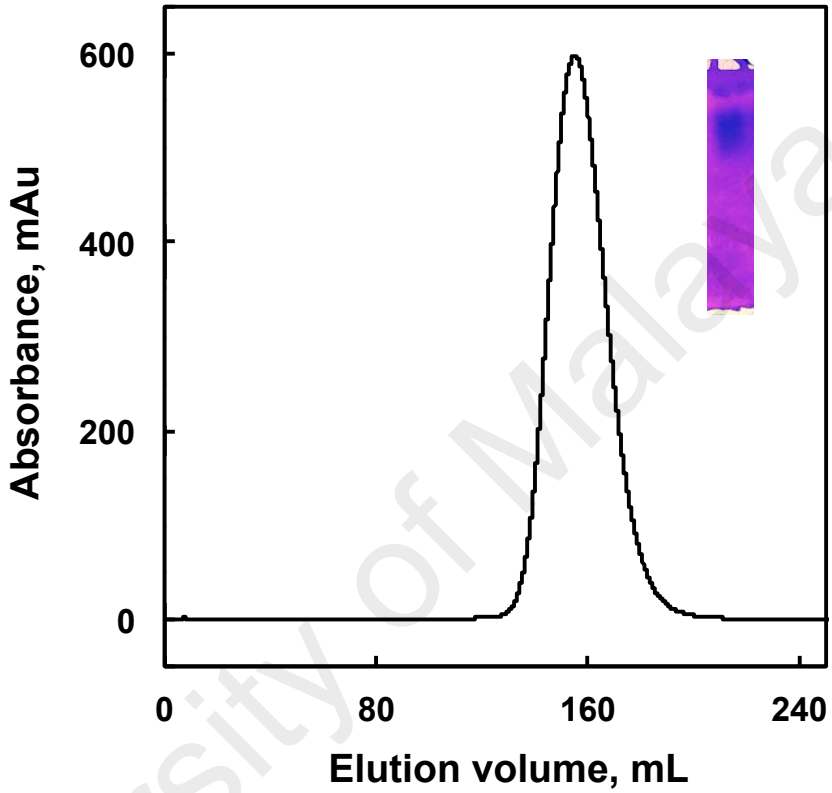


Figure 3.1: Gel chromatographic profile of the purified CGB lectin on a HiPrep 26/60 Sephacryl S-100 HR column in PBS (pH 7.2) at 25°C. Inset shows the electrophoretogram of CGB lectin (sample size: 1.6 μg/10 μL) on 8% polyacrylamide gel. The gel was stained with Coomassie blue following the method of Brunelle and Green (2014).

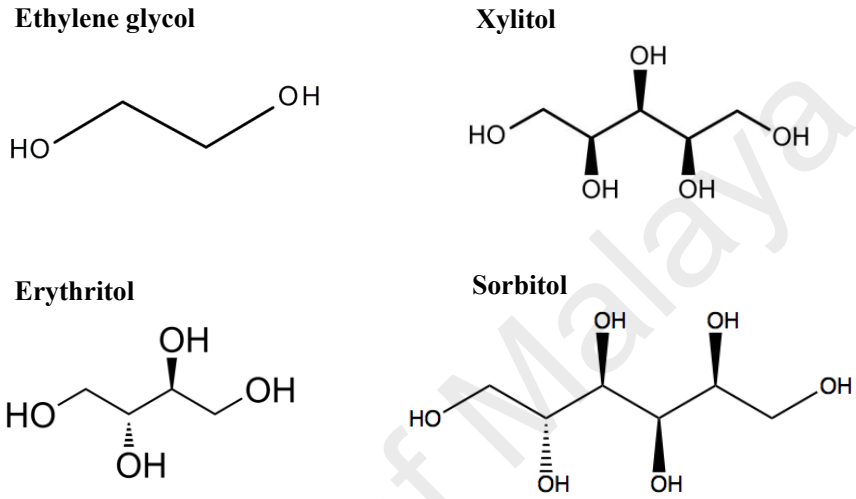


Figure 3.2: Chemical structures of four linear polyols, namely, ethylene glycol, erythritol, xylitol and sorbitol.

3.1.5. Reagents used in gel chromatography

HiPrep 26/60 Sephacryl S-100 HR column and Sepharose-4B were acquired from GE Healthcare, UK. BioGel P-100 was procured from BioRad Laboratories Inc., USA. Blue dextran 2000 was purchased from Amersham Pharmacia Biotech AB, Sweden.

3.1.6. Other reagents

D-galactose (≥98%, type G0625) and divinyl sulfone (type V3700) was purchased from Sigma-Aldrich, Co., USA. Analytical grade samples of potassium dihydrogen phosphate and sodium chloride were procured from Merck KGAa, Germany and Sigma-Aldrich, Co., USA, respectively. Standard buffers of pH 4.0 and pH 7.0 were purchased from Reagecon, Ireland.

3.1.7. Miscellaneous

Cellulose nitrate membrane filters (0.45 μm) and nylon syringe filters (0.22 μm) were supplied by GE Healthcare, UK. Parafilm 'M' was the product of Bemis Company Inc., USA.

Ultrapure water (type 1), obtained from Milli-Q system (Merck Millipore, Germany), was used throughout this study.

3.2. Methods

3.2.1. pH measurements

All pH measurements were performed on a Sartorius Basic Meter PB-10, connected to a Sartorius pH/ATC electrode (Sartorius AG, Germany). The pH meter had a least count of 0.01 pH unit. It was routinely calibrated using standard buffers of pH 4.0 and pH 7.0 at 25°C.

3.2.2. Analytical procedures

3.2.2.1. Determination of protein concentration

Lectin solution was prepared in phosphate-buffered saline (PBS, pH 7.2) and its concentration was determined following the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. A fixed volume (250 μL) of the Bradford reagent was pipetted into a 96-well flat-bottomed plate. Five microlitres of the protein solution were mixed into each well. The mixture was incubated for 30 min at 25°C in dark. The standard as well as the lectin samples were prepared in triplicates. The absorbance values of the solutions were measured at 595 nm on a BioTek Synergy H1 Multi-Mode Reader (BioTek Instruments Inc. USA). The average values were taken and corrected with the blank, which was prepared in the same way except that ultrapure water or buffer was used to replace the protein solution.

3.2.2.2. Determination of ANS concentration

ANS stock solution was prepared fresh by dissolving a small amount (~2.5 mg) of ANS crystals into 5 mL of working buffer in dark. ANS concentration was determined spectrophotometrically on a Shimadzu UV-2450 double-beam spectrophotometer (Shimadzu Corp., Japan), using a molar extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm (Mulqueen & Kronman, 1982).

3.2.2.3. Determination of denaturant concentration

The stock solutions of urea (~11 M) and GdnHCl (~7 M) were prepared fresh as described by Pace and Scholtz (1997). A calculated amount of urea or GdnHCl in grams (*U*) was weighed and dissolved into the respective buffers. The denaturant solution was also weighed and its weight in grams was represented as *Δ*. The weight fraction of the denaturant in solution (*W*) was obtained using the following equation:

$$W = U / \Delta \quad (1)$$

The ratio of the density of urea stock solution (d) to the density of water (d_o) was calculated according to the following equation:

$$d/d_o = 1 + 0.2658\ W + 0.0330\ W^2 \quad (2)$$

Similarly, the d/d_o value for the GdnHCl stock solution was obtained using the following equation:

$$d/d_o = 1 + 0.2710\ W + 0.0330\ W^2 \quad (3)$$

Values of Δ and d/d_o, as obtained above were used to calculate the volume (V) of the stock denaturant solutions (in mL) in the following way:

$$V\ (\text{mL}) = \Delta\ /\ d/d_o \quad (4)$$

The concentrations of the stock denaturant solutions (in M) were then determined according to the following equation:

$$\text{Concentration (M)} = (U / M_r) /\ (V / 1000) \quad (5)$$

where, M_r is the molar mass of the respective denaturants (g/mol), which were taken as 60.056 and 95.533 for urea and GdnHCl, respectively.

3.2.3. Spectral measurements

3.2.3.1. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed on a Jasco J-815 spectropolarimeter, equipped with a Jasco PTC-423S/15 Peltier-type temperature controller (Jasco Corp., Japan) under constant nitrogen flow. The instrument was calibrated with (+)-10-camphorsulfonic acid. Far-UV CD spectra were recorded in the wavelength range, 200−250 nm, using 0.2 μM protein concentration in a 1 mm

pathlength cuvette and a response time of 1 s. The scan speed was 100 nm/min and each collected spectrum was the average of four scans. The CD measurements were made at 25°C, unless otherwise stated. All CD spectra were corrected with their respective blanks.

Secondary structural changes in the lectin were monitored by analyzing the CD values at 218 nm ($CD_{218\ nm}$). If required, the CD values were transformed into relative CD values by taking the $CD_{218\ nm}$ value of the native CGB lectin as 100.

3.2.3.2. Fluorescence spectroscopy

Fluorescence measurements were carried out on a Hitachi F-2500 spectrofluorometer (Hitachi Ltd., Japan) at 25°C, using a 1 cm pathlength quartz cuvette. The excitation and emission slits were set at 10 nm each. Different parameters were fixed as: scanning speed 300 nm/min; response time 0.08 s; PMT voltage for all fluorescence measurements 400 V except ANS fluorescence measurements for urea denaturation studies at 700 V.

Intrinsic fluorescence spectra of the lectin (0.1 μM) were recorded in the wavelength range, 300−400 nm, using an excitation wavelength of 280 nm (to excite both Tyr and Trp residues) or 295 nm (to excite Trp residues only). Values of the fluorescence intensity were monitored at the emission maximum. If required, these values were transformed into relative fluorescence intensity by taking the fluorescence intensity of the native CGB lectin at the emission maximum as 100.

ANS fluorescence spectra of the lectin (0.1 μM) were recorded in the wavelength range, 400−600 nm, after exciting the samples at 380 nm. The ANS fluorescence intensity was monitored at the emission maximum.

All fluorescence spectra were corrected with their respective blanks.

3.2.4. Gel chromatography

BioGel P-100 column (1.6 × 35 cm), attached to AKTAprime plus system (GE Healthcare, UK) was used to perform gel chromatography. The column was equilibrated with the respective buffers according to the sample pH. The protein sample (12 μg/0.5 mL) was injected into the column and the chromatography was carried out at a flow rate of 0.4 mL/min. All samples were incubated at the respective pH at 25°C for 14 h before application. The peak elution volume was recorded as V_e. A small volume of blue dextran (0.75 mg/0.5 mL) was also injected into the column in the respective buffers to obtain the void volume, V_o of the column. Various elution peaks obtained from different samples were compared with one another by their V_e/V_o values.

3.2.5. Denaturation studies

Denaturation studies were carried out using increasing urea concentrations (0−9.0 M), decreasing pH (pH 7.0−1.0) or increasing temperatures (20−95°C). The concentration of CGB lectin used was 0.2 μM for far-UV CD measurements and 0.1 μM for fluorescence measurements. All experiments were replicated at least thrice and carried out at 25°C, unless otherwise stated. The denaturation curves were drawn freehanded according to the data points, as a guide to the eyes.

3.2.5.1. Urea denaturation

Chemical denaturation experiments were carried out according to the method described earlier (Muzammil et al., 2000). Requisite volumes of the stock urea solution were added into different volumes of the working buffer solution taken in different tubes. A fixed volume (25 μL) of the lectin solution was then added to these tubes, to obtain the desired concentrations of the protein and the denaturant. The final volume of the incubation mixture was 1.5 mL for far-UV CD measurements and 3 mL for fluorescence measurements. The samples were then incubated for 14 h at 25°C for equilibration attainment before spectral measurements (Gaikwad & Khan, 2003).

For denaturation experiments involving ANS fluorescence measurements, freshly prepared ANS stock solution (50 μL) was added to the above incubated protein samples in dark and mixed well. A further incubation for 30 min in dark at 25°C was made before spectral measurements. The molar ratio of the ANS concentration to the lectin concentration was kept as 100:1 (Naseem & Khan, 2005). The fluorescence and the far-UV CD spectra were recorded in the same way as described in Section 3.2.3.

Analysis of the urea denaturation results was made by plotting values of the relative $CD_{218\ nm}$, emission maximum, Trp fluorescence intensity at 333 nm or ANS fluorescence intensity at 461 nm against denaturant concentration. Characterization of the native and urea-denatured states of CGB lectin was carried out using the far-UV CD spectra, Trp fluorescence spectra and ANS fluorescence spectra in the absence and presence of 5.0 M and 9.0 M urea.

3.2.5.2. Acid denaturation

Acid denaturation experiments were performed by incubating CGB lectin in buffers of different pH but same molarity (10 mM). The different buffers used along with their pH ranges were: sodium phosphate buffer (pH 7.0−6.0), sodium acetate buffer (pH 5.5−3.75), glycine-HCl buffer (pH 3.5−2.0) and KCl-HCl mixture (pH 1.75−1.0). To a constant volume of different buffer solutions taken in different tubes, 144 μL (far-UV CD measurements) / 72 μL (fluorescence measurements) of CGB lectin was added and the contents were mixed well. The solution mixtures (2 mL) were left to equilibrate for 14 h at 25°C (Gaikwad & Khan, 2003). For experiments involving ANS fluorescence measurements, similar procedure was followed as described in Section 3.2.5.1. The fluorescence and the far-UV CD spectra were then recorded following standard procedures (Section 3.2.3). The pH values of the solution mixtures were checked both before and after spectral measurements and were found to lie within ± 0.1 pH units.

Acid denaturation results were analyzed by plotting the intrinsic fluorescence intensity at 330 nm or ANS fluorescence intensity at 468 nm against pH values. Characterization of the native and the acid-denatured states of CGB lectin was carried out using intrinsic fluorescence spectra, ANS fluorescence spectra and far-UV CD spectra, as well as size exclusion chromatography and thermal denaturation curves at different pH values. For experiments involving GdnHCl, buffers of different pH values contained the requisite amount of GdnHCl.

3.2.5.3. Thermal denaturation

Thermal denaturation experiments were performed on a Jasco J-815 spectropolarimeter, equipped with a Jasco PTC-423S/15 Peltier-type temperature controller, attached to a water bath. The CD values of the protein solution (pH 7.0 and pH 2.5) were recorded at 218 nm in the temperature range, 20−95°C after equilibrating the protein sample at the start temperature for 5 min. The ramp rate was set at 1°C/min. The data obtained were corrected with the respective blanks.

Thermal denaturation curves were transformed into F_D (fraction denatured) curves using the following equation:

$$F_D = (Y - Y_N) / (Y_D - Y_N) \quad (6)$$

where Y denotes the observed $CD_{218\ nm}$ value at a particular temperature, Y_N represents the value of the native state and Y_D of the denatured state (Pace and Scholtz, 1997). The midpoint temperature of the transition, T_m, was taken as the temperature corresponding to the F_D value of 0.5.

3.2.6. Hemagglutination assay

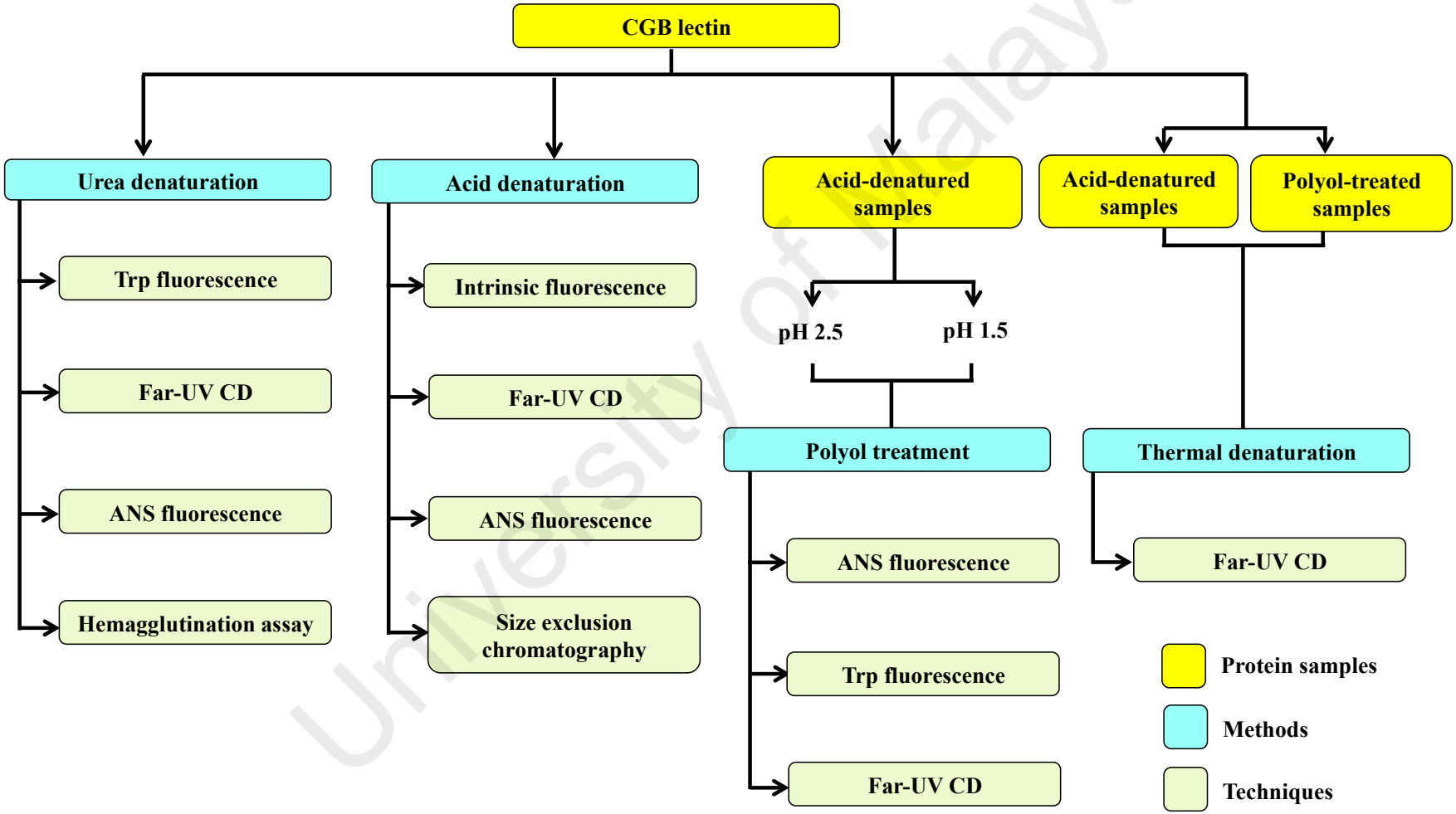
The sugar binding activity of CGB lectin was checked by hemagglutination assay using rabbit erythrocytes. Rabbit blood was obtained from a local rabbit farm, located in Gombak, Kuala Lumpur. Rabbit erythrocytes were separated from the plasma by

centrifugation at 3000 × g at 25°C and washed thrice with 0.15 M sodium chloride before being suspended in PBS (pH 7.2) to obtain 2% erythrocyte suspension. The CGB lectin was incubated at different urea concentrations (0−4.0 M) for 14 h. The assay was carried out by adding 50 μL of the lectin (26 pM) sample and 50 μL of 2% erythrocyte suspension into each well of a 96-well V-bottomed microtitre plates. The plates were incubated at room temperature for 3 h and the highest concentration of urea, which still permitted agglutination, was recorded. Positive agglutination was considered if the erythrocytes remained in suspension after the incubation time, whereas negative hemagglutination was confirmed if the erythrocytes settled down at the bottom of the well. Prior to the hemagglutination assay, a control run was made without CGB lectin to ensure absence of hemolysis caused by urea.

3.2.7. Structural transition studies with polyols

Acid-denatured states of CGB lectin were treated with four different polyols, namely, ethylene glycol, erythritol, xylitol and sorbitol. Acid-denatured samples of the lectin at pH 2.5 and pH 1.5 were prepared in the same way as described in Section 3.2.5.2. The protein samples containing a final polyol concentration of 30% (w/v) were incubated for 14 h at 25°C before recording the CD / fluorescence spectra. The final protein concentration was 0.1 μM for fluorescence spectral measurements and 0.2 μM for far-UV CD spectral measurements and thermal denaturation experiments.

FLOW CHART OF THE RESEARCH WORK



4. RESULTS AND DISCUSSION

4.1. Urea-induced denaturation of CGB lectin

Urea-induced denaturation of CGB lectin was studied using three different spectral probes, namely, far-UV CD spectral signal, Trp fluorescence and ANS fluorescence. Biological activity was also employed as a probe to monitor denaturation using hemagglutination assay.

4.1.1. Far-UV CD

The far-UV CD spectra of CGB lectin (0.2 μM), obtained in PBS (pH 7.2) both in the absence and presence of increasing concentrations (0−9.0 M) of urea are shown in Figure 4.1. CD spectrum of native CGB lectin was characterized by the presence of a negative peak at 218 nm, which is the characteristic feature of β-structure found in proteins (Greenfield, 2006). This agreed well with the three-dimensional structure of CGB lectin, showing abundance of β-structure (Gabrielsen et al., 2014). Addition of increasing concentrations of urea to the protein solution produced an initial increase in the –CD spectral signal, reaching to a maximum (inset of Figure 4.1), followed by a continuous decrease (Figure 4.1). CD spectra could not be obtained between the urea concentrations, 2.0 M and 4.25 M due to significant precipitation. Use of a relatively higher protein concentration (0.2 μM) in CD measurements seems to be responsible for visible protein aggregation. Values of the far-UV CD spectral signal at 218 nm ($CD_{218\ nm}$) were obtained from the CD spectra (Figure 4.1) and transformed into relative $CD_{218\ nm}$ as described in Section 3.2.3.1. A plot of the relative $CD_{218\ nm}$ value against urea concentration yielded the urea denaturation curve, as shown in Figure 4.2.

Urea denaturation curve of CGB lectin was characterized by a two-step, three-state transition, showing a gradual increase in the $-CD_{218\ nm}$ spectral signal up to 4.5 M urea, which remained unchanged up to 5.75 M urea and showed a continuous decrease within 5.75−7.5 M urea concentration range (Figure 4.2). Increase in the $-CD_{218\ nm}$ value up to

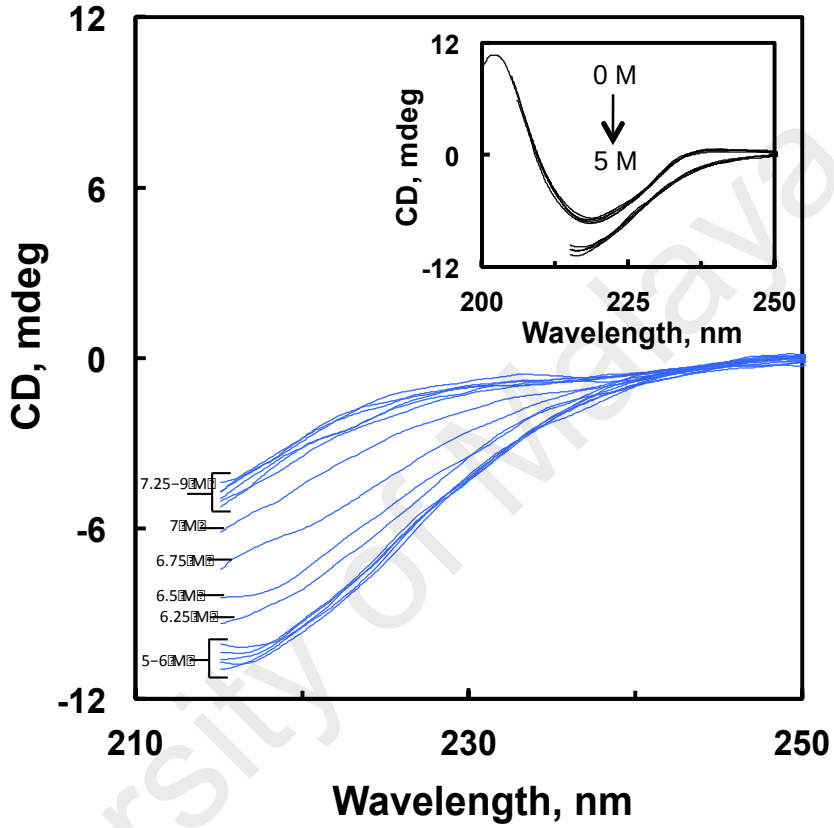


Figure 4.1: Far-UV CD spectra of CGB lectin (0.2 μM), obtained in PBS (pH 7.2) in the absence and presence of increasing concentrations (0−9.0 M) of urea. Arrows show increasing (🡻) and decreasing (🡹) trends of the −CD spectral change, obtained at different urea concentrations. Blue lines depict the CD spectra, obtained at 5.0−9.0 M urea, while the inset shows the CD spectra, acquired at 0−5.0 M urea. CD measurements could not be made within the urea concentration range, 2.0−4.5 M due to significant precipitation.

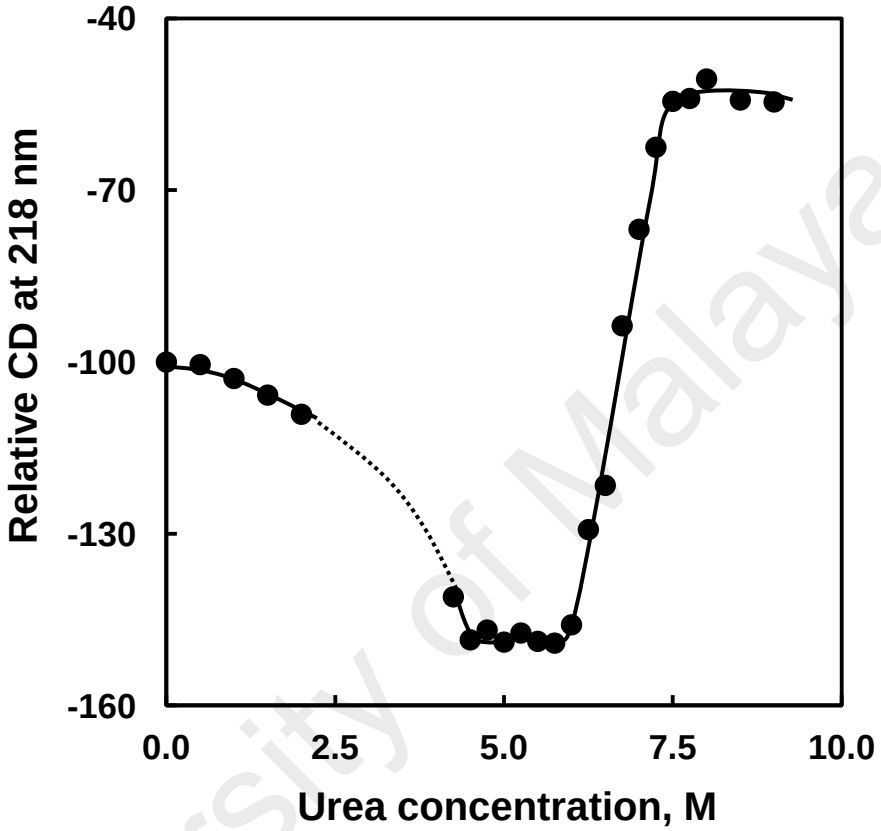


Figure 4.2: Urea denaturation curve of CGB lectin (0.2 μM) in PBS (pH 7.2), as studied by far-UV CD measurements at 218 nm. CD values were transformed into relative CD values by taking the CD value of the native CGB lectin at 218 nm as 100. CD measurements could not be made within the urea concentration range shown by the dotted line due to precipitation.

4.5 M urea clearly suggested formation of additional β structures in the protein. The tetrameric structure of CGB lectin is stabilized by two different interfaces between these subunits, involving both hydrogen bonds and hydrophobic interactions (Gabrielsen et al., 2014). Loss of these interfacial contacts between different subunits in the presence of urea seems to allow the release of structural restrictions, experienced by these subunits in the tetrameric form of the protein. This may be responsible for the formation of additional β structures in the protein. The $CD_{218\ nm}$ value remained constant between 4.5 M and 5.75 M urea, suggesting formation of a stable conformational state of the protein. In view of the tetrameric nature of CGB lectin, it appears that all subunits might have undergone dissociation into monomers at 4.5 M urea. Similar dissociation of tetrameric proteins into structured monomers in the presence of urea has also been reported earlier in concanavalin A and soybean agglutinin (Ghosh & Mandal, 2001; Chatterjee & Mandal, 2003).

A second transition, observed within the urea concentration range, 5.0−9.0 M was marked by a decrease in the $-CD_{218\ nm}$ value. The start- and the end-points of the transition were observed at 5.75 M and 7.5 M urea, respectively (Figure 4.2, Table 4.1). The second transition seems to represent the denaturation of the structured monomers into their unfolded forms due to the loss in the secondary structures, as reflected from the decrease in the $-CD_{218\ nm}$ value. Furthermore, this was supported by the absence of any change in the $CD_{218\ nm}$ value above 7.5 M urea. These results were similar to those shown earlier with concanavalin A, representing unfolding of monomer in the second transition (Chatterjee & Mandal, 2003).

4.1.2. Tryptophan fluorescence

Figure 4.3 shows the Trp fluorescence spectra of CGB lectin (0.1 μM), obtained in PBS (pH 7.2), both in the absence and presence of increasing concentrations (0−9.0

Table 4.1: Characterization of urea denaturation curves of CGB lectin in PBS (pH 7.2), as studied by different probes.

Probe	Urea (M)			
	First transition		Second transition	
	Start-point	End-point	Start-point	End-point
$CD_{218\ nm}$	-	-	5.75	7.50
Relative fluorescence intensity at 333 nm	2.60	4.50	5.75	7.50
Emission maximum	2.00	4.50	5.75	7.75
ANS fluorescence intensity at 461 nm	1.50	4.20	4.20	6.80

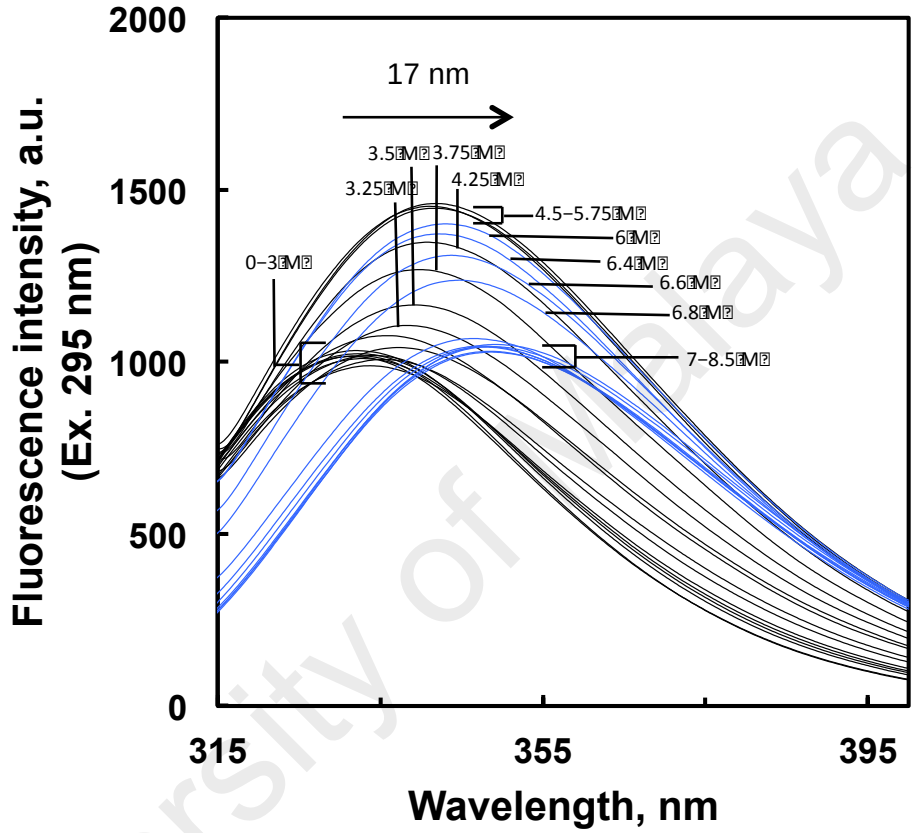


Figure 4.3: Tryptophan fluorescence spectra of CGB lectin (0.1 μM), obtained in PBS (pH 7.2) in the absence and presence of increasing concentrations (0−9.0 M) of urea, upon excitation at 295 nm. Arrows show increasing (🡅) and decreasing (🡇) trends of the spectral change, obtained at different urea concentrations. Black lines show the fluorescence spectra, obtained at 0−5.0 M urea, whereas blue lines depict the fluorescence spectra, obtained at 5.0−9.0 M urea.

M) of urea. Native CGB lectin produced a fluorescence spectrum with an emission maximum at 333 nm due to the presence of Trp residues (Gabrielsen et al., 2014).

Addition of increasing urea concentrations to CGB lectin led to a gradual red shift in the emission maximum. A total of 17 nm red shift in the emission maximum was observed at the highest urea concentration, which was suggestive of protein denaturation. However, the fluorescence intensity gradually increased in the initial urea concentration range and showed a steady decrease thereafter (Figure 4.3). Red shift in the emission maximum of a protein is usually found upon denaturation, irrespective of the change in the quantum yield / fluorescence intensity (Eftink, 1994). In order to better understand these changes in the fluorescence characteristics of the protein, values of the emission maximum and relative fluorescence intensity at 333 nm (Section 3.2.3.2) were plotted against urea concentration (Figures 4.4 and 4.5).

As can be seen from Figure 4.4, the denaturation curve (emission maximum versus urea concentration) shows a two-step, three-state transition. No change in the emission maximum was noticed up to 2.0 M urea, beyond which a gradual increase in the emission maximum was observed up to 4.5 M urea. The second transition started at 5.75 M urea and was characterized by the end-point at 7.75 M urea (Figure 4.4, Table 4.1). Generally, red shift in the emission maximum indicates change in the microenvironment of Trp residues from nonpolar to polar (Lakowicz, 2006). Such a change in the microenvironment around Trp residues may be explained in the following two ways. Either surface exposure of Trp residues to aqueous environment or movement of charged groups in the vicinity of Trp residues can produce a red shift in the emission maximum of a protein (Lakowicz, 2006). Movement of charged groups in the vicinity of Trp residues seems to be responsible for the red shift, observed in the first transition, while surface exposure of Trp residues to aqueous environment can explain the second transition, which was characterized by the appearance of the

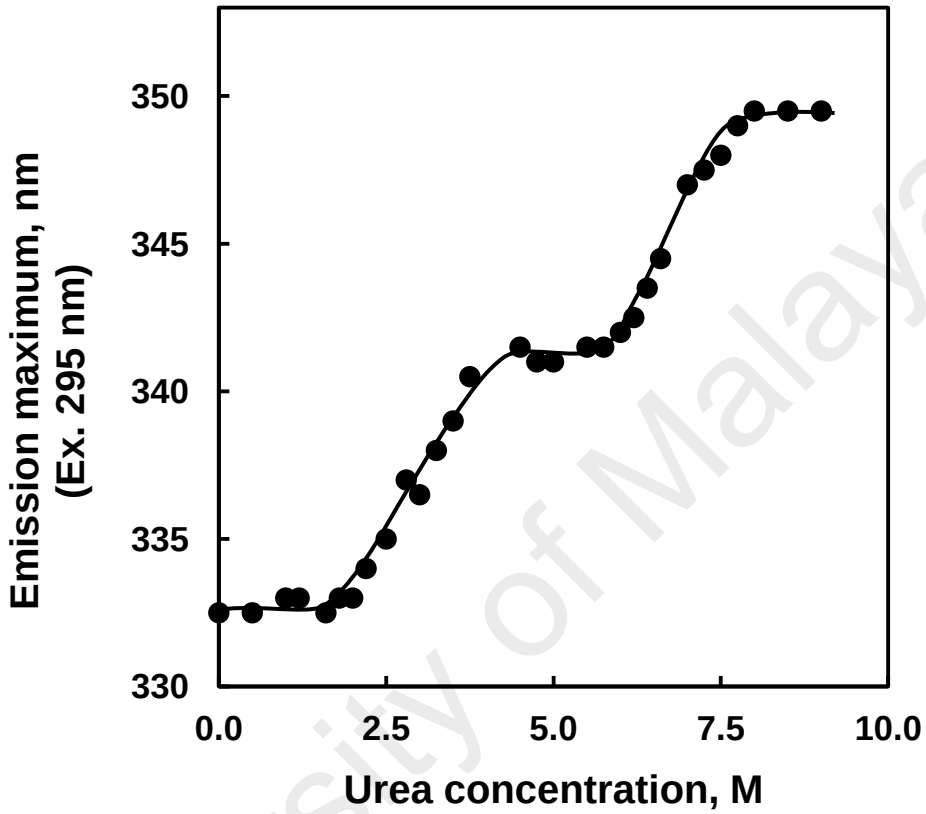


Figure 4.4: Urea denaturation curve of CGB lectin (0.1 μM) in PBS (pH 7.2), as studied by emission maximum measurements. The protein samples were excited at 295 nm.

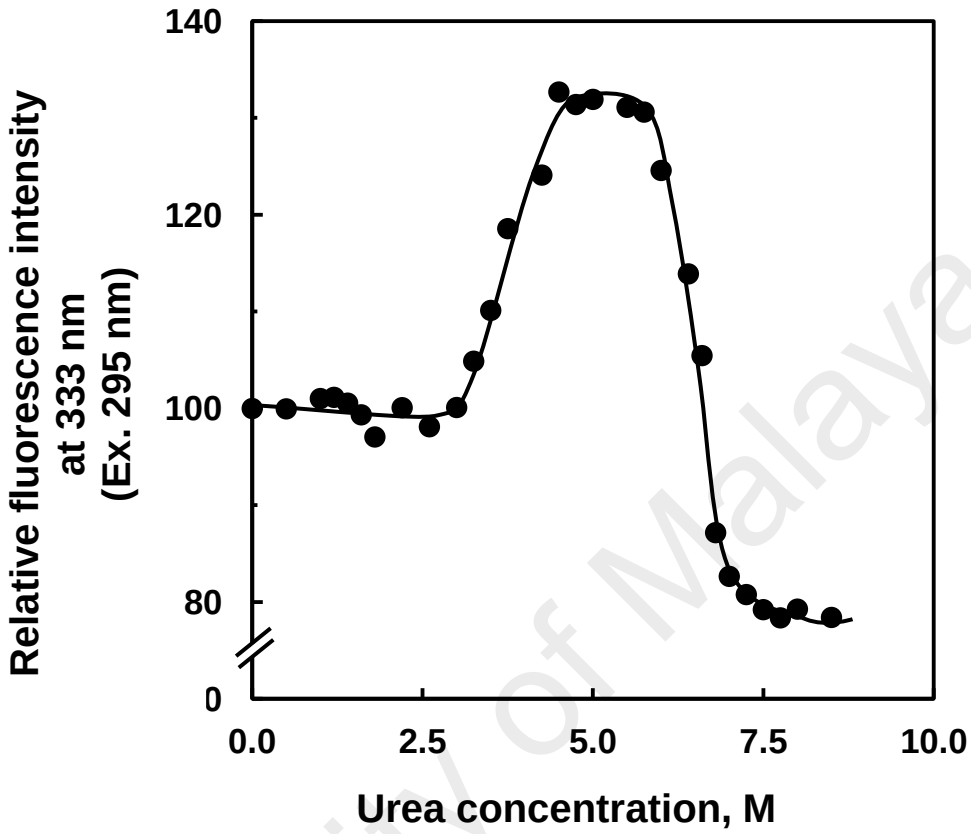


Figure 4.5: Urea denaturation curve of CGB lectin (0.1 μM) in PBS (pH 7.2), as studied by fluorescence intensity measurements at 333 nm. Values of the relative fluorescence intensity at different urea concentrations were obtained by taking the fluorescence intensity of the native CGB lectin at 333 nm as 100. An excitation wavelength of 295 nm was used to obtain the fluorescence intensity.

emission maximum at 350 nm. Tryptophan fluorescence peak usually occurs near 350 nm, when one or both faces of benzene rings are water-exposed (Eftink, 1994; Lakowicz, 2006), which is possible when protein is completely unfolded. These results were supported by our CD spectral results (Figure 4.2), showing formation of additional secondary structures during the first transition, followed by disruption of these structures at higher urea concentrations. Earlier studies on tetrameric lectins, i.e. concanavalin A, soybean agglutinin and peanut agglutinin have also shown a two-step, three-state transition in urea denaturation, when studied by emission maximum measurements (Ghosh & Mandal, 2001; Chatterjee & Mandal, 2003; Dev et al., 2006).

As evident from Figure 4.5, the fluorescence intensity remained constant up to 2.6 M urea, showed a gradual increase, reaching to a maximum value at 4.5 M urea and plateaued up to 5.75 M urea. Further increase in the urea concentration led to a progressive decrease in the fluorescence intensity up to 7.5 M urea, before leveling off (Figure 4.5). In other words, urea denaturation of CGB lectin was found to be a two-step transition. The first transition, marked by the increase in the fluorescence intensity was characterized by the occurrence of the start- and the end-points at 2.6 M and 4.5 M urea, respectively (Figure 4.5, Table 4.1). Such increase in the fluorescence intensity may be ascribed to the separation of quenching residues from the Trp residues (Ando et al., 2008). This seems understandable as monomerization of tetramer might have relieved such quenching of Trp fluorescence. The second transition displayed a decrease in the fluorescence intensity, starting from 5.75 M urea and ending at 7.5 M urea (Figure 4.5, Table 4.1). This decrease in the fluorescence intensity clearly indicated formation of the unfolded structure with surface-exposed Trp residues, which was well-supported by the results showing loss in the secondary structures at higher urea concentrations (Figure 4.2). Similar increase in the fluorescence intensity at lower urea concentrations and decrease at higher urea concentrations have also been observed in

the urea denaturation of concanavalin A (Chatterjee & Mandal, 2003). As evident from Figures 4.2, 4.4 and 4.5, a stable equilibrium intermediate seems to exist within the urea concentration range, 4.5−5.75 M.

4.1.3. ANS fluorescence

ANS binding was also used as a probe to observe urea denaturation of CGB lectin. ANS shows fluorescence upon binding to hydrophobic clusters (Horowitz & Butler, 1993), which may be more exposed in the denatured form. The ANS fluorescence spectra of CGB lectin in PBS (pH 7.2) at increasing concentrations (0−9.0 M) of urea are shown in Figure 4.6. No significant ANS fluorescence was observed with the native CGB lectin due to the absence of hydrophobic clusters at the protein's surface. However, addition of urea produced an increase in the ANS fluorescence intensity up to 4.2 M, followed by a decrease at higher urea concentrations (Figure 4.6). Values of the ANS fluorescence intensity at 461 nm were plotted against urea concentrations, as shown in Figure 4.7. Urea denaturation curve, monitored by ANS fluorescence measurements, was also characterized as a two-step transition. There was an increase in the ANS fluorescence intensity within 1.5−4.2 M urea concentration range (Figure 4.7, Table 4.1). Monomerization of the CGB lectin could be the determinant for such increase in ANS binding as it might have exposed the hydrophobic clusters otherwise concealed in the tetramer. For the second transition, the denaturation curve immediately plunged down from 4.2 M urea towards 6.8 M urea, showing complete abolishment of ANS binding (Figure 4.7, Table 4.1). Gradual loss of the ANS fluorescence intensity above 4.2 M urea can be attributed to the deterioration of the structured monomers (Varejão et al., 2011), which might have caused the hydrophobic clusters to disintegrate. Furthermore, urea itself might have also interrupted the ANS binding to hydrophobic clusters of the protein (Horowitz & Butler, 1993).

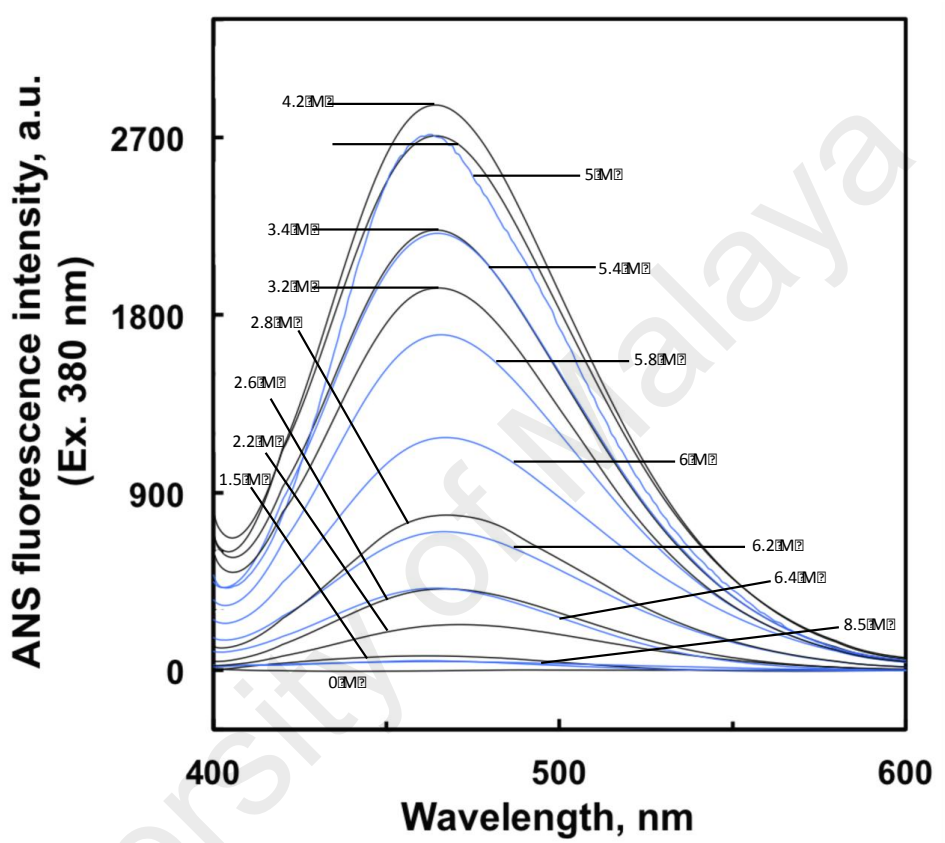


Figure 4.6: ANS fluorescence spectra of CGB lectin (0.1 μM), obtained in PBS (pH 7.2) in the absence and presence of increasing concentrations (0−9.0 M) of urea, upon excitation at 380 nm. Arrows show increasing (↑) and decreasing (↓) trends of the spectral change, obtained at different urea concentrations. Black lines show the fluorescence spectra, obtained at 0−4.2 M urea, whereas blue lines depict the fluorescence spectra, obtained at 4.2−9.0 M urea.

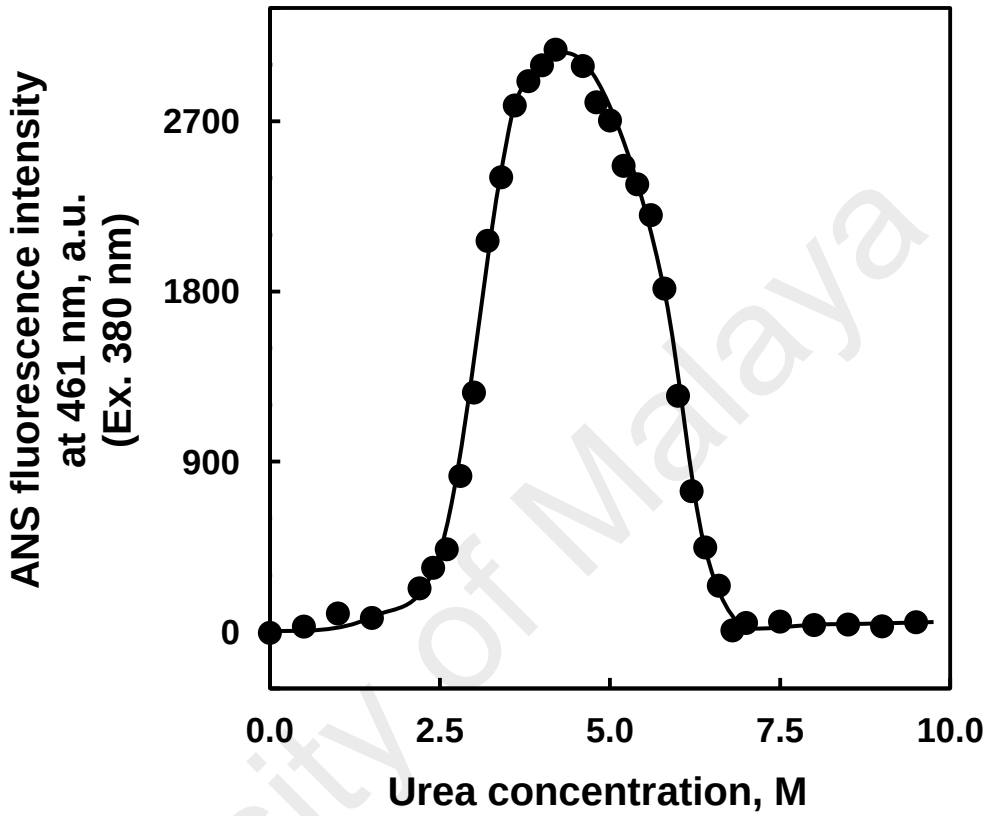


Figure 4.7: Urea denaturation curve of CGB lectin (0.1 μM) in PBS (pH 7.2), as studied by ANS fluorescence measurements at 461 nm. An excitation wavelength of 380 nm was used to obtain the ANS fluorescence intensity.

The denaturation curve followed by ANS binding (Figure 4.7) showed slight variation in the start- and the end-points for both first and second transitions, when compared to the denaturation curves, obtained using other probes (Figures 4.2, 4.4, 4.5), which exhibited almost similar start- and end-point values. This was not unusual, as several earlier reports have shown different transition characteristics of the denaturation curves, when studied using different probes (Muzammil et al., 2000; Halim et al., 2008; Kishore et al., 2012). Irrespective of the smaller variations, the latter three probes displayed the formation of a stable intermediate state in a relatively wide (4.5−5.75 M) urea concentration range.

4.1.4. Hemagglutinating activity

The hemagglutinating activity of CGB lectin was carried out in order to evaluate the sugar-binding ability as well as retention of the lectin tetrameric structure at increasing urea concentrations. Hemagglutination assay was carried out with CGB lectin in the presence of urea (0.5−4.0 M) to determine the point of denaturation, where the lectin loses its binding activity towards its ligand. Higher concentrations (>4.0 M) of urea could not be employed as it led to significant hemolysis of rabbit erythrocytes. Figure 4.8 shows rabbit erythrocyte suspension incubated with CGB lectin, which had earlier been incubated with increasing concentrations of urea (0.0−4.0 M) for 14 h. As can be clearly seen from the Figure 4.8, erythrocytes remained in suspension in the well, showing positive hemagglutinating activity of the lectin in the native form as well as up to 2.5 M urea-treatment. Up to this urea concentration, the lectin binding sites were conserved and retained their binding affinity towards the galactose residues, present on the erythrocytes' surface. At 3.0 M urea, the lectin showed reduced ability to agglutinate the erythrocytes and the agglutinating activity was totally lost at 4.0 M urea. Since this urea concentration falls in the range of the first transition, involving monomerization of the tetrameric lectin, absence of agglutination in the presence of 4.0

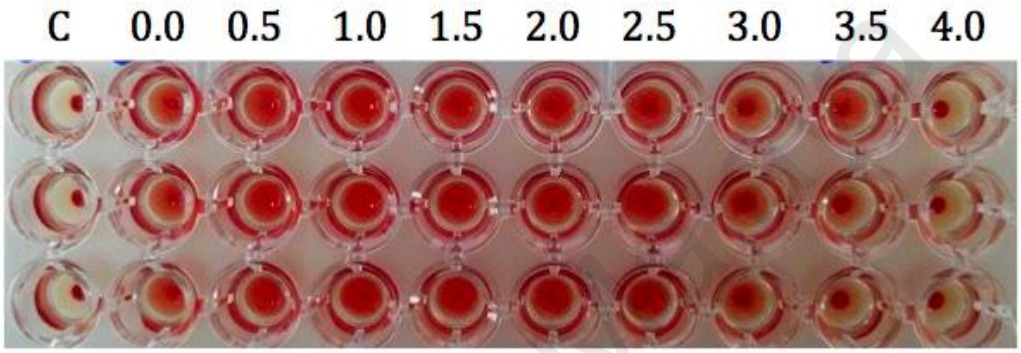


Figure 4.8: Hemagglutination assay of CGB lectin (26 pM) in the absence (0 M urea) and presence of 0.5–4.0 M urea. The control (C), representing the RBC suspension in PBS (pH 7.2), has been shown at the extreme left. The assay was carried out in triplicate.

M urea seems justifiable, as all molecules of the tetrameric lectin would have been converted into monomeric form at this urea concentration.

4.2. Characterization of the native and urea-denatured states of CGB lectin

In order to characterize the intermediate state of CGB lectin, spectral properties of the native state, the intermediate state at 5.0 M urea and the completely denatured state at 9.0 M urea were compared. Spectral characterization of these three states was made by analyzing their far-UV CD spectra, Trp fluorescence spectra and ANS fluorescence spectra.

4.2.1. Far-UV CD spectra

Figure 4.9 shows the far-UV CD spectra of the native, the intermediate and the completely denatured states of CGB lectin. The far-UV CD spectral characteristics of the native state were similar to a β-sheet protein due to the presence of a minimum at 218 nm (Greenfield, 2006). This agreed well with the presence of a high percentage of β-sheet structure in CGB lectin (Gabrielsen et al., 2014). Interestingly, the far-UV CD spectrum of the intermediate state showed ~53% increase in the $CD_{218\ nm}$ value compared to the native state (Table 4.2). This seems plausible due to the loss of intersubunit interactions during monomer dissociation at this urea concentration, which were supposed to govern the secondary structures of these subunits / monomers in the bound tetrameric state. Reformation of the secondary structures in the intermediate state induced by urea has also been reported in several other studies on tetrameric and dimeric proteins (Chattoraj et al., 1996; Chatterjee & Mandal, 2003; Varejão et al., 2011; Ghosh & Mandal, 2012). On the other hand, the far-UV CD spectrum of CGB lectin in 9.0 M urea showed ~50% decrease in the $CD_{218\ nm}$ value from that found in the native state (Table 4.2). Such decrease in the $CD_{218\ nm}$ value is expected, as 9.0 M urea

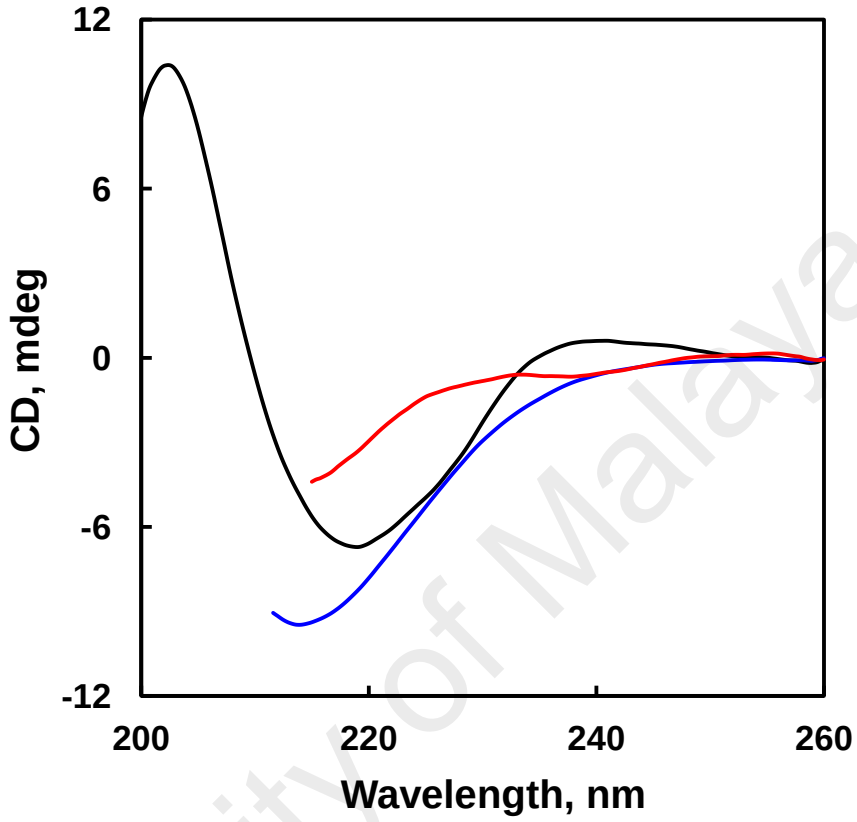


Figure 4.9: Far-UV CD spectra of CGB lectin (0.2 μM) in the absence (*black line*) and presence of 5.0 M urea (*blue line*) and 9.0 M urea (*red line*) in PBS (pH 7.2) at 25°C. The spectra could not be obtained below 218 nm in the presence of urea due to high signal-to-noise ratio.

Table 4.2: Spectral characteristics of CGB lectin in PBS (pH 7.2) at different urea concentrations, as monitored by different probes.

Probe	CGB lectin		
	Native	**+ 5.0 M urea**	**+ 9.0 M urea**
$CD_{218\ nm}$ (mdeg)	−6.89	−10.52	−3.48
Trp fluorescence intensity at emission maximum (a.u.)	1022	1468	1122
Emission maximum (nm)	333	341	350
ANS fluorescence intensity at 461 nm (a.u.)	0	2706	13

is sufficient to remove noncovalent interactions, required to form secondary structures (Bennion & Daggett, 2003).

4.2.2. Tryptophan fluorescence spectra

The fluorescence spectra of the native, the intermediate and the completely denatured states are shown in Figure 4.10. The fluorescence spectrum of the intermediate state showed a significant increase (~44%) in the fluorescence intensity at the emission maximum along with a red shift (8 nm) in the emission maximum, when compared to the fluorescence characteristics of the native state (Table 4.2). As discussed in Section 4.1.2, the fluorescence intensity of CGB lectin seems to be quenched in the native tetrameric state due to the presence of quenchers in the vicinity of Trp residues (Ando et al., 2008). Dissociation of these subunits into monomeric forms at 5.0 M urea might have distanced away the quenching residues from Trp residues, thus leading to an increase in the fluorescence intensity. Increase in the fluorescence intensity upon urea denaturation has also been observed in lysozyme and ficin (Ong et al., 2009; Sidek et al., 2013). In contrast, at higher urea concentration (9.0 M), the emission maximum was moved to 350 nm along with a decrease in the fluorescence intensity (Figure 4.10, Table 4.2), indicating exposure of Trp residues to the aqueous environment. In view of the occurrence of the emission maximum at 350 nm of the free Trp in aqueous buffer (Schiller, 1985), CGB lectin in 9.0 M urea is supposed to be in completely unfolded form with surface-exposed Trp residues.

4.2.3. ANS fluorescence spectra

Figure 4.11 shows the ANS fluorescence spectra of the native, the intermediate and the completely denatured states of CGB lectin, observed at 0.0 M, 5.0 M and 9.0 M urea, respectively. The native state showed negligible ANS fluorescence intensity along the emission wavelength range, suggesting absence of the exposed hydrophobic clusters in the native state. However, a marked increase in the ANS fluorescence intensity (2706

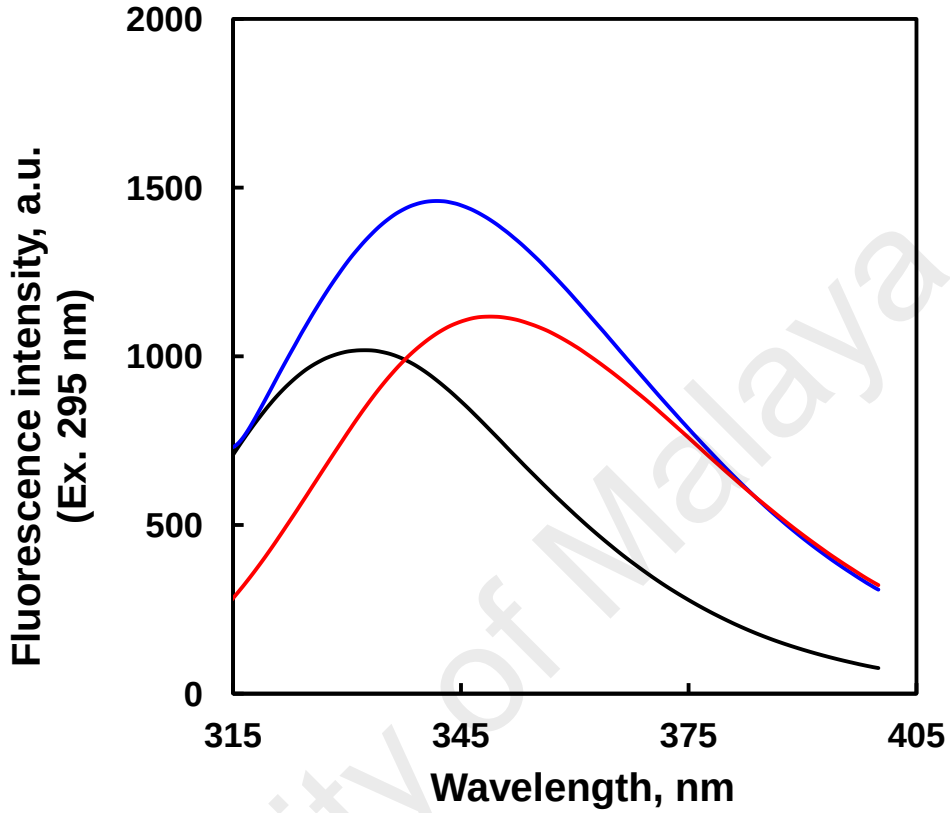


Figure 4.10: Tryptophan fluorescence spectra of CGB lectin (0.1 μM) in the absence (*black line*) and presence of 5.0 M urea (*blue line*) and 9.0 M urea (*red line*) in PBS (pH 7.2) at 25°C. The protein samples were excited at 295 nm.

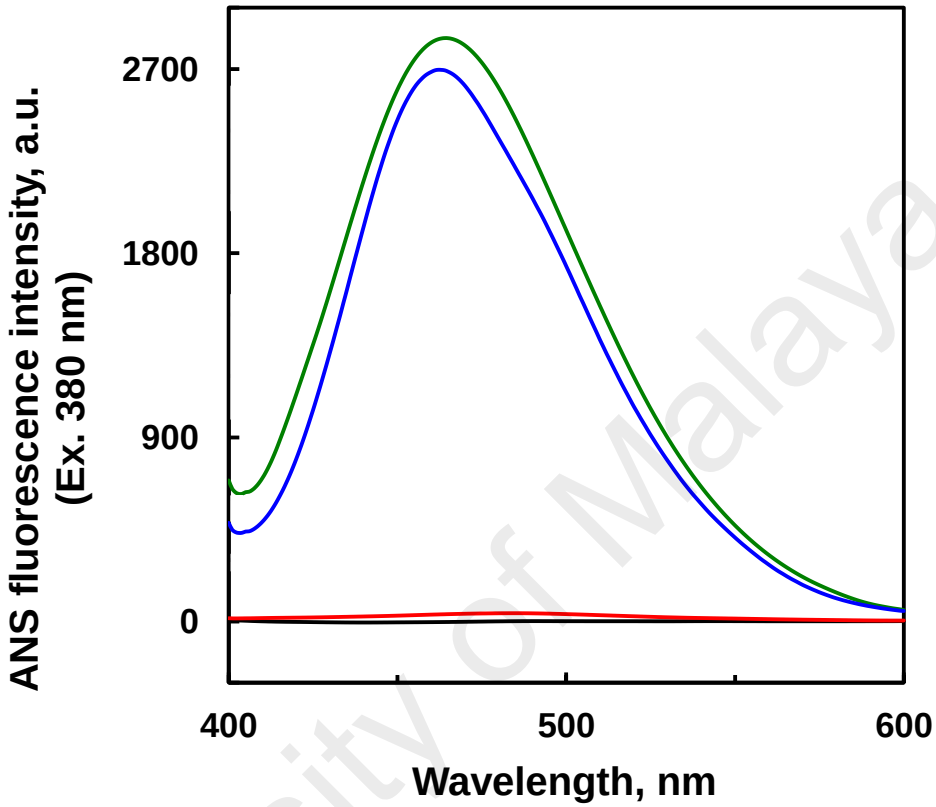


Figure 4.11: ANS fluorescence spectra of CGB lectin (0.1 μM) in the absence (*black line*) and presence of 4.2 M urea (*green line*), 5.0 M urea (*blue line*) and 9.0 M urea (*red line*) in PBS (pH 7.2) at 25°C. The protein samples were excited at 380 nm.

a.u.) was observed with the intermediate state, showing emission maximum at 461 nm (Figure 4.11, Table 4.2). This can be attributed to the dissociation of monomers of the CGB lectin in 5.0 M urea, thus exposing the hydrophobic clusters, which were involved in the interfacial contacts between the subunits to stabilize the quaternary structure (Wójciak et al, 2003). ANS fluorescence intensity was completely lost in the presence of 9.0 M urea (Figure 4.11, Table 4.2) due to disintegration of the hydrophobic clusters as well as weakening of the hydrophobic interactions, which are known to be involved in ANS binding to proteins (Hawe et al., 2008). In other words, CGB lectin seems to acquire the unfolded conformation in the presence of 9.0 M urea, showing lack of ANS binding, as observed with other urea-denatured proteins (Chatterjee & Mandal, 2003; Varejão et al., 2011). Since urea denaturation curve, as probed by ANS fluorescence (Figure 4.7) showed the mid-point between two transitions around 4.2 M urea, ANS fluorescence characteristics of CGB lectin in 4.2 M urea were also studied. As can be seen from Figure 4.11, the fluorescence spectrum was found similar (fluorescence intensity at 461 nm = 3080 a.u.) to the one observed at 5.0 M urea.

4.3. Acid-induced denaturation of CGB lectin

Acid-induced conformational changes in CGB lectin were studied in the pH range, 7.0–1.0, using intrinsic fluorescence and ANS fluorescence spectral signals as probes.

4.3.1. Intrinsic fluorescence

Figure 4.12 shows the fluorescence spectra of CGB lectin (0.1 μM), obtained at different pH values in the range, pH 7.0−1.0. The fluorescence spectrum of the native CGB lectin showed an emission maximum at 330 nm. The spectral signal showed a progressive decrease upon decreasing the pH from pH 7.0 to pH 1.0, without any change in the emission maximum. Plotting the values of the fluorescence intensity at 330 nm against pH yielded the acid denaturation curve of CGB lectin (Figure 4.13). The

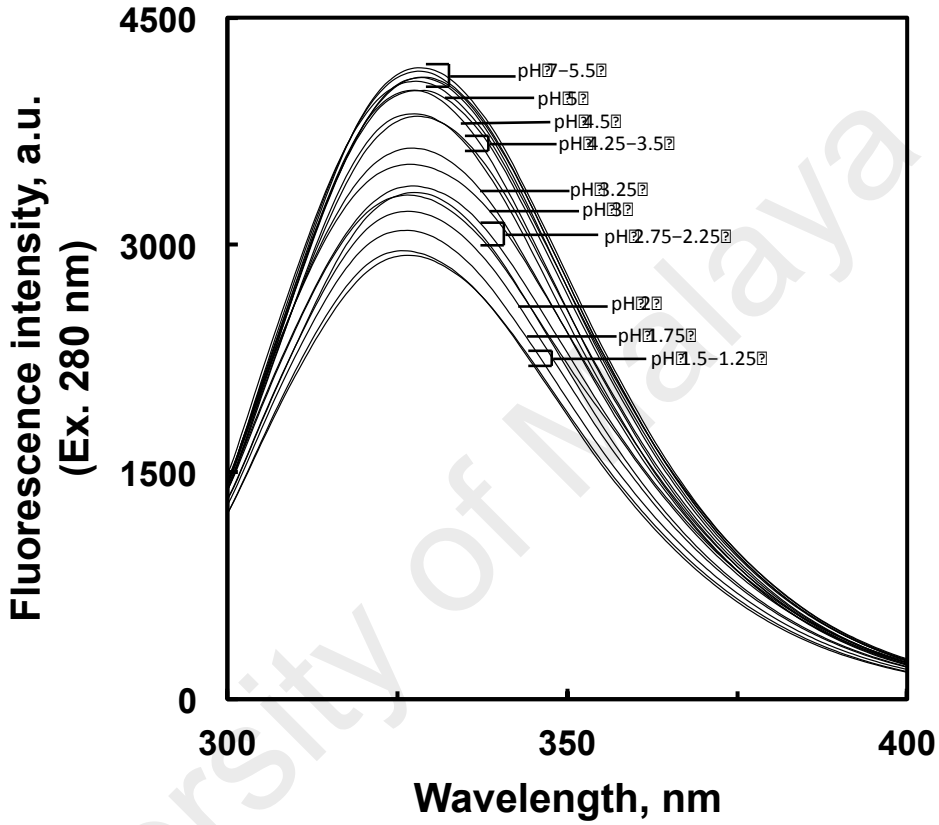


Figure 4.12: Fluorescence spectra of CGB lectin (0.1 μM), obtained at different pH values, upon excitation at 280 nm. Arrow shows the decreasing trend (🡻) of the spectra from top to bottom, obtained in the pH range, 7.0−1.0. Various buffers (10 mM) used to obtain different pH values were: sodium phosphate buffer (pH 7.0−6.0), sodium acetate buffer (pH 5.5−3.75), glycine-HCl buffer (pH 3.5−2.0) and KCl-HCl mixture (pH 1.75−1.0).

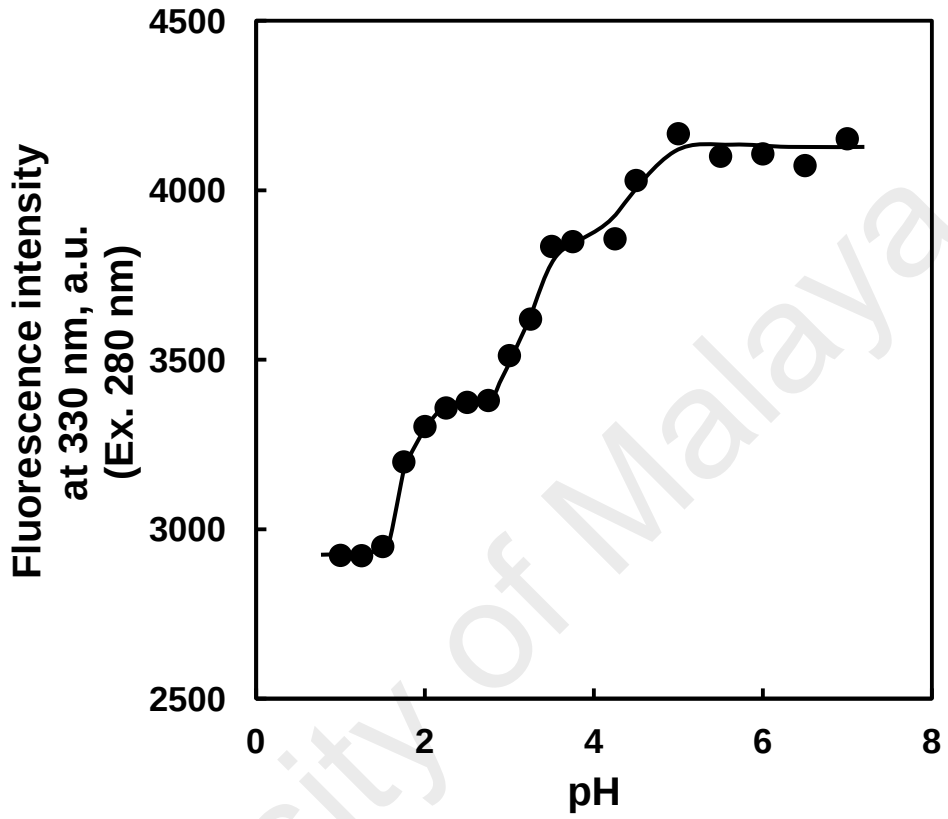


Figure 4.13: Acid denaturation curve of CGB lectin (0.1 μM), as studied by fluorescence intensity measurements at 330 nm. The protein samples were excited at 280 nm.

fluorescence intensity showed no significant variation in the pH range, 7.0−5.0. A smaller decrease in the fluorescence intensity was observed below pH 5.0, which became stagnant in the pH range, 4.5−3.5. Such change in the fluorescence intensity can be ascribed to the local disordering within each monomer of the tetrameric lectin, which might have changed the polarity around the aromatic fluorophores. Further decrease in the pH below pH 3.5 produced a conformational transition, which ended at pH 2.75 and was characterized by the significant decrease in the fluorescence intensity. Dissociation of the homotetrameric lectin into its individual subunits (monomers) might be responsible for this transition. Exposure of the aromatic fluorophores (Tyr and Trp), which were otherwise buried in the interface between the subunits of the lectin (Gabrielsen et al., 2014), to a polar environment could have led to a significant decrease in the fluorescence intensity. Another transition, marked by a further decrease in the fluorescence intensity occured between pH 2.25 and pH 1.5, which might be explained by the denaturation of the separated monomers into their unfolded forms. In a previous study, concanavalin A has also been shown to exhibit relatively stable fluorescence intensity in the pH range, 7.0−5.0, followed by significant loss in the fluorescence signal down to pH 2.0 (Khan et al., 2005).

4.3.2. ANS fluorescence

ANS binds to the hydrophobic clusters available at the surface of the protein (Horowitz & Butler, 1993). Treatment of a protein with acidic pH exposes buried hydrophobic clusters and results in an increase in ANS binding (Fink et al., 1994). Figure 4.14 shows the ANS fluorescence spectra of CGB lectin exposed to different pH values in the range, 7.0−1.0. There was an initial increase in the ANS fluorescence spectral signal upon decreasing the pH from pH 7.0 to pH 2.5, followed by a decrease in the ANS fluorescence up to pH 1.5. Decreasing the pH further from pH 1.5 to pH 1.0

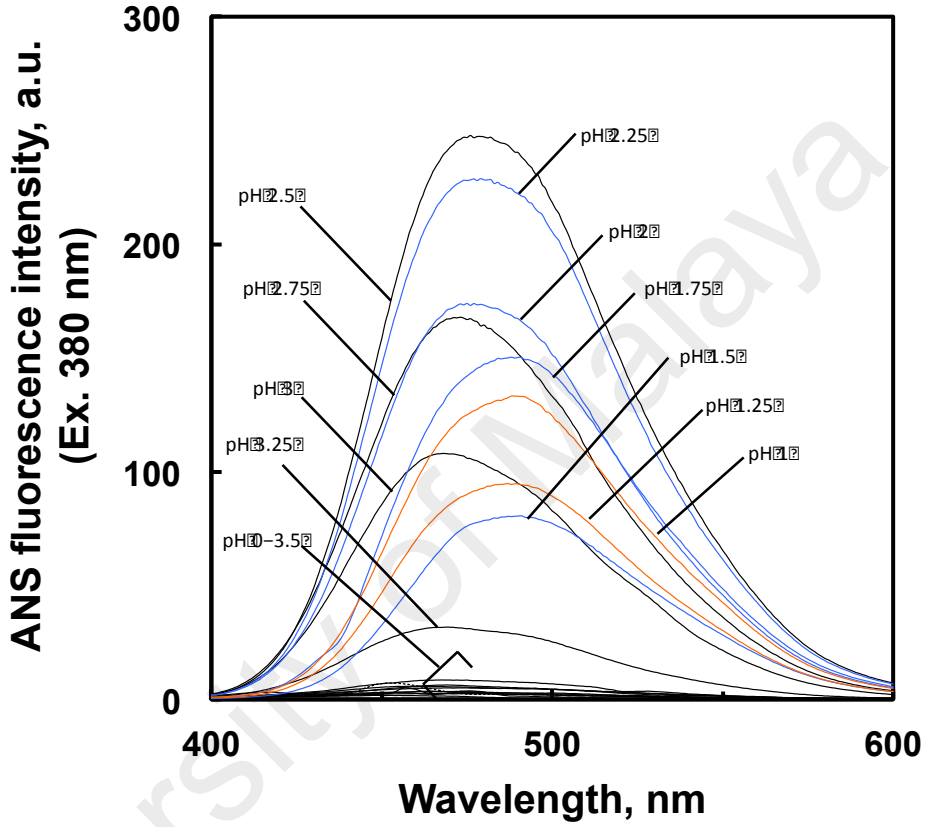


Figure 4.14: ANS fluorescence spectra of CGB lectin (0.1 μM), obtained at different pH values, upon excitation at 380 nm. Arrows show increasing (↑) and decreasing (↓) trends of the spectral change, obtained at different pH values. Various colors show the spectra, obtained in different pH ranges; black (pH 7.0−2.5), blue (pH 2.5−1.5) and orange (pH 1.5−1.0).

led to a significant recovery in the ANS spectral signal (Figure 4.14). Such variation in the ANS spectral signal with pH can be clearly seen from Figure 4.15, which shows the acid-induced conformational changes in CGB lectin, as probed by the change in the ANS fluorescence intensity at 468 nm (emission maximum observed at pH 7.0).

The acid denaturation curve of CGB lectin showed a two-step, three-state transition (Figure 4.15). This was slightly different from the denaturation behavior, observed with fluorescence intensity signal where a significant change in the spectral signal was noticed between pH 5.0 and pH 4.5 (Section 4.3.1, Figure 4.13). Such kind of difference in the denaturation pattern was not unusual, as many earlier studies have shown similar behavior (Muzammil et al., 2000; Halim et al., 2008; Kishore et al., 2012). Intrinsic fluorescence intensity signal characterizes the changes in the microenvironment around Tyr and Trp residues, which are quite evenly distributed throughout the molecule (Gabrielsen et al., 2014). In contrast, ANS fluorescence signal characterizes the appearance of hydrophobic clusters upon denaturation.

As seen from Figure 4.15, a significantly lower, almost negligible ANS fluorescence intensity was observed at pH 7.0, which remained constant down to pH 3.5. These results indicated the absence of the hydrophobic clusters at the protein surface, available for ANS binding. In other words, the protein seems to be fairly stable within the pH range, 7.0–3.5, with respect to the exposure of the hydrophobic clusters. This was in accordance to our intrinsic fluorescence results (Figure 4.13), suggesting local disordering within each monomer up to pH 3.5. Interestingly, further decrease below pH 3.5 produced a marked increase in the ANS fluorescence intensity up to pH 2.5. Such increase can be ascribed to the separation of subunits in the lectin tetramer, leading to structured monomers at pH 2.5. This seems understandable, as protonation of aspartic acid and glutamic acid residues of the protein might have disturbed the charge balance, leading to electrostatic repulsion, which was sufficient to separate monomers

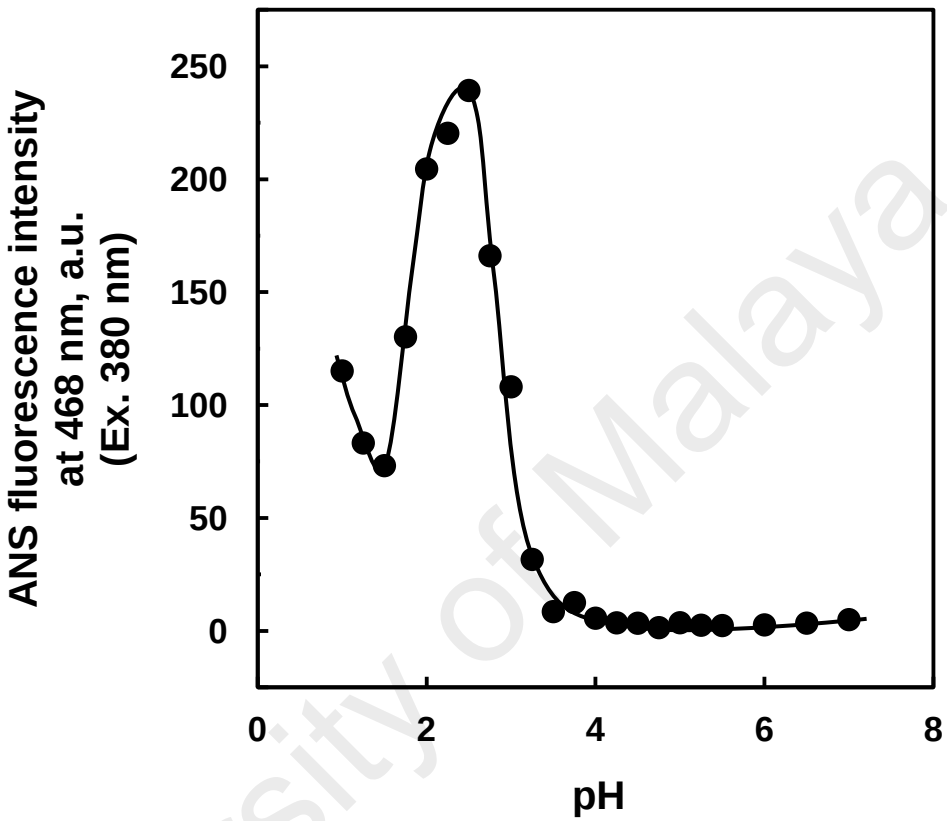


Figure 4.15: Acid denaturation curve of CGB lectin (0.1 μM), as studied by ANS fluorescence measurements at 468 nm. An excitation wavelength of 380 nm was used to obtain the ANS fluorescence intensity.

of the lectin without affecting their tertiary structures. Thus, large number of hydrophobic clusters in these monomers might have been exposed and made available for ANS binding, leading to an increase in the ANS fluorescence intensity. Lowering the pH from pH 2.5 to pH 1.5 produced the second transition, which was characterized by the decrease in the ANS fluorescence intensity. The loss in the ANS fluorescence intensity can be attributed to the unfolding of the structured monomers into the acid-denatured forms. Such unfolding might have disrupted the hydrophobic clusters, resulting in a decrease in the ANS binding and thus the ANS fluorescence intensity.

Similar denaturation curves have been reported with acid denaturation of other tetrameric lectins such as jacalin and soybean agglutinin (Sahasrabuddhe et al., 2004; Alam et al., 2015). Further increase in acidity up to pH 1.0 seems to induce refolding of the lectin, which might form hydrophobic pockets, available for ANS binding (Goto et al., 1990a). This can be seen from the increase in the ANS fluorescence intensity at pH values below pH 1.5.

4.4. Characterization of the native and acid-denatured states of CGB lectin

The native and various acid-denaturated states of CGB lectin were characterized by their fluorescence spectra, ANS fluorescence spectra, far-UV CD spectra, gel chromatographic profiles and thermal denaturation behavior.

4.4.1. Fluorescence spectra

Figure 4.16 depicts the intrinsic fluorescence spectra of different states of CGB lectin, obtained at pH 7.0 (native state) as well as at pH values 3.5, 2.5 and 1.5 (acid-denatured states). The fluorescence spectrum of CGB lectin in the native state at pH 7.0 produced an emission maximum at 330 nm. Free Trp in a polar environment typically exhibits an emission maximum above 350 nm, which is blue-shifted to a lower wavelength as the polarity of its microenvironment decreases (Eftink, 1994). In view of it, appearance of the emission maximum at 330 nm simply reflects the nonpolar

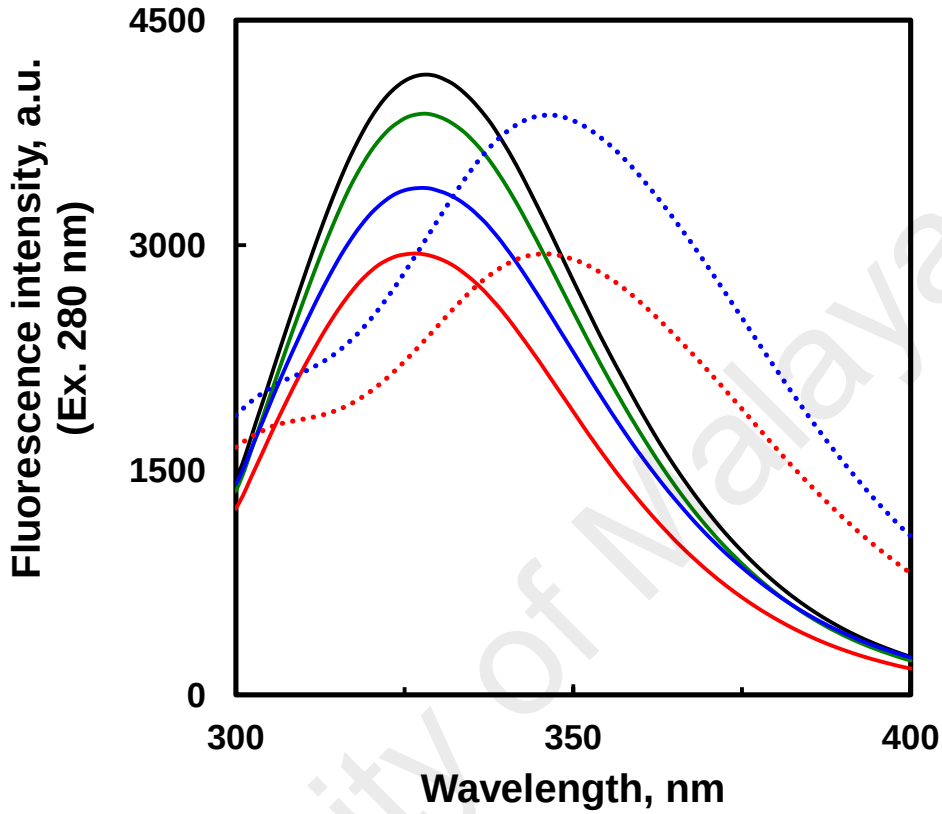


Figure 4.16: Fluorescence spectra of CGB lectin (0.1 μM) at pH 7.0 (*black line*), pH 3.5 (*green line*), pH 2.5 (*blue lines*) and pH 1.5 (*red lines*), as studied at 25°C. Fluorescence spectra of the protein samples containing 6 M GdnHCl are shown with dotted lines. The protein samples were excited at 280 nm.

environment of Trp residues in the native protein. In other words, these Trp residues seem to be buried in the protein interior. The fluorescence spectra, obtained with the acid-denatured states of the lectin showed relatively weaker fluorescence spectral signals, the decrease being more pronounced at pH 1.5. Quantitatively, around 30% decrease in the fluorescence intensity was observed from pH 7.0 to pH 1.5 (Table 4.3). Such a gradual change in the fluorescence intensity, obtained with the acid-denatured states, indicated progressive exposure of aromatic fluorophores, reflecting a stepwise denaturation process of the tetrameric lectin into their unfolded monomers. In order to check if the acid-denatured state at pH 1.5 represented the fully extended state of the protein, CGB lectin was treated with 6 M GdnHCl both at pH 2.5 and pH 1.5. Interestingly, 16 nm red shift in the emission maximum was observed with 6 M GdnHCl-treated both at pH 2.5 and pH 1.5 (Figure 4.16). Occurrence of the emission maximum at 346 nm clearly reflected far greater exposure of the Trp residues to polar environment compared to that observed in the absence of GdnHCl, thus suggesting incomplete denaturation of CGB lectin at pH 1.5.

4.4.2. ANS fluorescence spectra

The ANS fluorescence spectra of the native and various acid-denatured states of CGB lectin are shown in Figure 4.17. CGB lectin in the native state at pH 7.0 as well as at pH 3.5 showed negligible ANS fluorescence intensity (Table 4.3) due to the absence of hydrophobic clusters for ANS binding. This suggested that exposure of CGB lectin to pH 3.5 was not sufficient to bring major conformational change in the protein. Interestingly, a striking increase in the ANS fluorescence intensity at 468 nm was observed with CGB lectin at pH 2.5 (Figure 4.17, Table 4.3), which can be explained by the exposure of the hydrophobic clusters, supposed to be involved in the inter-subunit interactions in the native protein. A remarkable decrease in the ANS fluorescence intensity (~70% decrease relative to that observed at pH 2.5) characterized the acid-

Table 4.3: Spectral characteristics of different conformational states of CGB lectin at different pH values, as studied by different probes.

Probe	CGB lectin				
	pH 7.0	**pH 3.5**	**pH 2.5**	**pH 1.5**	**pH 1.0**
Fluorescence intensity at 330 nm (a.u.)	4153	3835	3375	2950	-
ANS fluorescence intensity at 468 nm (a.u.)	5	9	239	73	115
ANS emission maximum (nm)	468	470	476	489	489
$CD_{218\ nm}$ (mdeg)	−9.2	-	−10	−9.7	-

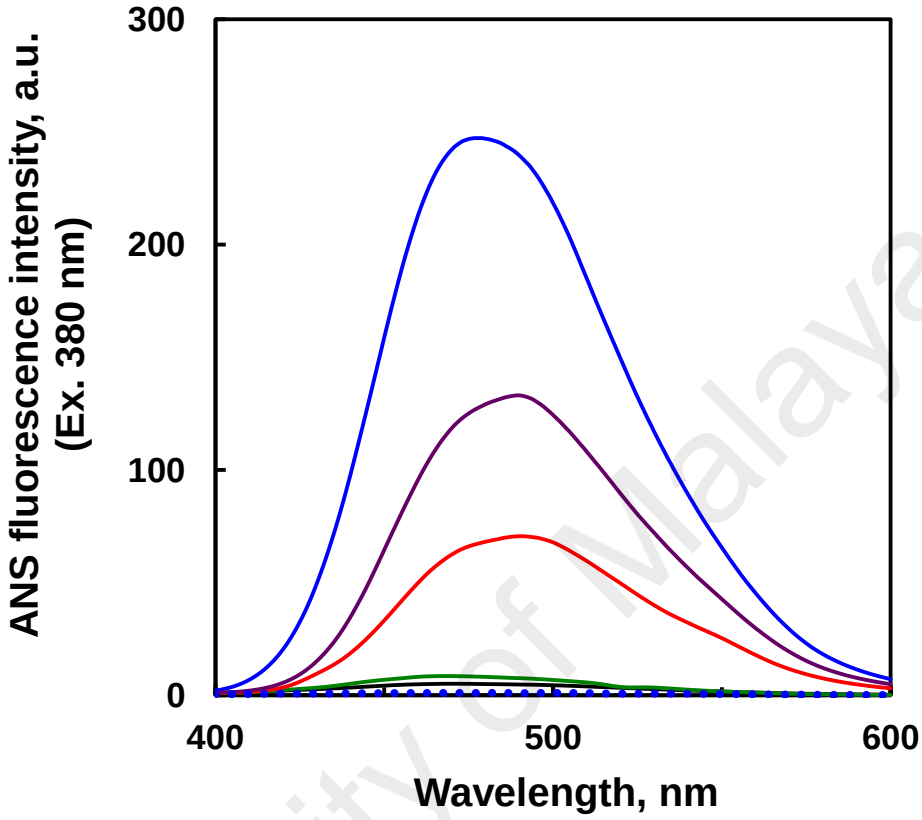


Figure 4.17: ANS fluorescence spectra of CGB lectin (0.1 μM) at pH 7.0 (*black line*), pH 3.5 (*green line*), pH 2.5 (*blue lines*), pH 1.5 (*red line*) and pH 1.0 (*purple line*), as studied at 25°C. ANS fluorescence spectrum of the protein sample at pH 2.5 in 6 M GdnHCl is shown with dotted line. The protein samples were excited at 380 nm.

denatured state of CGB lectin at pH 1.5 (Figure 4.17, Table 4.3). Such loss in the ANS binding characteristics indicated formation of the acid-unfolded forms of the lectin monomers. It is noteworthy that the loss in the ANS fluorescence intensity at pH 1.5 was not complete, indicating presence of some residual structure in the protein. This was verified by incubating the CGB lectin at pH 2.5 in the presence of 6 M GdnHCl, which is known to disrupt all noncovalent interactions in proteins (Emadi & Behzadi, 2014), for 14 h at 25°C. Absence of any significant ANS fluorescence spectral signal (blue dotted line, Figure 4.17) clearly indicated the absolute loss of hydrophobic clusters, available for ANS binding. This was in line with other reports, suggesting incomplete denaturation of proteins in low pH (Aune et al., 1967; Tanford, 1968; Fink et al., 1994). The ANS fluorescence spectrum of CGB lectin at pH 1.0 showed ~18% recovery in the ANS fluorescence intensity, compared to that observed at pH 2.5 (Figure 4.17; Table 4.3). Such regain in the fluorescence intensity was not surprising but indicated certain degree of refolding in CGB lectin at lower pH. This was not uncommon as anion-induced refolding has been well-documented in the literature (Goto et al., 1990a; 1990b; Muzammil et al., 2000; Uversky & Goto, 2009).

In addition to the decrease in the ANS fluorescence intensity, ANS emission maximum also showed a 21 nm red shift from 468 nm (for the native state at pH 7.0) to 489 nm at pH 1.5 (Table 4.3). The shift can be attributed to the dissociation of the hydrophobic clusters of the lectin, which in turn increased the polarity of the environment of ANS bound to the lectin (Stryer, 1965).

4.4.3 Far-UV CD spectra

Figure 4.18 shows the far-UV CD spectra of CGB lectin in the native state at pH 7.0 (black line) and the acid-denatured states at pH 2.5 (blue line) and pH 1.5 (red line). These spectra were typical of a β-structure protein, characterized by a negative peak at 218 nm. This was in agreement with the presence of ~62% β structure in CGB lectin

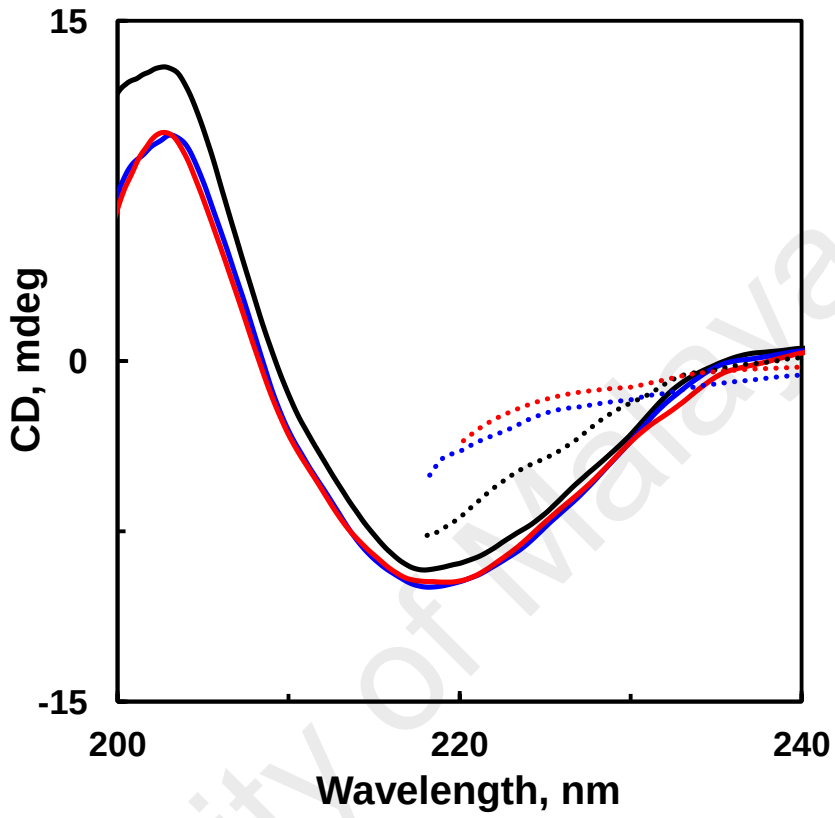


Figure 4.18: Far-UV CD spectra of CGB lectin (0.2 μM) at pH 7.0 (*black lines*), pH 2.5 (*blue lines*) and pH 1.5 (*red lines*), as studied at 25°C. Far-UV CD spectra of protein samples containing 6 M GdnHCl are shown with dotted lines.

(Gabrielsen et al., 2014). The CD spectra showed a small increase in the –CD values, when the sample pH was decreased from pH 7.0 to pH 2.5 or pH 1.5 (Table 4.3). These results suggested that the secondary structures of the lectin subunits remained largely unaffected in the monomerization as well as unfolding processes. Similar results were observed in soybean agglutinin upon conversion of the tetramer into monomers (Sinha & Surolia, 2005). However, GdnHCl treatment of the lectin tetramer at pH 7.0 and the lectin monomers at pH 2.5 and pH 1.5 produced interesting results. Whereas, lectin tetramer showed significant reduction in the secondary structures in the presence of 6 M GdnHCl, drastic loss in the secondary structures was observed at pH 2.5 and pH 1.5 upon addition of 6 M GdnHCl (Figure 4.18). These results further supported our intrinsic fluorescence and ANS fluorescence results, suggesting nearly complete denaturation at pH 2.5 in the presence of 6 M GdnHCl.

4.4.4. Gel chromatographic profiles

In order to verify the above results about the acid-denatured states of CGB lectin, obtained at pH 2.5 and pH 1.5, size exclusion chromatography of native and acid-denatured forms of CGB lectin was performed under different experimental conditions on a BioGel P-100 column (Figure 4.19). It is important to note that there is an inverse correlation between the elution volume of a protein on a gel chromatographic column and its hydrodynamic radius. Therefore, an increase in the elution volume of a protein is usually regarded as the decrease in its molecular size and vice versa (Lathe & Ruthven, 1956). CGB lectin in the native state at pH 7.0 (tetrameric form) showed a single elution peak (Figure 4.19A) with a V_e/V_o value of 1.56 (Table 4.4). Incubation of the lectin at pH 2.5 for 14 h shifted the elution peak to a higher elution volume (Figure 4.19B, Table 4.4), suggesting transformation of the protein to a structure with smaller hydrodynamic volume. The V_e/V_o value of this state of the protein was found to be 2.28. Such change in the elution volume of the tetrameric CGB lectin upon pH

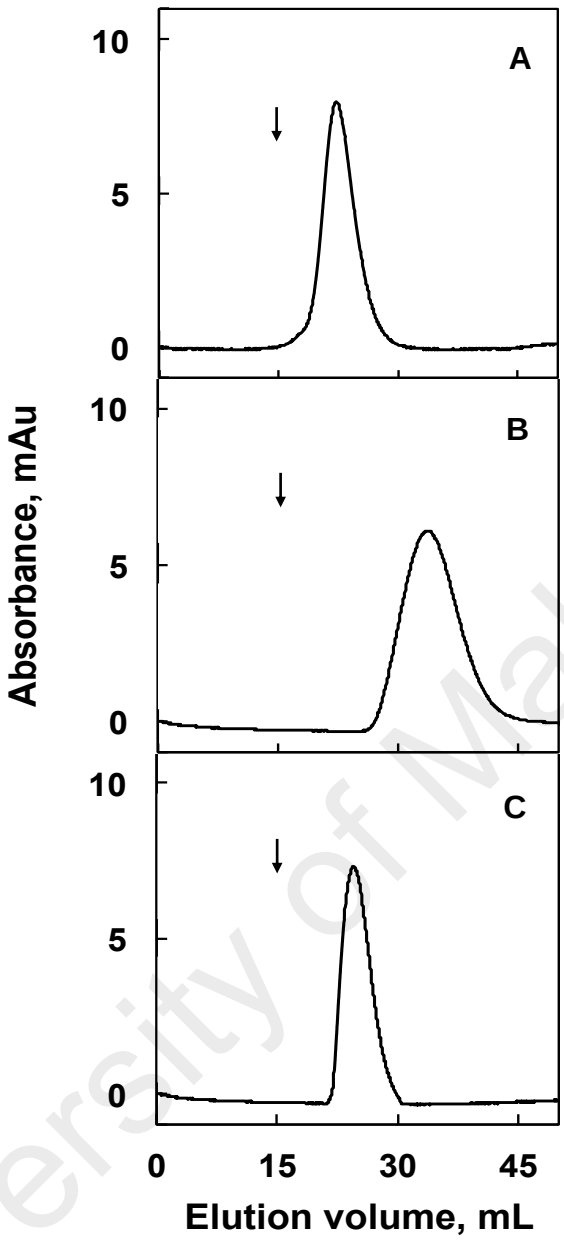


Figure 4.19: Gel chromatographic profiles of (A) native CGB lectin (pH 7.0); (B) acid-denatured CGB lectin (pH 2.5) and (C) acid-denatured CGB lectin (pH 1.5) on BioGel P-100 column (1.6 × 35 cm). The column was equilibrated with the respective buffers before applying the samples. The sample size was 12 μg/500 μL. Arrow shows the elution position of the blue dextran in each experiment.

Table 4.4: Elution characteristics of different conformational states of CGB lectin on BioGel P-100 column (1.6 × 35 cm).*

CGB lectin	Elution volume, V_e (mL)	V_e/V_o
Native (pH 7.0)	22.3	1.56
Acid-denatured (pH 2.5)	33.9	2.28
Acid-denatured (pH 1.5)	24.5	1.69

*The column was equilibrated with the respective buffers.

treatment (pH 2.5) reflected separation of noncovalently-associated monomers. It is plausible to think the separation of the lectin subunits into their monomeric forms at pH 2.5 due to weakening of the electrostatic interactions operative at the interfaces between the lectin monomers. Dissociation of subunits of other oligomeric proteins at low pH has also been demonstrated in several published reports (MacKenzie, 1970; Biswas & Kayastha, 2002; Khan et al., 2013). Interestingly, the elution peak of CGB lectin at pH 1.5 moved towards lower elution volume (Figure 4.19C) and had a V_e/V_o value of 1.69 (Table 4.4). Such decrease in the V_e/V_o value of CGB lectin from 2.28 (acid-denatured state at pH 2.5) to 1.69 (acid-denatured state at pH 1.5) can be ascribed to the molecular expansion due to acid-induced unfolding of monomers.

4.4.5. Thermal denaturation

Figure 4.20 shows thermal denaturation curves of the native and the acid-denatured (pH 2.5) states of CGB lectin in the temperature range, 20−95°C. Being a predominantly β-structured protein (Gabrielsen et al., 2014), changes in the secondary structures of the lectin upon thermal denaturation were monitored at 218 nm. As can be seen from the figure, the $-CD_{218\ nm}$ value of the native protein remained constant within the temperature range, 20−50°C. The value showed a slight decrease at 55°C, beyond which, visible aggregation impeded the CD measurements. Such aggregation may be the result of the clustering of the heat-denatured molecules (Mulvihill & Donovan, 1987). On the other hand, acid-denatured state at pH 2.5 showed a cooperative transition within the temperature range, 50−75°C, characterized by the increase in the $-CD_{218\ nm}$ value. Such increase reflected the formation of β-structures upon thermal denaturation. Formation of structured monomers at pH 2.5 might have led to the exposure of the amino acid residues, which were initially involved in the intersubunit interactions in the tetrameric conformation of CGB lectin (Gabrielsen et al., 2014). Since most of these residues, i.e. Leu, Phe, Ile, Trp, Val and Thr, are known to

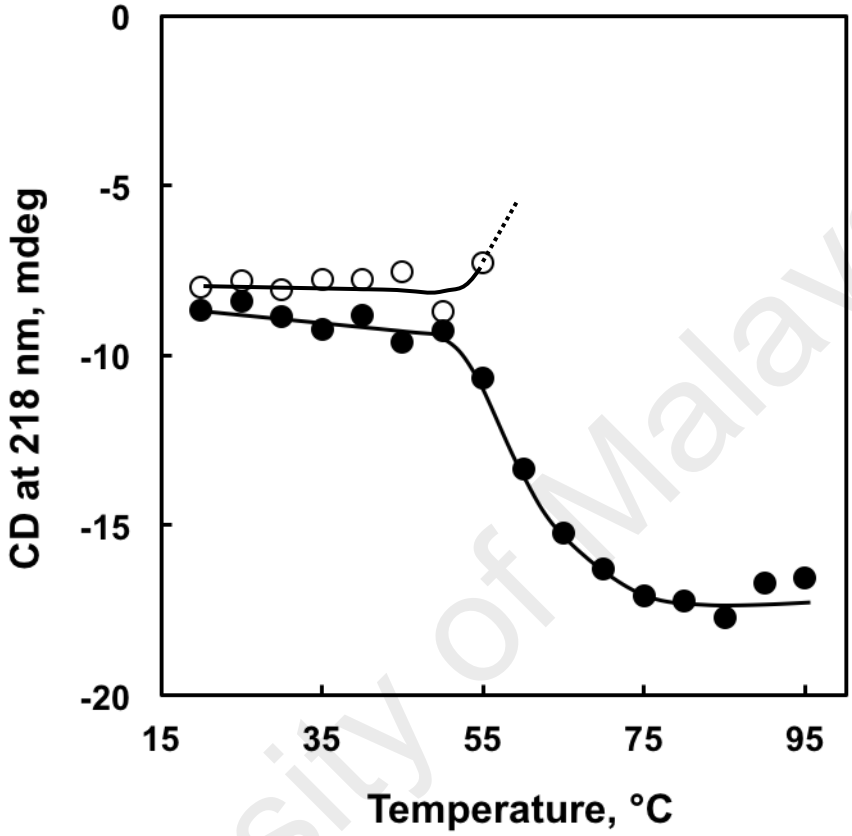


Figure 4.20: Thermal denaturation curves of CGB lectin (0.2 μM) at pH 7.0 *(open circles)* and pH 2.5 *(closed circles)* in the temperature range, 20−95°C, as monitored by far-UV CD measurements at 218 nm. The CD values of CGB lectin at pH 7.0 could not be obtained at temperatures beyond 55°C (represented by the dotted line) due to significant precipitation.

possess high propensity for the β-structure formation (Fujiwara et al., 2012), increase in the $CD_{218\ nm}$ value, representing high β-structure content upon thermal denaturation was not surprising. In an earlier report, Kato and Takagi (1988) also showed formation of β-structures upon thermal denaturation of ovalbumin.

4.5. Polyol-induced thermal stabilization of the acid-denatured states of CGB lectin

Polyols have been used to increase protein stability under various denaturing environments (Kamiyama et al., 1999; Athès et al., 1999; Singh & Singh, 2003; Zaroog et al., 2013; Fonin et al., 2016). However, the degree of stabilization conferred to a protein seems to depend on the structure of the polyol, i.e. size or chain length of the polyol (Gekko & Morikawa, 1981; Kamiyama et al., 1999). Furthermore, different proteins have also shown varying effects, when incubated with a particular polyol (Singh et al., 2011). Thus, it became of interest to study the effect of various polyols on the acid-denatutred states of CGB lectin.

The stabilizing potentials of four cosolvents (polyols) on the thermal stability of the acid-denatutred states of CGB lectin were investigated using far-UV CD spectral signal. The four polyols selected in this study were different in the number of hydroxyl groups such as ethylene glycol (2 hydroxyl groups), erythritol (4 hydroxyl groups), xylitol (5 hydroxyl groups) and sorbitol (6 hydroxyl groups). Since higher temperature produced significant precipitation of native CGB lectin at pH 7.0 (Section 4.4.5), study of the effect of these polyols was restricted to only acid-denatured states, accumulated at pH 2.5 and pH 1.5. Both thermal denaturation curves and far-UV CD spectra were employed to study the stabilizing effect of these polyols. For ease of comprehension, the acid-denatured states of CGB lectin at pH 2.5 (structured monomers) and pH 1.5 (unfolded monomers) are denoted as ADL 2.5 and ADL 1.5, respectively.

4.5.1. Thermal denaturation

Figures 4.21−4.24 show normalized thermal denaturation curves (F_D curves) of ADL 2.5 both in the absence and presence of different polyols, i.e. ethylene glycol, erythritol, xylitol and sorbitol, respectively, as studied by CD measurements at 218 nm. As evident from the figures, denaturation curves showed a single-step, two-state transition, characterized by the start-point, the mid-point and the end-points, values of which are summarized in Table 4.5. Thermal transition of ADL 2.5, which represented the structured monomer, started at 50.6°C and ended at 77.0°C with the mid-point (T_m) occurring at 60.9°C. Presence of polyols in the incubation mixture produced a shift in the transition curve towards either the lower temperature range or the higher temperature range. Among the four polyols studied, ethylene glycol was found unique as it shifted the whole transition curve towards lower temperature range (Figure 4.21), showing the start-, the mid- and the end-points, occurring at 43.6°C, 50.0°C and 57.0°C, respectively (Table 4.5). In other words, the structured monomer of CGB lectin (ADL 2.5) had undergone thermal denaturation at a much lower temperature range in the presence of 30% (w/v) ethylene glycol than in its absence. These results suggested that ethylene glycol destabilized the structured monomer against thermal denaturation. This was in accordance with the destabilizing potential of ethylene glycol, reported in earlier studies (Back et al., 1979; Gekko & Morikawa, 1981; Kumar et al., 2011; Zaroog et al., 2013). Interestingly, the other three polyols, namely, erythritol, xylitol and sorbitol delayed the onset of transition (Figures 4.22−4.24), as reflected from the higher values of the start-, the mid- and the end-points of the transition in the presence of these polyols (Table 4.5). Quantitatively, values of the start-, the mid- and the end-points were changed by a difference of +(5.7−8.9)°C, +(4.3−8.5)°C and +(5.5−6.5)°C, respectively, compared to those obtained in their absence (Table 4.5), which suggested

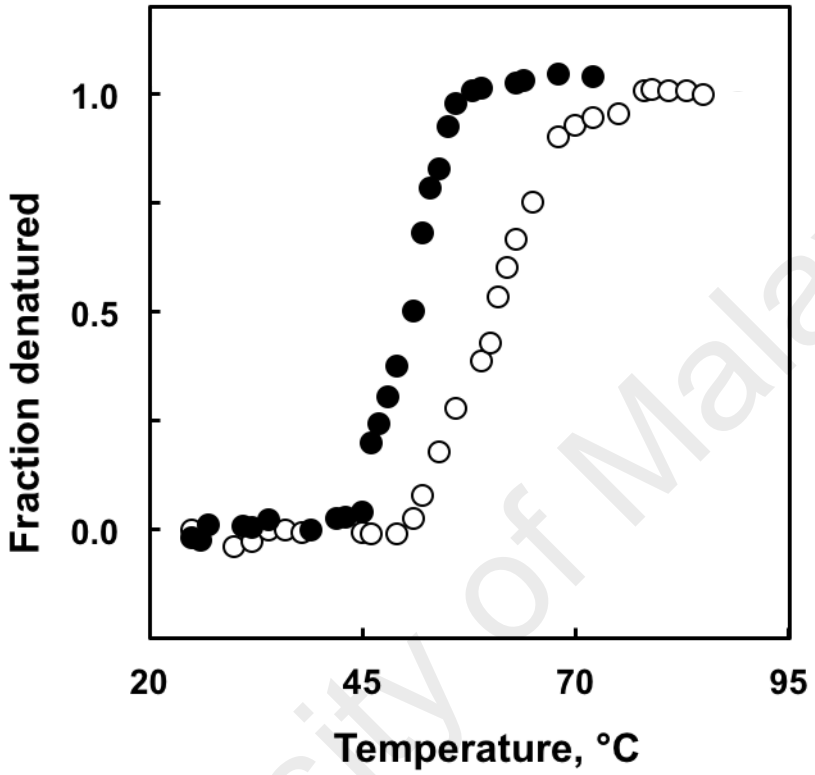


Figure 4.21: Normalized thermal denaturation curves of ADL 2.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) ethylene glycol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

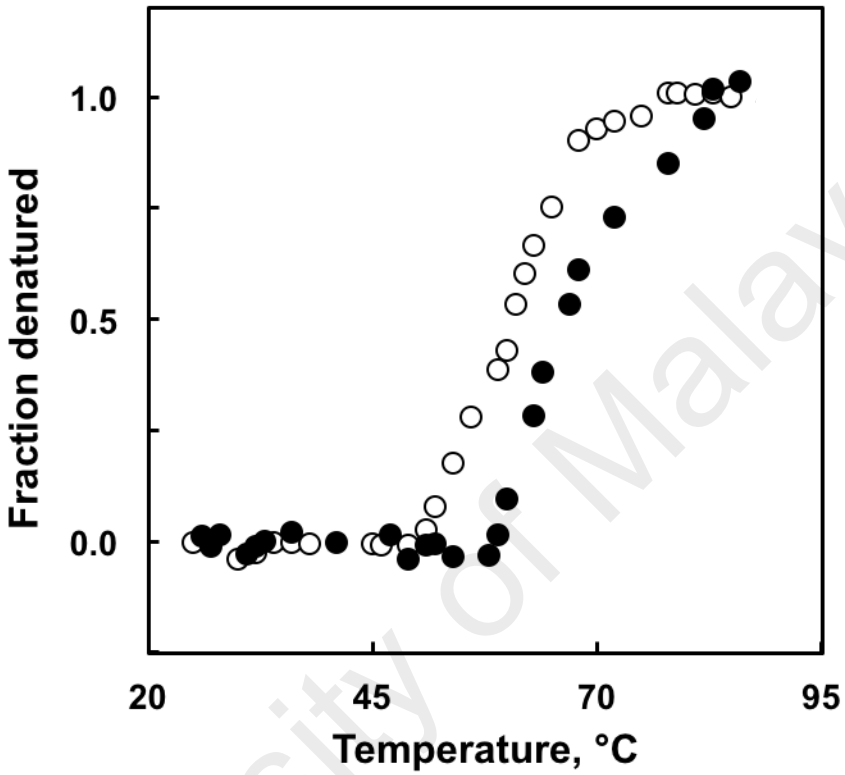


Figure 4.22: Normalized thermal denaturation curves of ADL 2.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) erythritol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

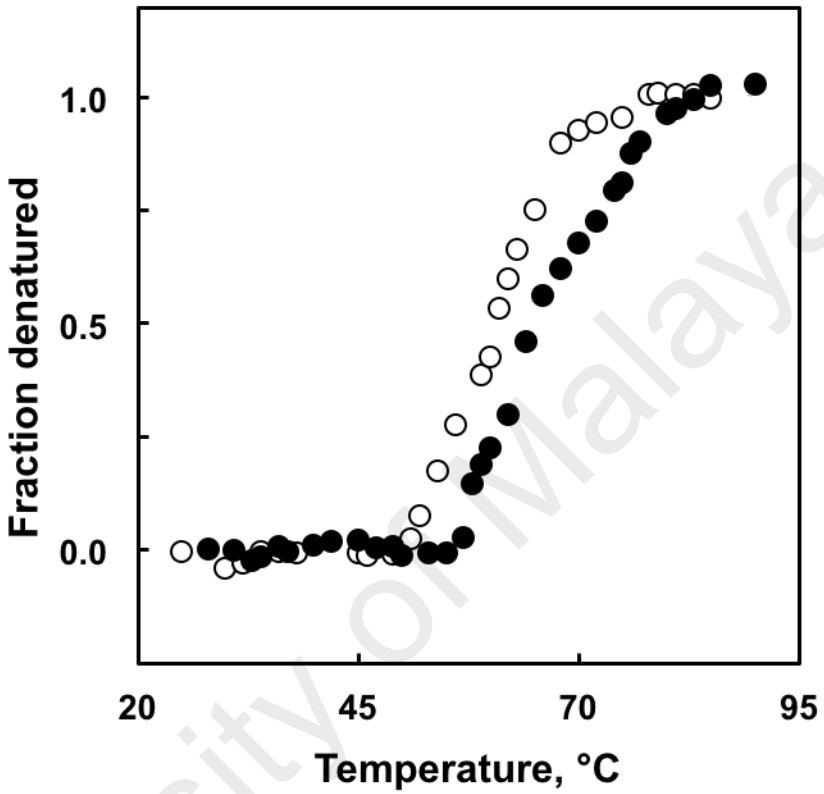


Figure 4.23: Normalized thermal denaturation curves of ADL 2.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) xylitol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

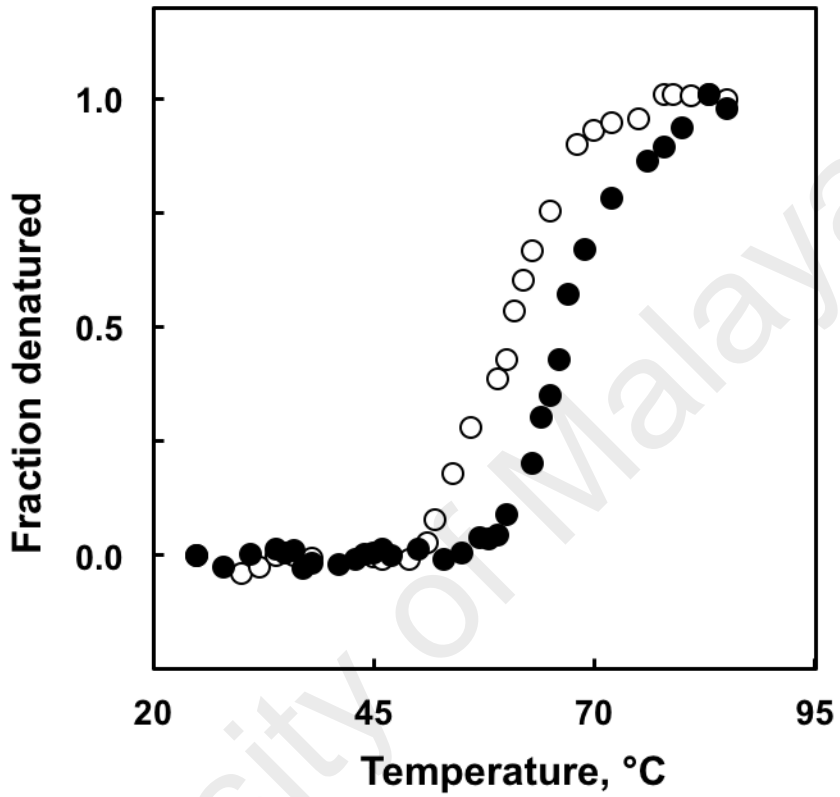


Figure 4.24: Normalized thermal denaturation curves of ADL 2.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) sorbitol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

Table 4.5: Characteristics of thermal denaturation curves of ADL 2.5, obtained in the absence and presence of different polyols, as monitored by $CD_{218\ nm}$ measurements.

Sample	Temperature (°C)		
	Start-point	Mid-point	End-point
CGB lectin (X)	50.6 ± 0.6	60.9 ± 0.3	77.0 ± 1.0
X + 30% Ethylene glycol	43.6 ± 1.5	50.0 ± 0.2	57.0 ± 1.0
X + 30% Erythritol	58.6 ± 1.5	69.4 ± 1.4	83.5 ± 0.5
X + 30% Xylitol	56.3 ± 0.6	65.7 ± 0.7	82.5 ± 0.5
X + 30% Sorbitol	59.5 ± 0.5	65.2 ± 0.2	82.5 ± 0.5

significant thermal stabilization of the structured monomer in the presence of these polyols.

Since the acid-denatured state at pH 1.5 of the protein represented the unfolded monomer of CGB lectin, which was indistinguishable from the structured monomer of CGB lectin (acid-denatured state at pH 2.5) in terms of CD spectral characteristics (Section 4.4.3), we thought it worthwhile to study thermal denaturation of ADL 1.5 using $CD_{218\,nm}$ signal. Normalized thermal denaturation curves of the ADL 1.5, obtained in the absence and presence of these polyols are depicted in Figures 4.25−4.28. All thermal transitions were characterized as single-step, two-state transitions and their values of the start-, the mid- and the end-points are listed in Table 4.6. It is important to note that the transition curves obtained with ADL 1.5 were restricted to lower temperature range, compared to those observed with ADL 2.5 (Table 4.6). This seems understandable, as presence of structural elements in the structured monomer (ADL 2.5) would have offered protection against thermal denaturation. Similar effects of these polyols were noticed on the thermal transition of the ADL 1.5. Ethylene glycol produced destabilizing effect by shifting the transition curve towards lower temperature range (Figure 4.25), showing the start- and the mid-points at 39.6°C and 48.6°C, respectively, against 46.0°C and 51.2°C, respectively, observed in its absence (Table 4.6). Anomalous behavior in the values of the end-points of the transition curves (Table 4.6) can be ascribed to the continuous increase in the formation of secondary structures in the presence of ethylene glycol at higher temperatures. The other three polyols produced stabilizing effect by shifting the transition curves towards a higher temperature range (Figures 4.26−4.28). The change in the start-, the mid- and the end-points was found to be +(4.3−7.0)°C, +(7.0−9.8)°C and +(5.5−11.5)°C, respectively. Although ethylene glycol also destabilized ADL 1.5 against thermal denaturation, this destabilizing effect (Figure 4.25) was not as pronounced as observed with the structured

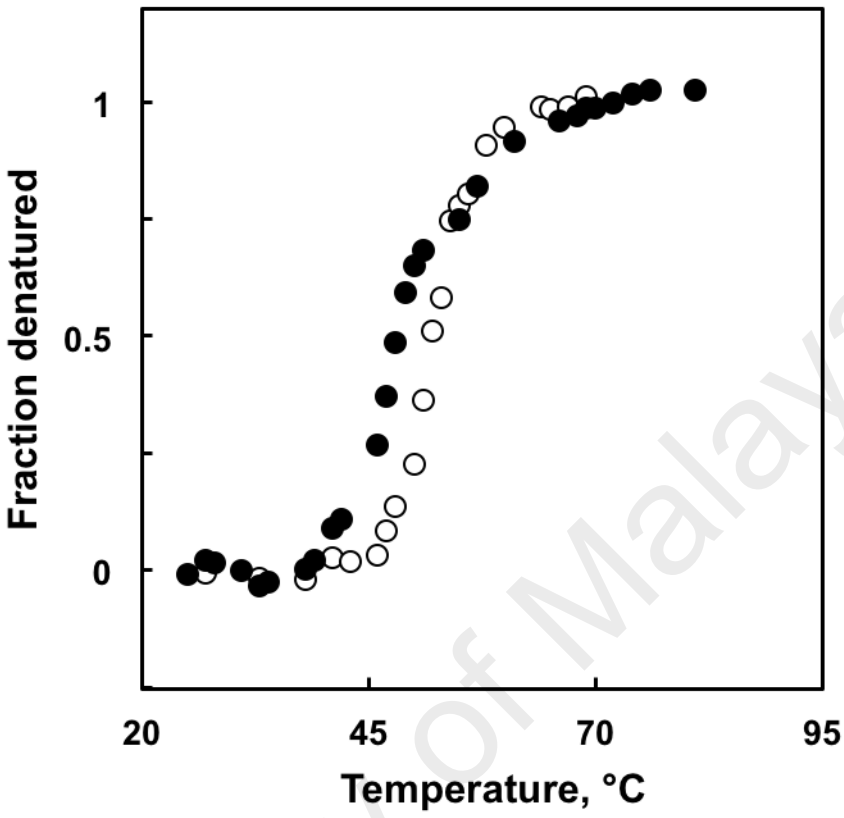


Figure 4.25: Normalized thermal denaturation curves of ADL 1.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) ethylene glycol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

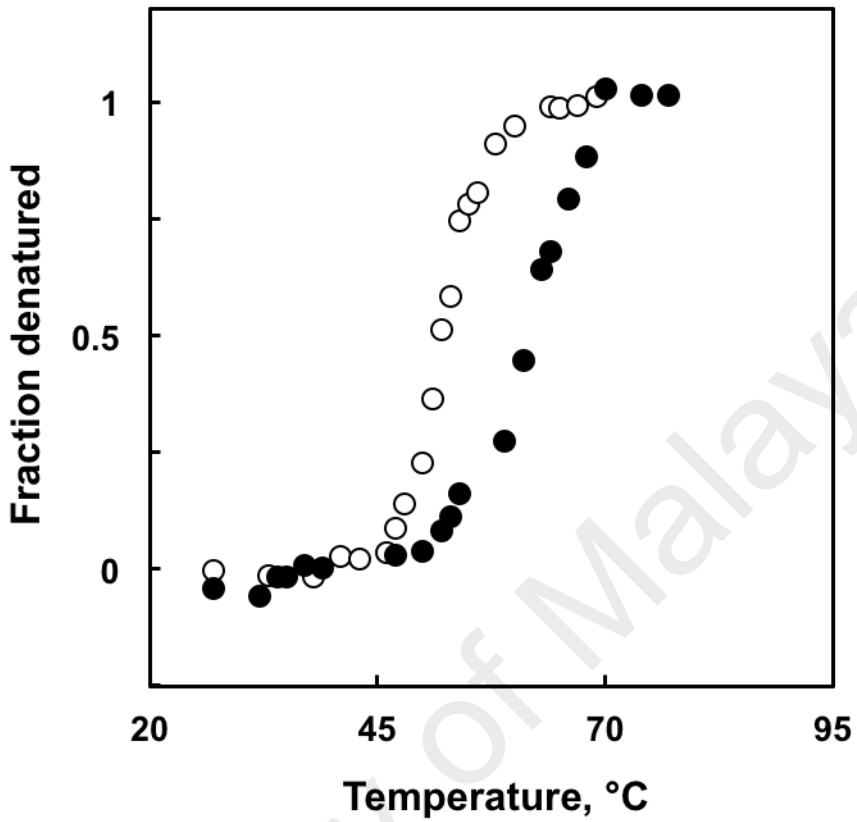


Figure 4.26: Normalized thermal denaturation curves of ADL 1.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) erythritol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

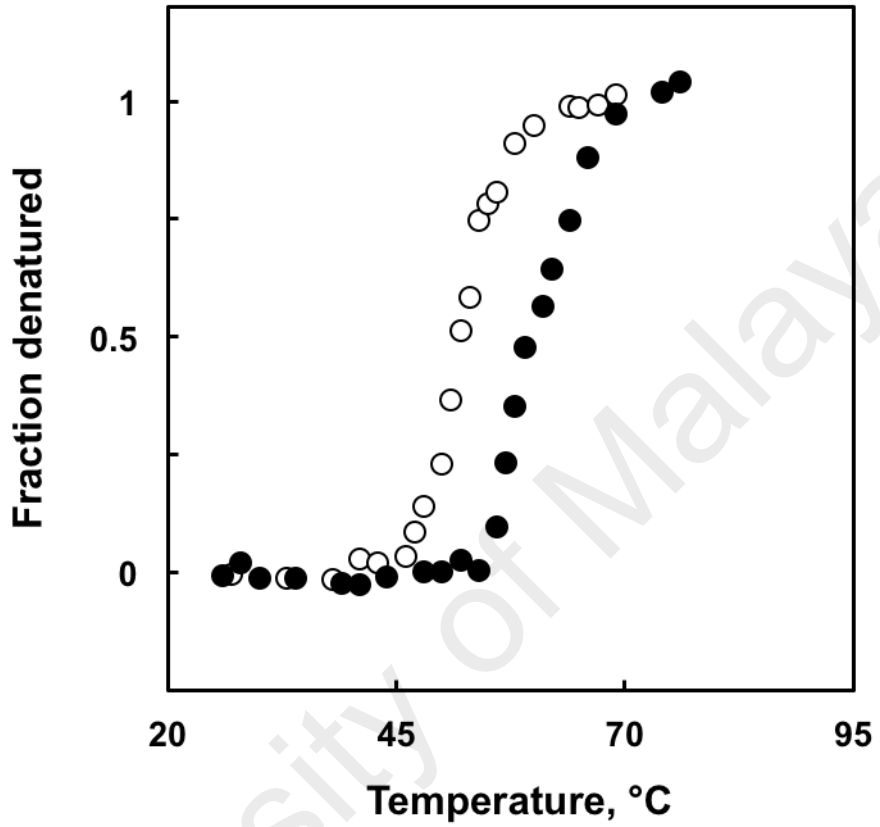


Figure 4.27: Normalized thermal denaturation curves of ADL 1.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) xylitol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

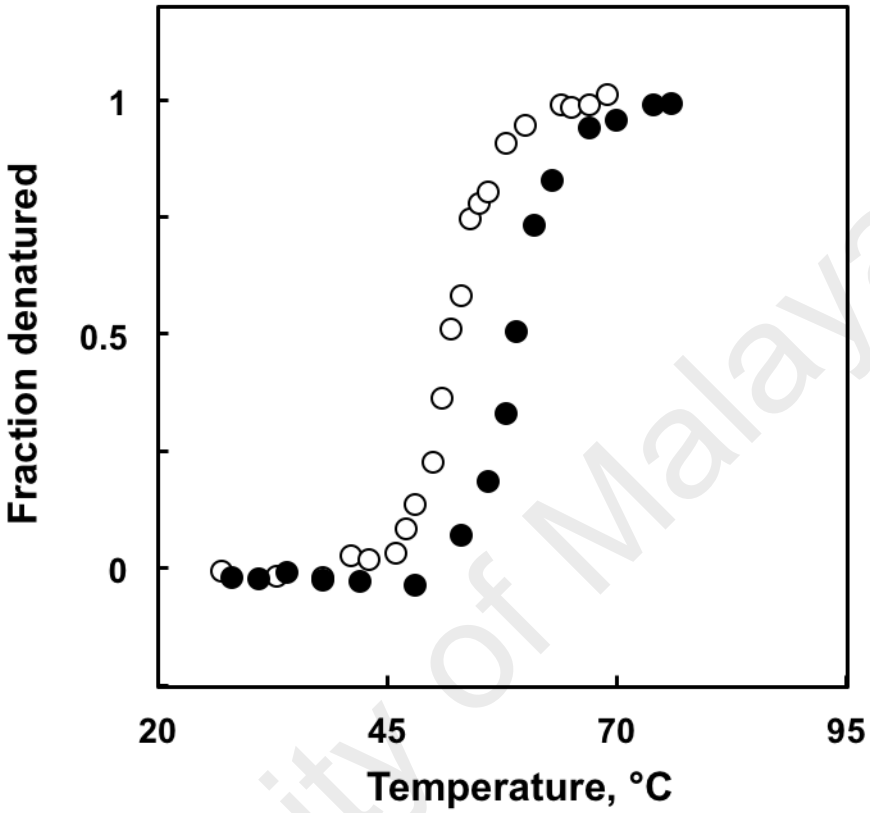


Figure 4.28: Normalized thermal denaturation curves of ADL 1.5 (0.2 μM) in the absence (*open circles*) and presence (*closed circles*) of 30% (w/v) sorbitol, as studied by far-UV CD measurements at 218 nm. Values of the fraction denatured, F_D were obtained by treating the data according to equation (6), as described in Section 3.2.5.3.

Table 4.6: Characteristics of thermal denaturation curves of ADL 1.5, obtained in the absence and presence of different polyols, as monitored by $CD_{218\ nm}$ measurements.

Sample	Temperature (°C)		
	Start-point	**Mid-point**	**End-point**
CGB lectin (X)	46.0 ± 1.0	51.2 ± 0.8	64.0 ± 0.0
X + 30% Ethylene glycol	39.6 ± 1.2	48.6 ± 0.6	71.3 ± 1.2
X + 30% Erythritol	50.3 ± 0.6	61.0 ± 0.8	71.5 ± 1.5
X + 30% Xylitol	53.0 ± 1.0	59.3 ± 1.0	69.5 ± 0.5
X + 30% Sorbitol	52.0 ± 1.0	58.2 ± 1.0	75.5 ± 1.5

monomer (acid-denatured state at pH 2.5) (Figure 4.21). As can be seen from Table 4.6, the difference in the mid-points of the transition curves of these two states were found to be −2.6°C for ADL 1.5 compared to −10.9°C, obtained for the structured monomer (pH 2.5) (Table 4.5). On the other hand, stabilizing effect of the other three polyols on ADL 1.5 was found to be relatively better than that observed with ADL 2.5 (Figures 4.22−4.24, 4.26−4.28; Tables 4.5, 4.6). Similarly, a previous report has also shown better stabilization of RNase A and cytochrome c at lower pH values (pH 4.0 and pH 2.5) compared to pH 7.0, in the presence of trehalose (Kaushik & Bhat, 2003).

Polyol-induced stabilization of protein against thermal denaturation can be explained by the increase in the surface tension of water and preferential hydration of proteins (Timasheff & Arakawa, 1988; Kaushik & Bhat, 1998). The interaction between the added polyols and water molecules prevents these water molecules from permeating the protein structure, promoting hydrophobic interactions within the protein interior, leading to an increase in structural stability (Back et al., 1979). Although stabilizing effect of linear polyols has been reported to be positively correlated with the number of hydroxyl groups in certain proteins (Gekko & Morikawa, 1981; Kamiyama et al., 1999), exceptions to this rule has also been documented in the literature (Athès et al., 1999; Rashid et al., 2006).

Contrary to the stabilizing effect of polyols as mentioned above, some polyols have also shown destabilizing effect on certain proteins under specific conditions (Obon et al., 1996; Faber-Barata et al., 2005; Romero et al., 2009; Zaroog et al., 2013). This has been detailed in an earlier review (Singh et al., 2011) discussing the dual role of various osmolytes in both stabilizing and destabilizing proteins. Unlike the preferential exclusion of polyols from the protein surface as observed in the polyol-induced stabilization of proteins, preferential binding of polyols to protein has been suggested to be the prime factor which shifts the denaturation equilibrium in the direction of the

denatured state (Timasheff, 2002; Singh et al., 2011). Since these polyols tend to accumulate at the polar surface of the protein, susceptibility of the protein to polyol-induced destabilization depends on the extent of exposure of polar protein backbone as well as degree of polyol interaction with the peptide groups (Auton & Bolen, 2005). Destabilization of protein by preferential binding of polyol might be resulted from lesser exposure of protein backbone on protein surface and increased side chain interactions upon polyol binding (Singh et al., 2011). Since the number of polyol molecules occupying the surface of the protein is one of the factors determining preferential binding (or preferential exclusion) (Timasheff, 2002), it is speculated that smaller molecules might act as a stronger destabilizing agent due to their clustering at protein surface in higher numbers. In view of this, ethylene glycol-induced destabilization of CGB lectin against thermal denaturation might be the result of increased binding or accumulation of the ethylene glycol molecules at the protein's surface.

4.5.2. Far-UV CD spectra

Since the two acid-denatured states of CGB lectin were distinguished by their T_m values of 61°C (ADL 2.5) and 51°C (ADL 1.5) (Tables 4.5, 4.6), effect of the aforementioned polyols on the CD spectra of these states at their T_m values was also studied and the results were compared with the CD spectra, obtained at 25°C. Figure 4.29 shows the far-UV CD spectra of ADL 2.5 at 61°C both in the absence and presence of different polyols. The far-UV CD spectrum of the same acid-denatured state at 25°C is also included for comparison. Values of the CD spectral signal at 218 nm, obtained under these conditions are given in Table 4.7. As can be seen from Figure 4.29 and Table 4.7, presence of polyols in the incubation mixture significantly affected the CD spectra. A comparison of these CD spectra showed a 65% increase in the $-CD_{218\ nm}$ signal at 61°C compared to that obtained at 25°C, which was suggestive of thermal

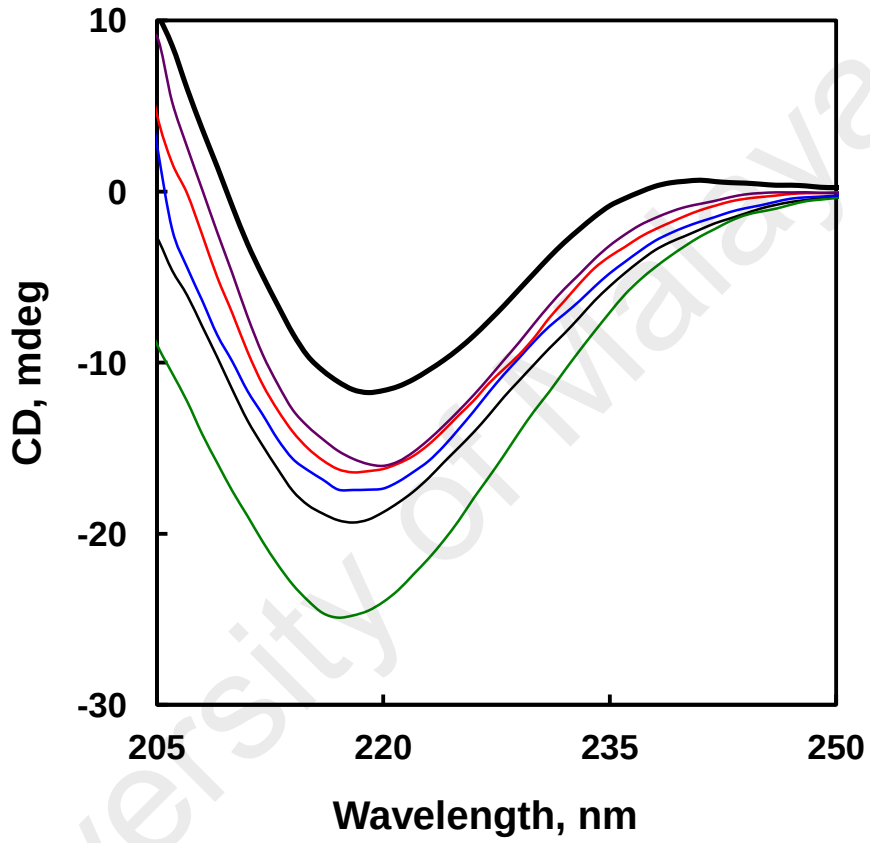


Figure 4.29: Far-UV CD spectra of ADL 2.5 (0.2 μM) at 25°C (*thick black line*) and 61°C (*thin black line*). The far-UV CD spectra of the heat-denatured (61°C) CGB lectin, obtained in the presence of 30% (w/v) polyols are represented as: ethylene glycol (*green line*), erythritol (*blue line*), xylitol (*red line*) and sorbitol (*purple line*).

Table 4.7: Effect of various polyols on the secondary structural characteristics of the acid-denatured states of CGB lectin at different temperatures.

Sample	pH 2.5		pH 1.5	
	$CD_{218\ nm}$ (mdeg)	$\Delta\ CD_{218\ nm}$ (%)	$CD_{218\ nm}$ (mdeg)	$\Delta\ CD_{218\ nm}$ (%)
CGB lectin at 25°C	−11.7	-	−12.1	-
Thermally-denatured CGB lectin (Y)*	−19.3	+65	−18.9	+56
Y + 30% Ethylene glycol	−25.2	+115	−23.5	+94
Y + 30% Erythritol	−18.0	+54	−13.5	+12
Y + 30% Xylitol	−16.8	+44	−13.7	+13
Y + 30% Sorbitol	−15.6	+33	−13.8	+14

*CGB lectin was thermally-denatured at T_m values of 61°C and 51°C for pH 2.5 and pH 1.5, respectively.

denaturation of the structured monomer by inducing more β-structures. This was not unusual, as several other proteins have shown formation of non-native β-structures upon thermal denaturation (Kato & Takagi, 1988; Fink, 1998; Pearce et al., 2007). Stabilizing effect of erythritol, xylitol and sorbitol on the thermally-denatured lectin was evident from the lesser change in the spectral signal, being ~54%, ~44% and ~33% in the presence of erythritol, xylitol and sorbitol, respectively, in comparison to 65% noticed in their absence (Figure 4.29; Table 4.7). Contrary to these results, ethylene glycol induced formation of more non-native β-structures, as reflected by a much larger increase (115%) in the spectral signal, suggesting destabilizing effect. Induction of secondary structures upon addition of ethylene glycol has also been observed with acid-denatured trypsinogen (Naseem & Khan, 2003). These results were similar to the results shown in Figures 4.21−4.24 and Table 4.5. Resistance against thermal-induced structural changes in the protein in the presence of polyols clearly reflected their stabilization potential against thermal denaturation.

Stabilizing effect of these polyols on ADL 1.5 at 51°C was found to be more pronounced compared to that observed with ADL 2.5 at 61°C. The far-UV CD spectra of ADL 1.5 at 51°C both in the absence and presence of these polyols along with the spectrum of the protein at 25°C are shown in Figure 4.30 and the values of the $CD_{218\,nm}$ spectral signal are given in Table 4.7. About 56% increase in the $-CD_{218\ nm}$ value was noticed at 51°C compared to that observed at 25°C, suggesting thermal denaturation of ADL 1.5, which was marked by the formation of non-native β-structures. The three polyols, namely, erythritol, xylitol and sorbitol markedly reduced the increase in the $-CD_{218\ nm}$ value to ~12%, ~13% and ~14%, respectively, indicating stabilization of the acid-denatured monomer against thermal denaturation (Figure 4.30; Table 4.7). The destabilizing effect of ethylene glycol was also noticed on the acid-denatured monomer

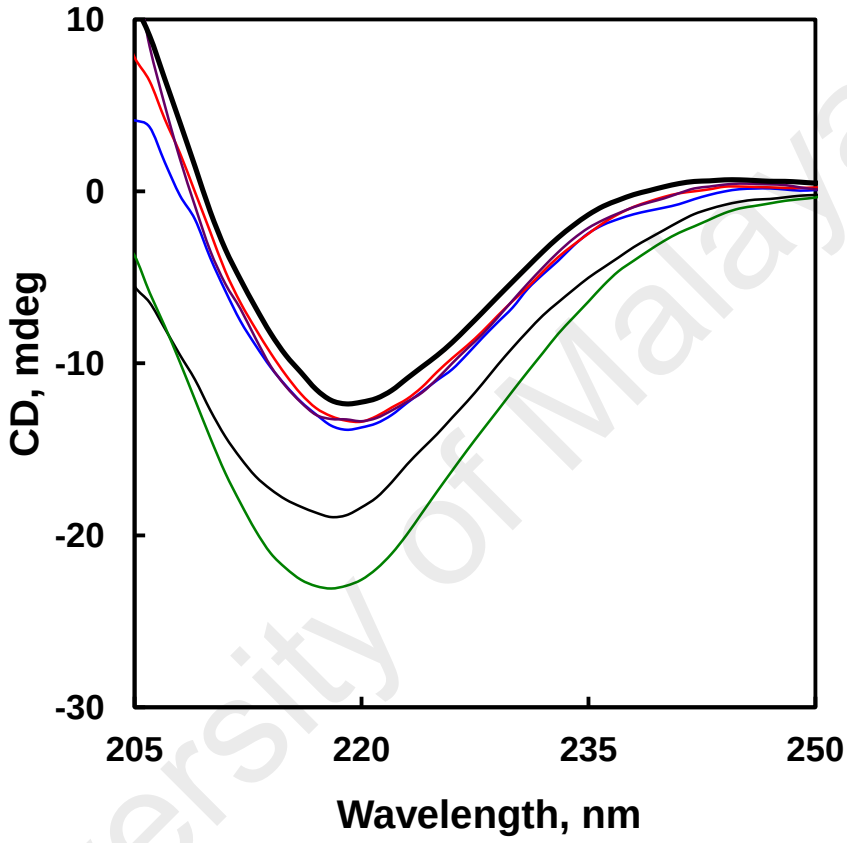


Figure 4.30: Far-UV CD spectra of ADL 1.5 (0.2 μM) at 25°C (*thick black line*) and 51°C (*thin black line*). The far-UV CD spectra of the heat-denatured (51°C) CGB lectin, obtained in the presence of 30% (w/v) polyols are represented as: ethylene glycol (*green line*), erythritol (*blue line*), xylitol (*red line*) and sorbitol (*purple line*).

as it produced greater increase in the $-CD_{218\ nm}$ value (94%), compared to 56% observed in its absence (Figure 4.30; Table 4.7).

4.6. Effect of polyols on the acid-denatured states of CGB lectin

Influence of different polyols on ADL 2.5 and ADL 1.5 at 25°C were studied using ANS fluorescence spectra, Trp fluorescence spectra and far-UV CD spectra.

4.6.1. ANS fluorescence spectra

ANS fluorescence spectra are commonly used to monitor changes in the protein conformation under various experimental conditions, by comparing the magnitude of the spectral signal at ~480 nm (Albani, 2004). Figures 4.31−4.34 show the effect of various polyols on the ANS fluorescence spectra of ADL 2.5 and ADL 1.5. The ANS fluorescence spectra of these states of CGB lectin displayed emission maxima at 476 nm (ADL 2.5) and 489 nm (ADL 1.5). Furthermore, ANS fluorescence intensity of CGB lectin was found significantly higher for ADL 2.5 compared to that obtained with ADL 1.5 due to the presence of more organized hydrophobic clusters in the structured monomers (ADL 2.5), which were otherwise involved in the subunit-subunit interaction to form lectin tetramer (native state) at pH 7.0 (Section 4.4.2). Lesser magnitude of the ANS fluorescence signal at pH 1.5 reflected disruption of these organized hydrophobic clusters upon acid treatment at pH 1.5.

Addition of 30% (w/v) ethylene glycol to CGB lectin at both pH 2.5 and pH 1.5 produced significant increase in the ANS fluorescence intensity. However, the increase was more marked (78%) at pH 2.5 compared to that observed (27%) at pH 1.5 (Figure 4.31; Table 4.8). Such enhancement in the ANS fluorescence intensity indicated presence of more hydrophobic clusters upon ethylene glycol treatment. Induction of non-native β-structures in the presence of ethylene glycol might have promoted formation of hydrophobic clusters in the protein, which may be responsible for increased ANS binding. In view of the greater induction of β-structures at pH 2.5

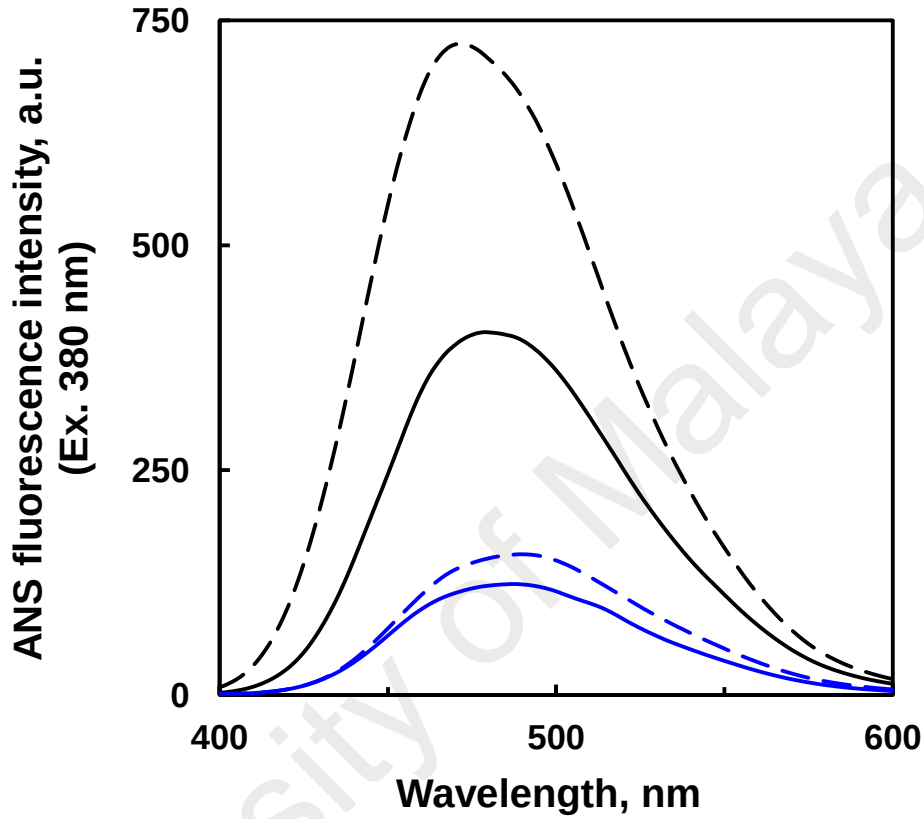


Figure 4.31: ANS fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) ethylene glycol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 380 nm.

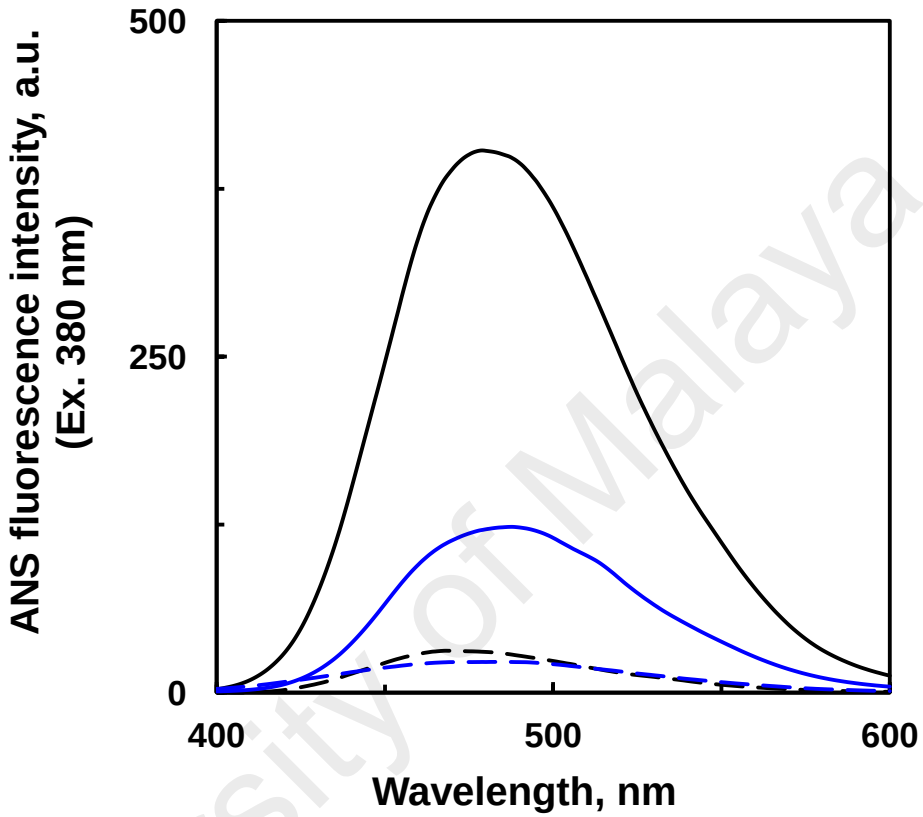


Figure 4.32: ANS fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) erythritol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 380 nm.

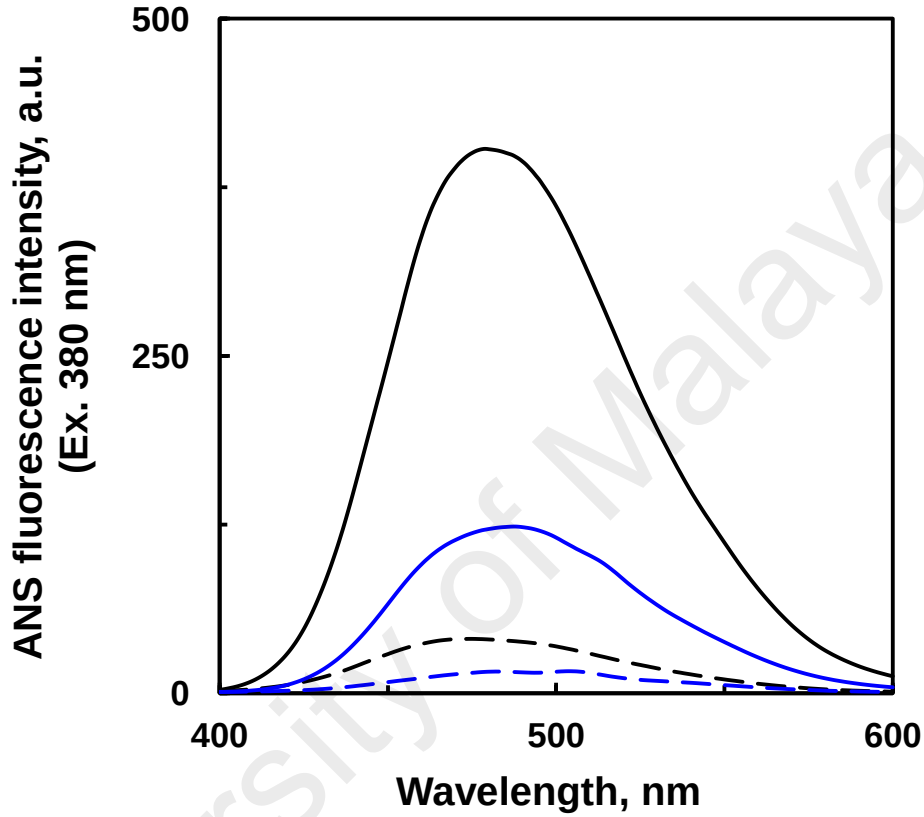


Figure 4.33: ANS fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) xylitol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 380 nm.

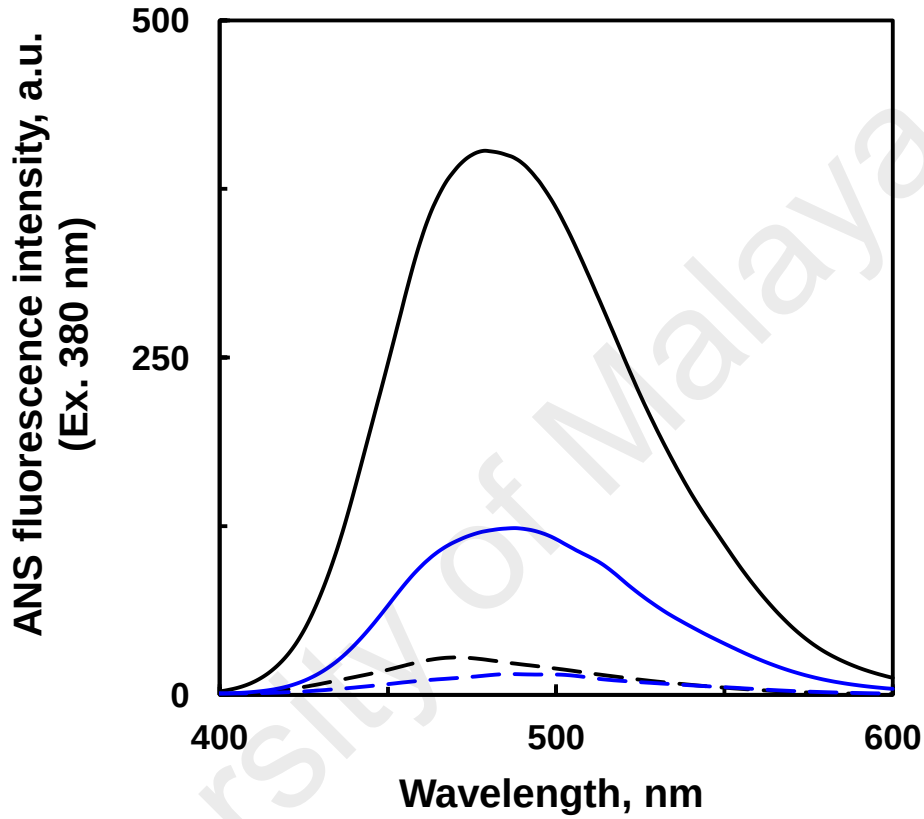


Figure 4.34: ANS fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) sorbitol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 380 nm.

Table 4.8: Effect of various polyols on the ANS fluorescence spectral characteristics of the acid-denatured states of CGB lectin at 25°C.

Sample	pH 2.5		pH 1.5	
	ANS FI*$_{476\ nm}$ (a.u.)	Δ ANS FI (%)	ANS FI$_{489\ nm}$ (a.u.)	Δ ANS FI (%)
CGB lectin (X)	403	-	124	-
X + 30% Ethylene glycol	719	+78	156	+27
X + 30% Erythritol	31	−92	23	−82
X + 30% Xylitol	40	−90	16	−87
X + 30% Sorbitol	27	−93	15	−88

* FI = Fluorescence intensity

compared to that observed at pH 1.5 in presence of ethylene glycol (as shown later in Figures 4.39 and 4.40), greater increase in ANS fluorescence intensity at pH 2.5 seems understandable. Similar ethylene glycol-induced change in the ANS fluorescence intensity has also been reported earlier (Naseem & Khan, 2003). In contrast to the positive influence of ethylene glycol on the ANS fluorescence intensity of ADL 2.5 and ADL 1.5, three other polyols (erythritol, xylitol and sorbitol) produced remarkable decline in the ANS fluorescence signal (Figures 4.32−4.34). However, magnitude of the loss in the ANS fluorescence signal was slightly higher (90−93%) at pH 2.5 compared to 82−88%, observed at pH 1.5 (Table 4.8). Such decrease in the ANS fluorescence intensity was suggestive of protein's structural reformation with buried hydrophobic clusters. These results were similar to earlier reports, where polyols were shown to decrease the ANS fluorescence intensity (Miroliaei et al., 2007; Xia et al., 2007; Devaraneni et al., 2012).

4.6.2. Tryptophan fluorescence spectra

Tryptophan fluorescence spectra were also used to probe conformational changes in the protein induced by these polyols. Although both characteristics of the Trp fluorescence spectra, i.e. fluorescence intensity and emission maximum are usually affected by the microenvironmental changes around Trp residues, shift in the emission maximum alone is considered to be a reliable parameter to monitor conformational changes in the protein. Many published reports on protein denaturation studies have shown red shift in the emission maximum with either increase or decrease in the fluorescence intensity upon denaturation (Yao et al., 1984; Eftink, 1994; Chatterjee & Mandal, 2003). Figures 4.35−4.38 show Trp fluorescence spectra of ADL 2.5 and ADL 1.5 both in the absence and presence of different polyols, while values of the emission maximum under these conditions are given in Table 4.9. Whereas ethylene

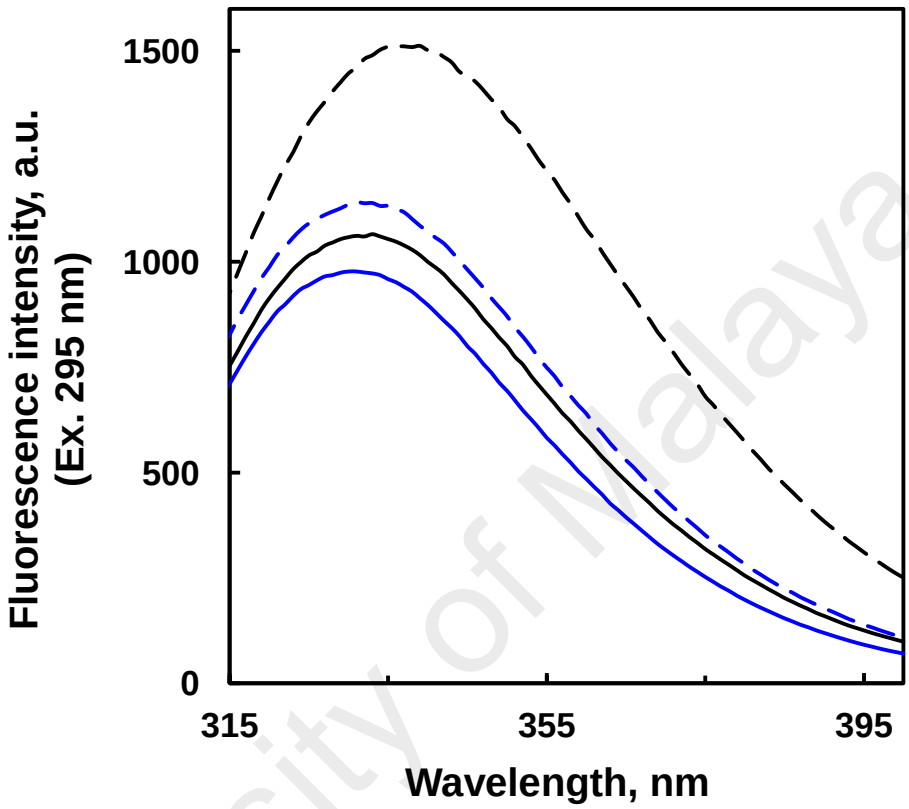


Figure 4.35: Tryptophan fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) ethylene glycol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 295 nm.

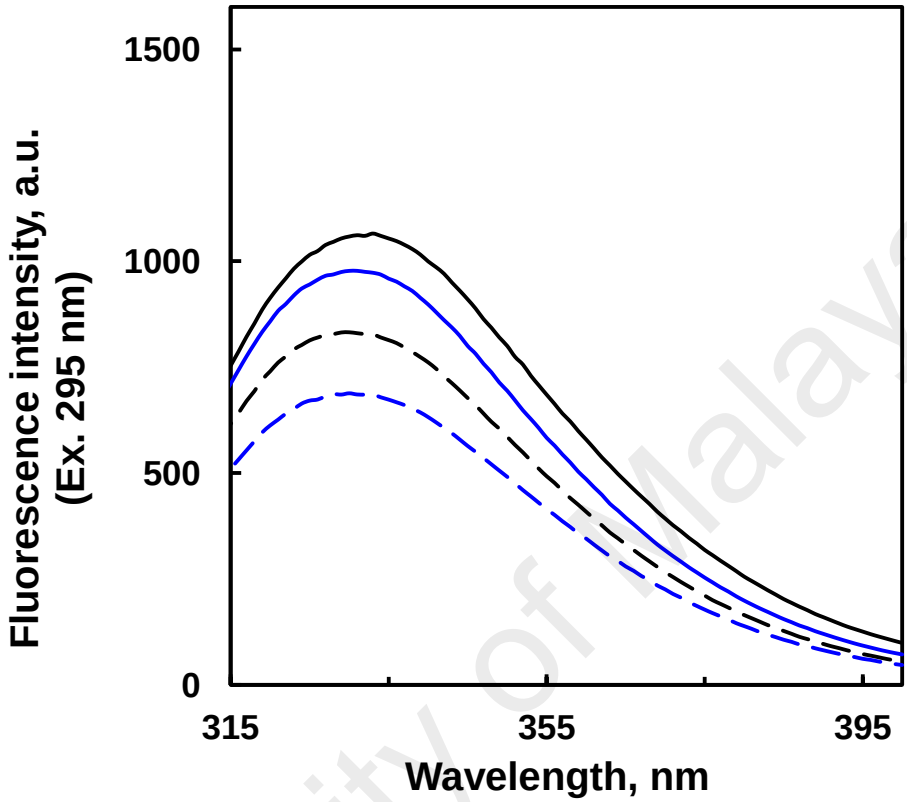


Figure 4.36: Tryptophan fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) erythritol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 295 nm.

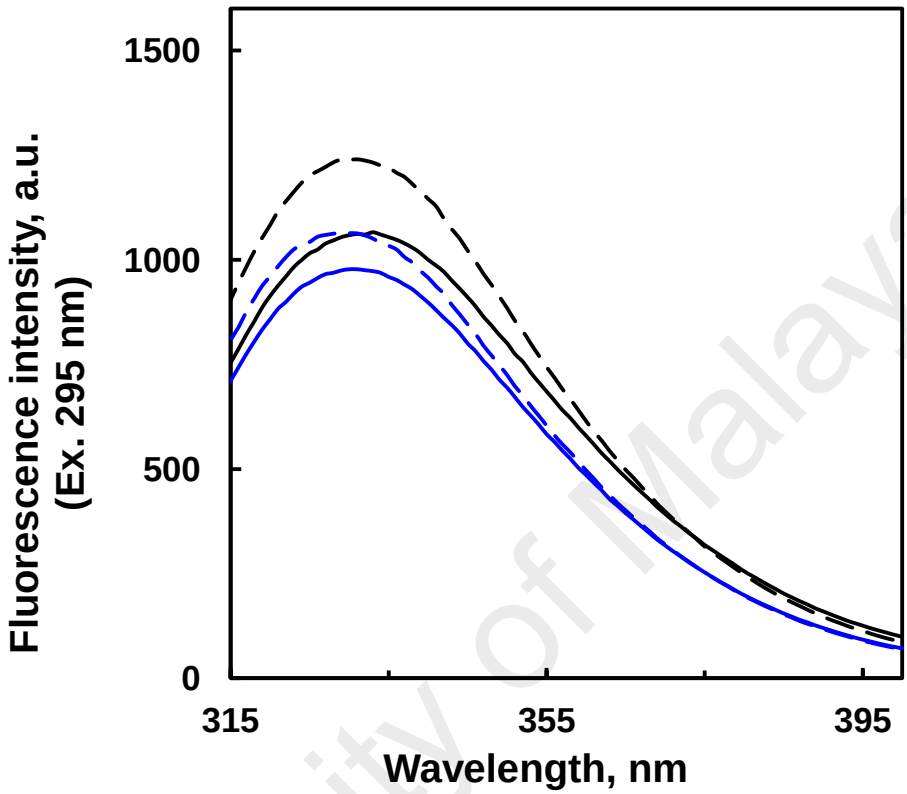


Figure 4.37: Tryptophan fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) xylitol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 295 nm.

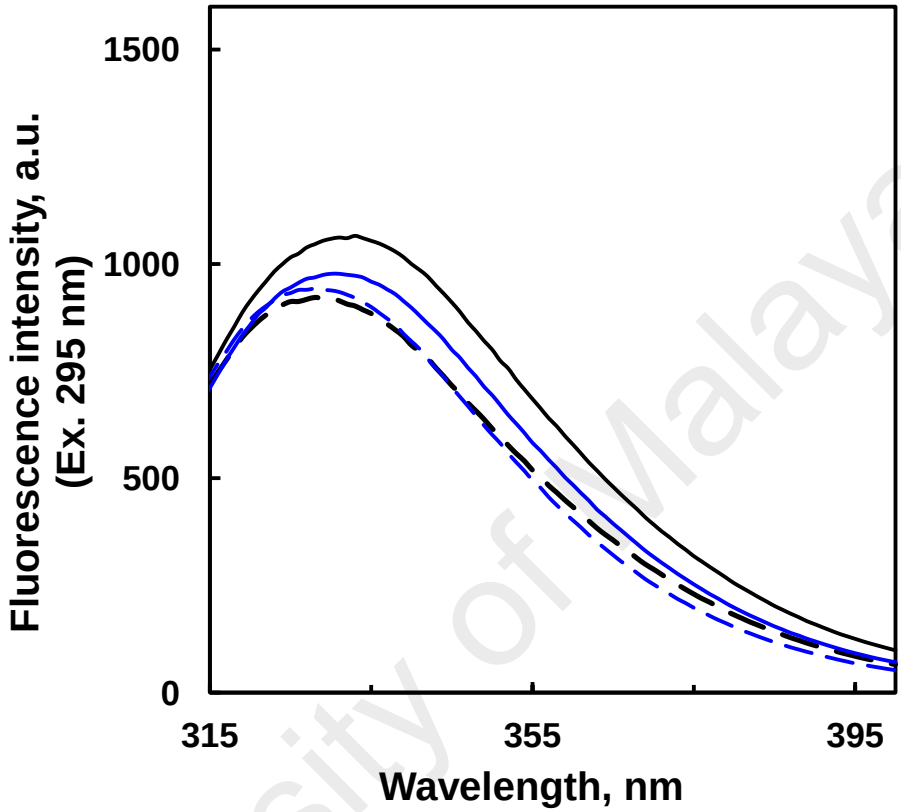


Figure 4.38: Tryptophan fluorescence spectra of ADL 2.5 (*black lines*) and ADL 1.5 (*blue lines*), obtained in the absence (*solid lines*) and presence of 30% (w/v) sorbitol (*dashed lines*) at 25°C. The protein concentration used was 0.1 μM and samples were excited at 295 nm.

Table 4.9: Effect of various polyols on the emission maxima of the acid-denatured states of CGB lectin at 25°C.

Sample	Emission maxima (nm)	
	pH 2.5	pH 1.5
CGB lectin (X)	333	331
X + 30% Ethylene glycol	339	331
X + 30% Erythritol	330	330
X + 30% Xylitol	331	329
X + 30% Sorbitol	328	328

glycol produced significant red shift of 6 nm in the emission maximum of ADL 2.5, other three polyols shifted the fluorescence spectra towards lower wavelength side (Figures 4.35−4.38; Table 4.9). On the other hand, ADL 1.5 showed no variation in the emission maximum in the presence of ethylene glycol (Table 4.9). Significant shift in the emission maximum upon ethylene glycol treatment observed with ADL 2.5 clearly suggested ethylene glycol-induced destabilization of the structured monomers of CGB lectin, leading to increased polarity around Trp residues (Vivian & Callis, 2001; Lakowicz, 2006). Lack of any change in the emission maximum at pH 1.5 was not surprising as the conformational states of CGB lectin at these pH values were totally different. These results were similar to those shown in Figure 4.31 and Table 4.8, where presence of ethylene glycol produced significant increase in the ANS fluorescence intensity, suggesting protein destabilization.

Contrary to the results obtained with ethylene glycol, other three polyols showed significant stabilization as reflected from the blue shift in the emission maximum. About 2−5 nm blue shift was noticed in the presence of these polyols (Table 4.9). Such blue shift in the emission maximum was indicative of the transfer of Trp residues from polar to nonpolar environment (Vivian & Callis, 2001; Lakowicz, 2006). Among these polyols, sorbitol was found to possess relatively better stabilizing effect than erythritol and xylitol. This was in line with the results of ANS fluorescence intensity (Table 4.8). Earlier studies have also reported blue shift in the Trp emission maximum of proteins in the presence of polyols (Lee et al., 2002; Gangadhara et al., 2009).

4.6.3. Far-UV CD spectra

Far-UV CD spectral measurements were employed to study the effect of polyols on the secondary structures of the acid-denatured states of CGB lectin. Figures 4.39 and 4.40 show the far-UV CD spectra of ADL 2.5 and ADL 1.5, respectively, both in the absence and the presence of 30% (w/v) ethylene glycol or xylitol. As evident from these

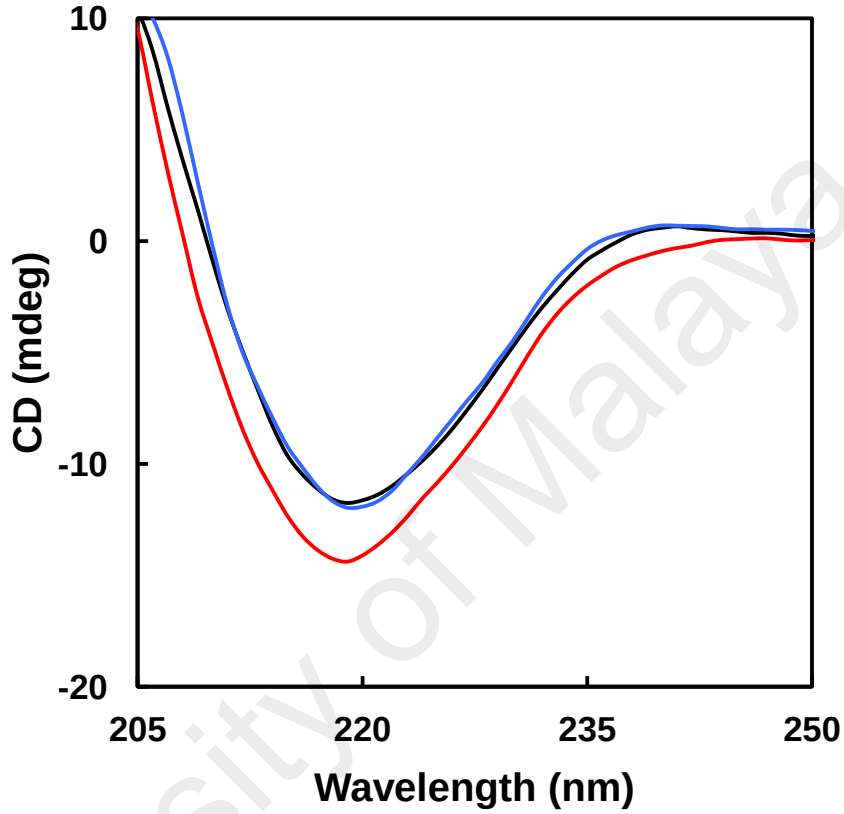


Figure 4.39: Far-UV CD spectra of ADL 2.5 (0.2 μM), obtained in the absence (*black line*) and presence of 30% (w/v) ethylene glycol (*red line*) and xylitol (*blue line*) at 25°C.

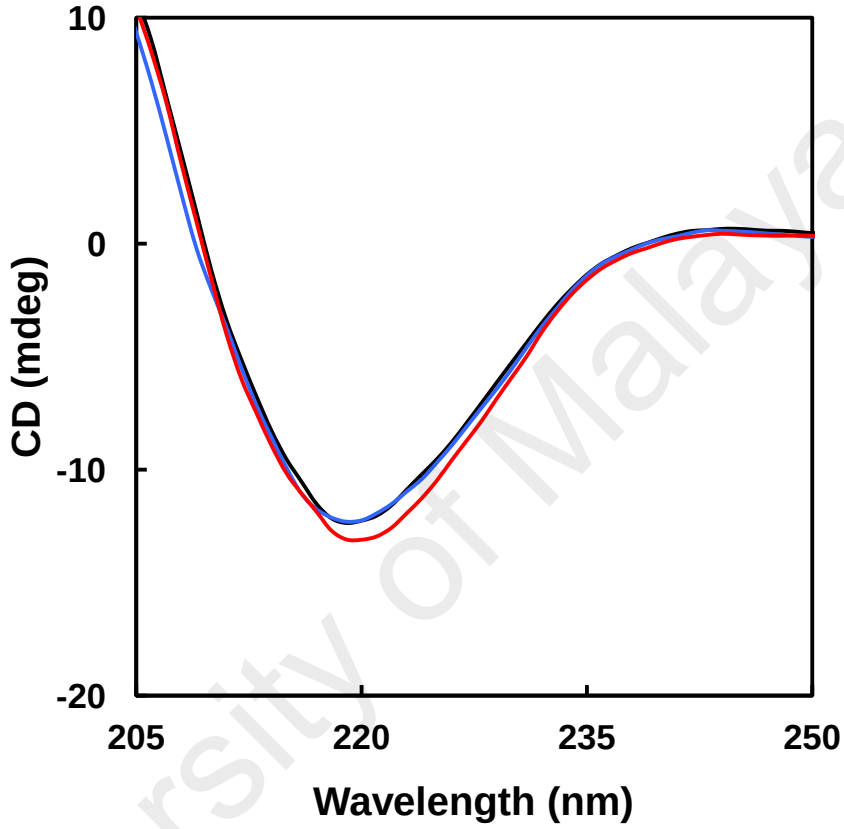


Figure 4.40: Far-UV CD spectra of ADL 1.5 (0.2 μM), obtained in the absence (*black line*) and presence of 30% (w/v) ethylene glycol (*red line*) and xylitol (*blue line*) at 25°C.

figures, both ADL 2.5 and ADL 1.5 were indistinguishable from each other in terms of CD spectral characteristics. Interestingly, differential effects of polyols were noticed on the CD spectral characteristics of ADL 2.5 and 1.5. Whereas ethylene glycol increased the CD spectral signal at 218 nm, no significant change in the spectral signal was observed in the presence of xylitol. Two other polyols (erythritol and sorbitol) also showed similar behavior as observed with xylitol (spectra omitted for clarity). A comparison of the CD spectra of ADL 2.5 and ADL 1.5 (Figures 4.39 and 4.40) suggested relatively greater induction of β-structures in ADL 2.5 than ADL 1.5, in the presence of ethylene glycol. These results, showing differential effects of ethylene glycol with other three polyols, were similar to those shown earlier in Figures 4.31−4.38. Increase in the non-native β-structures in the presence of ethylene glycol might be responsible for the destabilizing effect of ethylene glycol on ADL 2.5 and ADL 1.5.

5. CONCLUSIONS

In summary, the results presented in this thesis describe the structural stability of CGB lectin against urea, acid and thermal denaturations. Urea denaturation of CGB lectin was found to be a two-step, three-state transition, involving a structured monomeric intermediate with increased secondary structures as well as exposed Trp residues and hydrophobic clusters, compared to the native state. A gradual decrease in the hemagglutinating activity of CGB lectin was noticed with increasing urea concentrations, which was completely lost at ~4.0 M urea due to lectin monomerization. Similarly, CGB lectin also showed a two-step, three-state transition upon acid denaturation. A monomeric folded intermediate was characterized at pH 2.5 with increased exposure of Tyr and Trp residues as well as hydrophobic clusters, when compared to the native state at pH 7.0. Further decrease in the pH up to pH 1.5 led to unfolding of the monomer; however, a significant residual structure was retained in the protein at pH 1.5. Thermal denaturation of the acid-denatured CGB lectin (pH 2.5) resulted in the formation of non-native β-structures. Interestingly, polyols such as erythritol, xylitol and sorbitol stabilized the lectin against thermal denaturation at both pH 2.5 and pH 1.5, as revealed by the shift of the thermal transition curve towards a higher temperature range. Furthermore, these polyols also stabilized the tertiary structure of the lectin at both pH 2.5 and pH 1.5, whereas secondary structures of the lectin remained unaffected in the presence of these polyols. On the other hand, ethylene glycol showed destabilizing effect. The work presented in this thesis adds knowledge to understand the folding mechanism of CGB lectin via characterization of the protein intermediates. Furthermore, possible use of specific polyols in stabilizing CGB lectin for future applications has also been explored.

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LIST OF PUBLICATIONS / PRESENTATIONS

Publications

1. **Kameel, N. I. A.**, Wong, Y. H., Shuib, A. S., & Tayyab, S. (2016). Conformational analysis of champedak galactose-binding lectin under different urea concentrations. *Plant Physiology and Biochemistry*, *98*, 57-63.

2. **Kameel, N. I. A.,** Shuib, A. S., & Tayyab, S. (2016). Acid-induced unfolding of champedak galactose-binding lectin. *Protein and Peptide Letters*, *23*(12), 1111-1117.

3. **Kameel, N. I. A.,** Shuib, A. S., & Tayyab, S. (2018). Effect of various polyols on the acid-denatured states of champedak galactose-binding lectin. *Protein and Peptide Letters*, *25*, 1-11.

Presentations

1. **Kameel, N. I. A.**, Wong, Y. H., Shuib, A. S., & Tayyab, S. (2015, June). *Urea-induced structural transitions in champedak galactose-binding lectin.* Poster session presented at the 40th Annual Conference of the Malaysian Society for Biochemistry & Molecular Biology, Sepang, Malaysia.

2. **Kameel, N. I. A.,** Shuib, A. S., & Tayyab, S. (2015, December). *Acid-induced conformational changes in champedak galactose-binding lectin*. Poster session presented at the 20th Biological Sciences Graduate Congress, Bangkok, Thailand.

3. **Kameel, N. I. A.,** Shuib, A. S., & Tayyab, S. (2016, December). *Conformational states of champedak galactose-binding lectin obtained under various denaturing conditions*. Poster session presented at the 2nd Peptides and Proteins Symposium, Nanyang Avenue, Singapore.