

The primary structure of the acidic lectin from winged bean (*Psophocarpus tetragonolobus*): insights in carbohydrate recognition, adenine binding and quaternary association

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Abstract The amino acid sequence of the winged bean acidic lectin (WBA II) was determined by chemical means and by recombinant techniques. From the N- and C-terminal sequence, obtained chemically, primers were designed for PCR amplification of the genomic DNA. The PCR product was cloned and sequenced to get the complete primary structure of WBA II. Peptide fragments for sequencing were also obtained by tryptic cleavages of the native lectin. The WBA II sequence showed a high degree of homology with that of WBA I and *Erythrina corallodendron* lectin (ECoRL), especially in the regions involved in subunit association, where there is a very high conservation of residues. This perhaps implies the importance of this particular region in subunit interactions in this lectin. In addition, many of the residues, involved in carbohydrate binding in legume lectins, appear to be conserved in WBA II. The distinct differences in anomeric specificity observed amongst WBA I, WBA II, ECoRL and peanut agglutinin (PNA) may be explained by subtle differences in sequence/structure of their D-loops. WBA II binds adenine quite strongly; a putative adenine binding sequence has been identified.

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Key words: Lectin; Primary structure; Sugar recognition

1. Introduction

The seeds of the tropical legume, winged bean (*Psophocarpus tetragonolobus*) contain two homodimeric glycoprotein lectins, with similar monosaccharide specificities, but different isoelectric points [1]. The winged bean basic lectin (WBA I) has a $pI \sim 10$ and recognizes blood group A₁ and B substances, but not H-antigen [2,3]. Its complete amino acid sequence was determined earlier [4], and the gene encoding it has also been cloned [5]. The acidic lectin from winged bean (WBA II) has a similar molecular weight (54 kDa), but a $pI \sim 5.5$ and is specific for the H-antigenic sugars, Fuc α 1-2Gal and 2'-fucosyllactose [6,7]. Quite unusually, the binding of these fucosylated sugars is more entropically driven than their non-fucosylated counterparts (viz. galactose and lactose) suggesting an extended binding site with perhaps a hydrophobic region that

recognizes the fucosyl moiety [7,8]. In addition, the thermal unfolding of WBA II suggests that its denaturation occurs independent of its subunit dissociation. In this respect it is similar to the unfolding of both *Erythrina corallodendron* lectin (ECoRL) and WBA I, and differs from that of Con A and the pea lectin [9–11]. These differences in unfolding, between the latter lectins and ECoRL and winged bean lectin reflect differences in their quaternary association [9–11].

In this paper we report the primary structure of WBA II. The sequence of WBA II thus determined is indispensable not only for the elucidation of its three-dimensional structure [12] but also explains the molecular basis of its carbohydrate specificity and quaternary association, in addition to identifying potential glycosylation sites and the possible role of bound carbohydrate, we also discuss some of the general features that may be important in the recognition of adenine and related ligands.

2. Materials and methods

2.1. Materials

TPCK-treated trypsin, guanidinium hydrochloride, ammonium acetate, ammonium bicarbonate, phenyl isothiocyanate, adenine, adenosine diphosphate, 5-anilino naphthalene sulphonate and lactose were from Sigma, USA. Trifluoroacetic acid (TFA), carboxypeptidase Y, HPLC grade acetonitrile and methanol were from Pierce, USA. All the sequencing grade chemicals were from Shimadzu, Japan. Restriction enzymes and DNA modifying enzymes were obtained from Amersham, New England Biolabs, Pharmacia and Stratagene. DNA sequencing reagents were from US Biochemical Corp. WBA II was purified, by affinity chromatography, as described [6,13] and was found pure by sodium dodecyl sulphate polyacrylamide gel electrophoresis [14,15].

2.2. Chromatographic methods

For chromatofocusing WBA II was applied in water to a Mono P (HR 5/20) FPLC column, pre-equilibrated with 25 mM bis-tris buffer pH 6.7. After washing with one bed volume, the isolectins were eluted over a period of 60 min with Polybuffer 74, diluted 15 times and adjusted to pH 4.3 with 2 N HCl. The fractions were lyophilized and desalted on a Sephadex G-50 column to remove Polybuffer. Peak 4, isolectin-IV (IL-IV) was rechromatographed and used for sequencing. Tryptic peptides were separated by reverse phase FPLC on a pep RPC column (Pharmacia) using a gradient of acetonitrile (0–75%) in 0.1% TFA. Agarose gel (1 mm thickness) isoelectric focusing was carried out at pH 4–6 under native conditions using Pharmalytes according to manufacturer's directions.

2.3. Carboxypeptidase digestion

IL-IV in 0.05 M sodium citrate buffer, pH 5 and 4 M guanidinium hydrochloride was incubated with 0.5% (w/w) of carboxypeptidase Y. Aliquots were taken at 0, 2, 5, 10, 20, 30 and 60 min, reaction terminated with 0.4 MK-borate buffer, and the liberated amino acids were analyzed on HPLC as in [16].

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Abbreviations: WBA I and WBA II, winged bean basic and acidic agglutinin, respectively; PNA, peanut agglutinin; ECoRL, *Erythrina corallodendron* lectin; IL-IV, isolectin-IV; CIP, calf intestinal phosphatase

2.4. Sequencing

NH₂-terminal sequence analyses of the native IL-IV and its tryptic peptides were carried out on an automated Shimadzu (PSQ1) gas phase sequencer equipped with an on-line Shimadzu C-R4A120 chromatopac phenylthiohydantoin analyzer.

2.5. PCR amplification and cloning of WBA II

Total genomic DNA was isolated and purified from germinating winged bean [5]. The WBA II sequence was amplified using 100 ng of genomic DNA as the template and the primers 5'-GGAATTC-CATGGAAACCCAATCGTTTAAC-3' and 5'-CGAATTCCTGTT-TGTTTCTTGCAACGA-3'. The PCR conditions used were 95°C, 45 s; 51°C, 1 min; 72°C, 2 min. This cycle was repeated 30 times, followed by a 10 min extension at 72°C in a Pharmacia GENE-ATAQ thermal cycler. The PCR product was digested with *Eco*RI and cloned into the *Eco*RI-digested and calf intestinal phosphatase (CIP)ed to pT7T3 (18U) vector of Pharmacia Biotech.

2.6. DNA sequencing

All sequencing was performed using Sequenase version 2.0 from US Biochemical Corp. according to the manufacturer's instructions. The entire sequencing was carried out on single-stranded DNA template, obtained from pT7T3 (18U) clones, subsequent to infection with the helper phage M13KO7 [17]. Both strands were sequenced using M13 (-40) universal and M13 reverse primers. Deletion clones, generated by cleaving at *Bam*HI and *Hind*III restriction sites, were used to obtain additional sequence information.

2.7. Adenine binding

Fluorescence titrations were performed on a Hitachi fluorescence spectrophotometer using 5 nm slit width on both the monochromators. Interactions between WBA II and adenine were detected by perturbation of protein intrinsic fluorescence on addition of the ligand. To 1 ml of lectin in PBS (0.1–0.3 mg/ml) small aliquots of the ligand (less than 3% v/v) were added. Fluorescence measurements were taken following mixing and equilibration for at least 1 min after each addition. The samples were excited at 290 nm and the corrections due to the optical density of the sample (inner filter effect) were made as described [18]. Association constants were calculated using the method of Chipman et al. [19]. The value of association constant K_a was obtained graphically using the relationship

$$\log S_f = -\log K_a + \log \Delta F_0 / \Delta F_\infty$$

where S_f is the free ligand ΔF_0 and ΔF_∞ are changes in the fluorescence for a given ligand concentration and at infinite ligand concentrations respectively.

3. Results

3.1. Isoelectric focusing and chromatofocusing

Isoelectric focusing on agarose gels between pH 4 and 6 yielded a pattern with four distinct bands with *pI* values of 5.2, 5.3, 5.5 and 5.57 (Fig. 1a). Chromatofocusing was performed to isolate a homogeneous isoelectin species (Fig. 1b). The most abundant peak (4; IL-IV) was rechromatographed on a RP C8 column using a TFA/acetonitrile gradient. Rechromatographed IL-IV was homogeneous by isoelectric focusing and was used for sequencing (Fig. 1c). This protein yielded a single N-terminal sequence up to 34 residues, similar

to that of isoelectin I of Kortt, whose N-terminus analysis was reported earlier [1] (Fig. 2b).

3.2. Tryptic peptides

The isoelectin IV was subjected to tryptic cleavage and the peptides thus obtained were purified by reverse phase FPLC (data not shown). Many of these peptides were sequenced by Edman degradation on the PSQ-1 gas phase sequencer (Fig. 2b).

3.3. C-terminal analysis

Carboxypeptidase digestion of IL-IV identified C-terminal residues to be SSLQETN (Fig. 2b). This was also confirmed by sequencing the C-terminal tryptic fragment, T₁₁, which ended at asparagine.

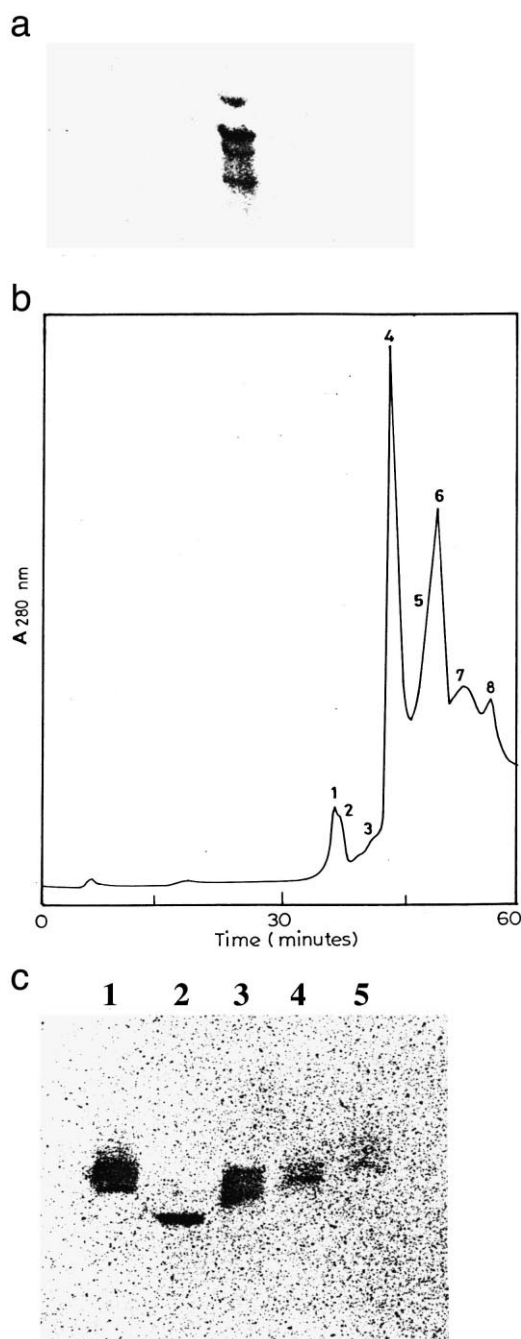


Fig. 1. a: Isoelectric focusing pattern of WBA II in the range of pH 4–6. b: FPLC chromatofocusing of WBA II for separation of isoelectins. The column (Mono P HR 5/20) was equilibrated with 25 mM bis-tris buffer (pH 6.7) before injecting the sample (10 mg) dissolved in the same buffer. The isoelectins were eluted with Poly-buffer 14, 15 times diluted and adjusted to pH 4.4 with 2 N HCl. Peak fractions were collected as indicated in the figure. c: Isoelectric focusing pattern, in pH 4–6 range, of the isoelectins obtained after chromatofocusing (1) peak 1 (2) IL-IV (3) peak 5 (4) peak 6 and (5) peak 7. IL-IV was used for sequence analysis.

a

N-Terminal primer:

E T Q S F N

WBA II: 5' GGAATTCATG GAA ACC CAA TCG TTT AAC 3'

WBA I: 5' AAA ACC ATA TCG TTT AAC 3'

K T I S F N

C-Terminal primer:

S L Q E T N

WBA II: 3' AGC AAC GTT CTT TGT TTG TCCTAAGC 5'

WBA I: 3' AGC AAC GGT CCT TGT TTG 5'

S L P G T N

b

ETQSFNFDHFEENSKELNLQRQASIKSSGVLELT KLTKNGVPVWKSTGR
 ──────────── NT ────────────
 ─── T₃ ─── ─── T₂ ─── ─── T₇ ───
 ALYAEPIKIWDSTGNVASFETRFSNITQPYAYPEPADGLTFMVPVNS
 ─── T₅ ─── ─── T₆ ───
 PGGEDGGNLGVFKSNPEGDNALAVEFDTFQNTWDPQVPHIGIDVNSIVSS
 ─── T₈ ───
 KTLHFQLENGGVANVVIKYDSPKILNVVLAFFHSVGTVYTLNIVDLKQE
 ─── T₉ ─── ─── T₁₂ ───
 FPNSEWVNVGLSATGYQKNAVETHEIISWKSFTSSLQETN
 ─── T₁ ─── ─── T₁₀ ─── ─── T₁₁ ───
 <<<<<<<<

c

A		B	
79	92	102	109
WBA II	QPYAYPEPADGL TF	WBA II	GEDGGNL G
WBA I	QPF P RHPADGLVF	WBA I	GEGGGYF G
ECorL	QPYT RPLPADGLVF	ECorL	AQGYGYL G
PNA	DI KDY -DPADGI IF	PNA	S I GGGTLG

C		D	
123	134	209	229
WBA II	VEFDTFQNTWD P	WBA II	GLSATTGY-----QKNA VETHE I I SW
WBA I	VEFDTRFNTWD P	WBA I	GFSAAATGDPGKQR NA TETHD I LSW
ECorL	VEFDTFSNQWD P	ECorL	GLSGATGA-----QR DA AETHDVYSW
PNA	VEFDTSYNSYND	PNA	GFSAS-GSLGGR Q-----IHL I RSW

Fig. 2. a: Sequence of the primers designed for PCR of WBA II. The DNA sequence of the corresponding regions of WBA I was modified by incorporating minimum changes (indicated in bold for WBA II). b: Complete amino acid sequence of WBA II. NT denotes the sequence determined by NH₂-terminal sequence analyses of IL-IV. T denotes tryptic peptides and subscript their order of elution from the pepRPC FPLC column. The peptide sequences obtained by both amino acid composition and sequence analysis are indicated by continuous solid lines, and those deduced by amino acid composition analysis by dashed lines. <<< – Residues also obtained by carboxypeptidase digestion. c: Residues that make up the four binding site loops of WBA I, ECorL and PNA compared with the putative loops of WBA II. The residues involved in binding are shown in bold.

3.4. Cloning of the WBA II coding region

Oligoprimers were designed corresponding to the N- and C-termini of WBA II using the codon degeneracy and the sequence of homologous WBA I (Fig. 2a). The N-terminal 29

nucleotide primer with the sequence 5'-GGAATTCATG-GAAACCCAATCGTTTAAC-3' consisted of 18 nucleotides corresponding to the first six amino acids and an additional 11 nucleotide overhangs containing sites for the restriction enzyme *EcoRI*. The C-terminal primer with the sequence 5'-CGAATTCCTGTTTGTTCCTTGCAACGA-3' containing 18 nucleotides corresponding to the last six amino acids was designed with an additional 9 nucleotide anchor sequence to incorporate sites for *EcoRI*. The single PCR product thus obtained, was about 700 bp in size, which is the size expected from the molecular weight of the protein. This suggested the absence of any intervening sequences. The PCR product was digested with *EcoRI* and cloned into the same site of plasmid pT7T3. *HindIII* and *BamHI* deletion clones were also generated to obtain additional sequence information. The complete sequence of WBA II, of the cloned PCR product and the deletion clones, was obtained by Sanger's dideoxy method. The amino acid sequence, derived from the DNA sequence, revealed that the protein was 240 residues long. This again is quite close to that expected for a protein with a *M_r* of 27 kDa. Also the sequence obtained from some of the tryptic peptides matched well with the DNA-derived protein sequence (Fig. 2b).

3.5. Adenine binding

The fluorescence of WBA II was quenched on interaction with adenine (Fig. 3a). The association constant, *K_a*, obtained from the plot in Fig. 3b, for adenine = $4.2 \times 10^5 \text{ M}^{-1}$ using a lectin molecular weight of 54 000 Da, with *n* being close to 1. For adenosine *K_a* was $1.5 \times 10^3 \text{ M}^{-1}$.

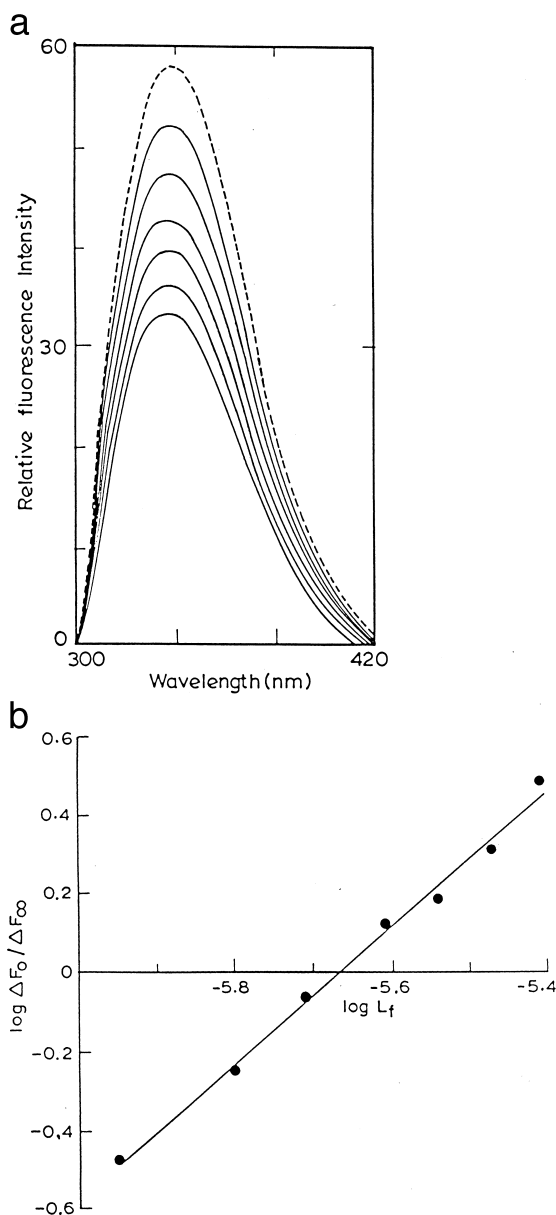
4. Discussion

Since legume lectin genes known so far do not possess introns, it was decided to attempt isolating the DNA coding region of WBA II by direct PCR amplification of the genomic DNA [5,20]. Earlier, the same strategy had been successfully employed for peanut agglutinin (PNA) and WBA I [5,20]. The sequence thus determined is consistent with its amino acid composition (data not shown), molecular weight as well as that obtained using Edman degradation of the native protein and most of its tryptic fragments. The primary structure of WBA II when compared with that of other legume lectins shows that most of the features characteristic of this family of proteins, namely the metal binding sites and the carbohydrate binding loops, are conserved (Fig. 4). In particular, it exhibits the highest identity with WBA I, at 64%; and ECorL at 60%. Thus it is in order to discuss some of the features of WBA I, ECorL and other related lectins sequences that may have a bearing on the structure and ligand binding properties of WBA II.

4.1. WBA II sequence and implications in ligand recognition

Most of the residues involved in metal binding in legume lectins appear to be conserved in WBA II. In WBA I, residues Glu-122 OE2, Asp-124 OD2, Asp-131 OD1, His-136 NE2 and two water molecules coordinate Mn²⁺, while Asp-124 OD1 and OD2, Phe-126 O, Asn-128 OD1, Asp-131 OD2 and two other water molecules coordinate Ca²⁺ [5,21]. The residues in WBA II that correspond to these are, Glu-124, Asp-126, Phe-127, Asn-129, Asp-132 and His-137.

The carbohydrate binding site of legume lectins is made up



C		
Lectins		Affinity
Tetrameric		
Lima bean lectin	VLITYDSST	H
PHA-E	VLITYDSST	H
Soybean lectin	VLITYDAST	H
Con A	- I I - YNSVD	-
Peanut lectin	V - I - YDSST	H
Dimeric		
ECoRL	VVIKYDASS	-
WBA I	VVIKYDAST	H
WBA II	VVIKYD SPT	H

Fig. 3. a: Fluorescence spectrum of WBA II excited at 290 nm and emission recorded at >300 nm. The dashed line represents the spectrum of WBA II, which is quenched on addition of adenine (solid lines). b: A typical plot for the evaluation of association constants for adenine binding. c: Homology between adenine binding peptides of legume lectins. (—) indicates non-binding of those lectins to adenine.

of four loops: A, B, C and D. Of these, the A, B and C loops are the most conserved providing the basic framework for carbohydrate binding [22,23]. On the other hand, the D loop is the most variable amongst the legume lectins, both in size and sequence, and determines their fine sugar specificities [23]. Gal/GalNAc specific lectins have a longer D loop than the Glc/Man specific ones [23,24]. In the Gal specific lectins, the O81 and O82 of Asp from loop A donate hydrogen bonds to 4-OH and 3-OH, respectively, while N82 of Asn and the main chain of Gly accept hydrogen bonds from the 3-OH of the sugar. In addition, Gal stacks with an aromatic residue. In WBA II residues corresponding to them are, Asp-88 that is expected to hydrogen bond the O3 and O4 of the sugar, and Gly-105 N and Asn-130 that hydrogen bonds with O3 of the sugar. The Gal ring in WBA I stacks against Phe-128 of the C-loop (Figs. 2c and 4).

An important feature of ligand specificity of WBA I is its pronounced preference for the α anomer of galactose. The ratio of the binding constant of the Me- α form to that of the Me- β form (K_{α}/K_{β}) is about 6.6 [9]. In contrast, ECoRL and PNA are much less discriminatory. Their K_{α}/K_{β} ratios are 1.77 [11] and 1.62 (Reddy, G.B. and Surolia, A., unpublished data) respectively. The crystal structure of WBA I, complexed to Me- α -Gal, provides an explanation for its higher selectivity for the α anomer [21]. While the α linkage is acceptable, the β linkage leads to an unacceptable steric contact with the D loop, particularly Ser-214. On the other hand, the smaller D loop size of ECoRL and PNA can accommodate both anomers, consequently, these lectins are less selective in their anomer specificity. WBA II with K_{α}/K_{β} 3.36 [7], is thus more specific towards the Me α Gal than either ECoRL or PNA, but less so than WBA I. It is therefore, conceivable that the smaller size of its D loop may make it more accommodating towards the β anomer than WBA I.

4.2. Implications of the sequence on the quaternary structure

All legume lectins, whose structures are known so far, appear to have a common tertiary structure – ‘the jelly roll’ fold [25]. Yet, legume lectins differ considerably in their quaternary associations; which appears to effect their unfolding properties, particularly their thermal stability [9–11,24]. While the canonically associated lectins, which possess a greater degree of inter-subunit interactions (nearly 1000 Å² area is buried at the subunit interface) possess very high unfolding cooperativity, with the entire oligomeric unit unfolding as a single entity, ECoRL and WBA I, where the inter-subunit interactions are much less (only 700 Å² area is buried at the interface) have a lower unfolding cooperativity, with the unfolding of each monomer occurring independently [11,24]. WBA II exhibits similar unfolding mechanisms as ECoRL and WBA I [26]. This suggests that, like ECoRL and WBA I, the monomers of WBA II may associate less tenaciously [26]. In the region that would correspond to the intersubunit interface (residues 161–194 of WBA I), there is about 70% sequence identity and

wba2	S	-E-TQS---FNFDFHEENSKELNLRQASIKSSGVLELTKLTKNGVPVWK
wba1	S	-K-TIS---FNFNQHFNQEEQLKLQRDARISSNSVLELTKVV-NGVPTWN
EcorL		VE-TIS---FSFESEFEPGNDNLTLQGAAALITQSGVLQLTKINQNGMPAWD
pna	ST	AE-TVS---FNFNSSFEGNPAINFGQDVTVLSNGNIQLTNLKNV-----N
pea	ST	T-ETTS---FLITKFSPDQQNLIFQGDG-YTTKEKLTLTAKVKNTV----
favin		TDEITS---FSIPKFRPDQPPLFIQGGG-YTTKEKLTLTAKVKNTV----
LOL I		T-ETTS---FSITKFGDPQQNLIFQGDG-YTTKERLTLTAKVRNTV----
LenL	S	T-ETTS---FSITKFPDQQNLIFQGDG-YTGKEGLTTLTKVSKETG----
conA	S	STHETNALHFMFNQFSKDQDKDLILQGDATTGTGNRLRTRVSSNGSQGS * .* **
wba2	S	STGRALYAEPKIWDSTTGNVASFETRFS-FNITQPYAYPEPADGLTFFM
wba1	S	STGRALYAKPVQWVDSTTGNVASFETRFS-FSIRQPFPRPHADGLVFFI
EcorL		STGRTLYAKPVIHWDMTGTVASFETRFS-FSIEQPYTRPLPADGLVFFM
pna	ST	SVGRVLYAMPVRIWSSATGNVASFLTDFS-FEMKDIKY-DPADGIIFI
pea	ST	--GRALYSSPIHIWDRDTGNVANFVTSF-TFVINAPNSY-NVADGFTFFI
favin		--GRALYSPLIIHDSETGNVADTTTTFFIFVIDAPNGY-NVADGFTFFI
LOL I		--GRALYSSPIHIWDSKTGNVANFVTSF-TFVIDAPNSY-NVADGFTFFI
LenL	S	--GRALYSTPIHIWDRDTVNVANFVTNGSQVFRESPNGY-NVEDGFTFFI
conA	S	SVGRALFYAPVHIWESSAV-VASFETF-TFLIHSPDSHP--ADGIAIFFI *** *. ** *
wba2	S	VPPNSP---GGEDGGLGVFKSNPE-GDN--ALAVEFDTF-QNTWDP-QV
wba1	S	APPNTQ---TGEGGGYFGIYNPL----SPYPFAVEFDTEFRNTWDP-QI
EcorL		GPTKS---PAQGYGGLGFNNFSKQ-DNSYQTLGVEFDTF-SNQWDDPPQ
pna	ST	APECTQIPAGSIGGTLGVSDTKGA-G--HFVGVEFDTYNSEYNDPPT
pea	ST	A-PVDTKPQT---GGGYLGVF-NSAEYDKTTQTVAVEFDTFYNAAWDSPNR
favin		AAPVDTKPQT---GGGYLGVF-NGKDYDKTAQTVAVEFDTFYNAAWDSPNG
LOL I		A-PVDTKPQT---GGGYLGVF-NSKDYDKTSQTVAVEFDTFYNTAWDSPNG
LenL	S	A-PVDTKPQT---GGGYLVGFYNGKEYDKTSQTVAVEFDTFYNAAWDSPNK
conA	S	SMIDSSIPSGSTGR-LGLFPDA---NADTIVAVELDTYPNTDIDIGDP * ..**.
wba2	S	P---HIGIDVNSIVSSKTLHFQLENGGVANVVIKYDSPTKILNVVLAFHS
wba1	S	P---HIGIDVNSIVSTKVPTFLDNGGIANVVIKYDASTKILHVVLVFPS
EcorL		P---HIGIDVNSIRSIKTPQFQLDNGQVANVVIKYDASSKILHAVLVYPS
pna	ST	D---HVGIDVNSVDSVKTVPWNVSUGAVVKVTIYDSSTKTLSVAVT-ND
pea	ST	DR-HIGIDVNSIKSVNT-SWLQNGEENVVIAFNAATNVLTVSLTYPN
favin		KR-HIGIDVNTIKSIKSWNLQNGEEAHVAISFNATTNVLTVSLTYPN
LOL I		DR-HIGIDVNSIKSINTKSWLKQNGKEANVVIAFNA-TNVLTVSLTYPN
LenL	S	ER-HIGIDVNSIKSVNTKSWNLQNG-----
conA	S	SSYPHGIDIKSVRSKKTAKWMNQNGKGVTAGHHIYNSVDKRLSAVVSYPN *...* * * *
wba2	S	VGTVYTLSNIVDLKQEFPNSEWWNVGLSATTCG---QKNAVETHESISW
wba1	S	LGTIYITIADVLDLKLQVLP--ESVNVGFSAAATGPSKGQRNATEHDILSW
EcorL		SGAIITYTIAEIADVVKQVLP--EWVDVGLSGATGA---QRDAETHDVDYSW
pna	ST	NGDIITTAIQVLDLKAQLP--ELRVKFGFSAS-GSLGGRQ-----IHLIRSW
pea	ST	V-TSYTLSDVVSCLKDVVP--EWRVIGFSATTG-A-----EYAAHEVLWSW
favin		L-TGYTLSEVVPLKDVVP--EWRVIGFSATTG-A-----EYATHEVLWSW
LOL I		V-TSYTLNEVVPLKDVVP--EWRVIGFSATTGGA-----EFAAHEVLWSW
LenL	S	V-TSYTLNEV-PLKDVVP--EWRVIGFSATTG-A-----EFAAQEVHSW
conA	S	ADSA-TVSYDVLDLNVLP--EWRVGLSASTGLYK-----ETNTILSW * * * *....* ..**
wba2	S	SFTSSLQETN-----
wba1	S	SFSASLPGTNE-----F
EcorL		SFAQASLPETNDAVIPTSNNHTFAI
pna	ST	SFTSTL-----ITTTRRS----
pea	ST	SFHSELSG-----TSSSKQAADA
favin		TFLSELTG-----PSN-----
LOL I		SFHSELGG-----TSGSQK----
LenL	S	SFNSQLG-----HTSKS
conA	S	SFTSKLKS----- * *

Fig. 4. Comparison of the amino acid sequence of WBA II with other legume lectins. The amino acids completely conserved in all the proteins are indicated (*). The glycosylation site for WBA II is at position 76, for WBA I at positions 44 and 219 and for ECorL at position 17. Plausible metal binding residues in WBA II are Glu-122, Asp-124, Asp-131, His-136, Phe-126 and Asn-128. Gaps (-) are introduced for maximum alignment.

92% similarity between WBA I and WBA II. In WBA II a single glycosylation site is identified at Asn-76 which is quite distant from that of ECorL (Asn-17). Consequently, glycosylation in WBA II should not then, in principle, prevent a canonical association. Thus the formation of ECorL/WBA I kind of quaternary structure for WBA II is intrinsic to the protein.

An additional factor that appears to affect the quaternary associations in legume lectins is the presence/absence of a charged residue in the region involved in the Con A-type dimeric interface. Lys-55 has been proposed, in addition to the glycosylation, as being responsible for the non-canonical association of ECorL [5,24]. A lysine is also found at corresponding position (K 53) in WBA I [5]. In addition, Glu-58 has been proposed to play an important role in the non-canonical association of GS IV [27]. On the other hand, in lectins with the canonical association, namely flavin, pea lectin and con A, the corresponding residues are uncharged; they are Leu-49, Ser-48 and Ala-177 respectively [28]. In WBA II, the residue at this position is Glu-54. Thus it seems probable that WBA II could display a non-canonical association in order to evade the burial of this charged group (i.e. Glu-54).

4.3. Adenine binding

Adenine binds to WBA II with a high affinity and was found to be independent of sugar binding. Also, hydrophobic ligands like 8-anilino naphthalene sulphonic acid did not bind to WBA II. The peptides from lima bean lectin (LBL) and *Phaseolus vulgaris* leucoagglutinin (PHA-E) that interact with adenine [29] are shown in Fig. 3c; aligned with the corresponding region in WBA II. Thus this region appears to be highly conserved amongst the adenine binding lectins. It may be noted that, both con A and pea lectin possess the adenine binding sequence, as does ECorL; and yet these lectins do not bind adenine. On the other hand although peanut lectin binds adenine, the sequence does not compare so well to the other adenine binding lectins [30]. It thus appears that the peptide sequence alone is insufficient to determine the adenine binding property of the lectin. This is confirmed, by the recent determination of the crystal structure of *Dolichos biflorus* lectin (DBL) complexed to adenine [25]. In DBL, the adenine binding site is present in two cavities, formed out of two interfaces of the tetramer. The adenine molecules are bound by hydrogen bonds from Leu-165, Thr-167 and Ser-178 and hydrophobic interactions with Leu-165, Val-176, Val-180, Ile-189, Leu-244 and 248. It appears that a very precise alignment of the monomers is essential for the formation of the adenine binding pocket, as was proposed by us earlier for WBA I [4]. This in turn perhaps explains why LBL binds adenine, but con A does not. In con A, the putative adenine binding region comprises a portion of the anti-parallel β structure, involved in contacts between the two dimers, to form the tetramer and is therefore unavailable for ligand binding. The requirement of very precise alignment of monomers, in order to form the adenine binding pocket at the monomer–monomer interface also, perhaps, explains why WBA I binds adenine, while ECorL does not. From Fig. 3c it is clear that there is just a single residue difference between the putative adenine binding sequence of WBA I and ECorL. While there are differences in two residues between this region of WBA I and that of WBA II (both of which bind adenine). Though ECorL and WBA I have the same quaternary structure, the angle between the

back sheets of the monomers, that associate to form the ‘hand-shake’ structure, is 47.6° in ECorL and 49.3° in WBA I [21,24]. Since the adenine binding sequence appears to be at the monomer–monomer interface, we propose that the slightly larger angle in WBA I results in greater exposure of this sequence. In WBA II, perhaps a similar exposure of the sequence results in its adenine binding.

In conclusion, the WBA II sequence possesses all the characteristic features of legume lectins in its metal ion and carbohydrate binding sites. It shows highest homology to ECorL and WBA I. Some of the specific ligand binding characteristics, such as anomeric specificity may be explained by features of its binding site loops, specifically the D loop. WBA II binds adenine, and based on the known adenine binding region of LBL and PHA-E, a putative adenine binding site has been identified. A precise alignment of monomers may be necessary to form the adenine binding site. Finally, similarities and differences in sequence, analogous to their thermal unfolding properties, exist between WBA II and the other legume lectins. This seems to imply that the quaternary association of WBA II, more so, the degree to which its subunits associate may be similar to that of ECorL.

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