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Unusual sugar specificity of banana lectin from *Musa paradisiaca* and its probable evolutionary origin. Crystallographic and modelling studies

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The crystal structure of a complex of methyl-α-D-mannoside with banana lectin from *Musa paradisiaca* reveals two primary binding sites in the lectin, unlike in other lectins with β-prism I fold which essentially consists of three Greek key motifs. It has been suggested that the fold evolved through successive gene duplication and fusion of an ancestral Greek key motif. In other lectins, all from dicots, the primary binding site exists on one of the three motifs in the three-fold symmetric molecule. Banana is a monocot, and the three motifs have not diverged enough to obliterate sequence similarity among them. Two Greek key motifs in it carry one primary binding site each. A common secondary binding site exists on the third Greek key. Modelling shows that both the primary sites can support 1–2, 1–3, and 1–6 linked mannosides with the second residue interacting in each case primarily with the secondary binding site. Modelling also readily leads to a bound branched mannopentose with the nonreducing ends of the two branches anchored at the two primary binding sites, providing a structural explanation for the lectin's specificity for branched α-mannans. A comparison of the dimeric banana lectin with other β-prism I fold lectins, provides interesting insights into the variability in their quaternary structure.

Key words: β-prism I fold lectin/evolution of carbohydrate specificity/lectin-branched sugar interaction/quaternary association/oligosaccharide modelling

Introduction

Lectins are an important class of carbohydrate binding proteins involved in a variety of biological processes (Lis and Sharon, 1998; Drickamer, 1999; Vijayan and Chandra, 1999; Loris, 2002). Plant lectins are the most thoroughly studied group among them. Plant lectins themselves can be broadly brought under five structural classes on the basis of their folds (http://cermav.cnrs.fr). One of these classes with a β-prism I fold was first characterized in this laboratory through the structure analysis of the galactose-specific tetramer, jacalin, one of the two lectins from jackfruit seeds (Shankarnarayanan *et al*., 1996). The other lectin from the same source, artocarpin, also adopts the same fold and quaternary structure, although it is mannose specific (Pratap *et al*., 2002). Subsequently, binding sites of the two lectins were thoroughly characterized (Jeyaprakash *et al*., 2002, 2003, 2004). They use separately, posttranslational proteolysis and variation in loop length as strategies for generating or modifying carbohydrate specificity. In the meantime, the structures of other lectins with a β-prism I fold also became available. They include *Maclura pomifera* agglutinin (Lee *et al*., 1998), heltuba (Bourne *et al*., 1999), and calsepa (Bourne *et al*., 2004). Although all of them have the same fold and similarly located single sugar binding site per monomer, they exhibit considerable variation in their quaternary association. Here we report the structure of a β-prism I fold lectin from banana (*Musa paradisiaca*). This is the first lectin to be x-ray analysed from Musaceae plant family.

Banana lectin was first isolated from *M. paradisiaca* and was shown to be dimeric and mannose specific in 1990 (Koshte *et al*., 1990). From studies on the lectin isolated from *Musa acuminata*, Peumans and others showed its subunit molecular weight to be 14–15 kDa (Peumans *et al*., 2000). From sequence comparison they concluded that the lectin has a jacalin-like structure with β-prism I fold. The carbohydrate binding properties of the lectin were further explored by Goldstein and others (Goldstein *et al*., 2001; Mo *et al*., 2001). They showed that this mannose-/glucose-specific lectin interacts with branched chain α-mannans and α-glucans. The results presented here provide a structural rationale for the specificity in terms of the two primary carbohydrate binding sites per subunit in banana lectin. Indeed, banana lectin is the only one among the lectins of known structure with β-prism I fold to exhibit more than one primary combining site per subunit. This observation has evolutionary implications as well. The structure of banana lectin, reported here, also provides insights into the variations in the quaternary structure of these lectins.

Results and discussion

Overall features

The structure of banana lectin was originally solved in trigonal crystals using room temperature data as described in our earlier crystallization article (Singh *et al*., 2004). Subsequently, the structure has been refined against data collected at low temperature (resolution 2.45 Å). The crystal asymmetric unit consists of a dimeric molecule

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with noncrystallographic two-fold symmetry. The two subunits superpose with an root mean square (rms) deviation of 0.26 Å in C^{α} positions. Thus, the two subunits have practically the same structure. Protein code AAM48480 against banana lectin in NCBI database (http://www.ncbi.nlm.nih.gov/entrez/) was used for model building. As in the case of other jacalin-like lectins, the 141 residue polypeptide chain in each subunit has a β-prism I fold in which the Greek keys roughly form the faces of a trigonal prism in such a way that the strands are parallel to the approximate three-fold axis of the prism (Figure 1). The polypeptide chain has a circular arrangement such that the C-terminal residues 120–141 and 5–20 (residues 1–4 are not defined in the structure) form Greek key I. Greek keys II and III are made up of residues 25–66 and residues 73–115 respectively. The loops in the Greek keys and those that connect them occur at the two ends of the prism. The carbohydrate binding sites are located at one of these ends.

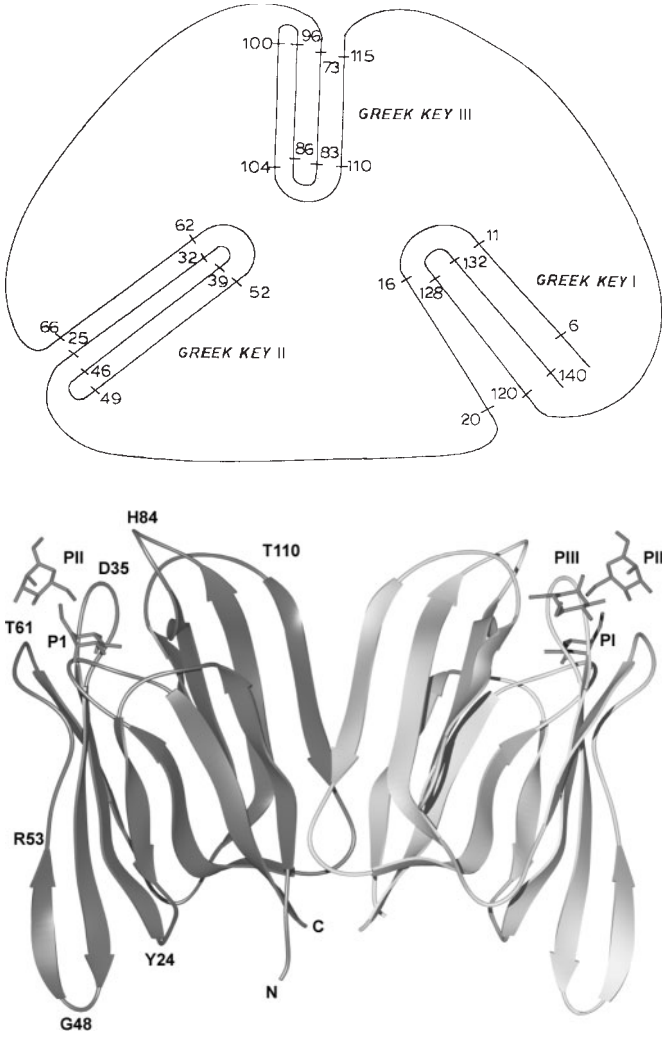


Fig. 1. (**a**) Schematic representation of the lectin fold (**b**) The structure of the banana lectin dimer viewed perpendicular to the molecular dyad. Bound sugar molecules (PI, PII, and PIII) are also indicated.

Structural variability and different modes of quaternary association

The crystal structures of three other mannose-specific β-prism I fold lectins, namely, artocarpin, heltuba, and calsepa, are currently available. Artocarpin is a tetramer, whereas heltuba is an octamer. Calsepa, like banana lectin, is a dimer. The sequence identity of banana lectin with the other three lectins, computed using a structure-based alignment, varies between 32 and 34%. The subunits of banana lectin superpose on those of the other lectins with rms deviations in the C^{α} positions in the range of 1.14–1.31 Å. Thus the subunit structures of the four lectins are essentially the same. Differences, however, occur in the loops. There is one major difference involving strands as well. As shown below, this difference is related to the quaternary association in the four lectins.

As illustrated in Figure 2, artocarpin is a tetramer with 222 symmetry, whereas heltuba is an octamer with 422 symmetry. The artocarpin tetramer has two significant inter-subunit interfaces: that between A and B and its symmetry equivalent and that between A and C and its symmetry equivalent. Heltuba also has sets of two interfaces: 1–2 and 1–3. The inter-subunit interface in dimeric banana lectin is similar to the AB interface in artocarpin and 1–2 in heltuba. The dimeric interface in calsepa has some similarity with the 1–3 interface in heltuba. If subunit 1 of heltuba and 1 of calsepa are superposed, the second subunit of calsepa can be brought into superposition on the subunit 3 of heltuba by a rotation of 113° about an axis roughly perpendicular to the molecular dyad of calsepa. Thus although the residues involved in the dimeric interface of calsepa and the 1–3 interface of heltuba have a great deal of commonality, the mutual orientation of the subunits about the interface is dissimilar.

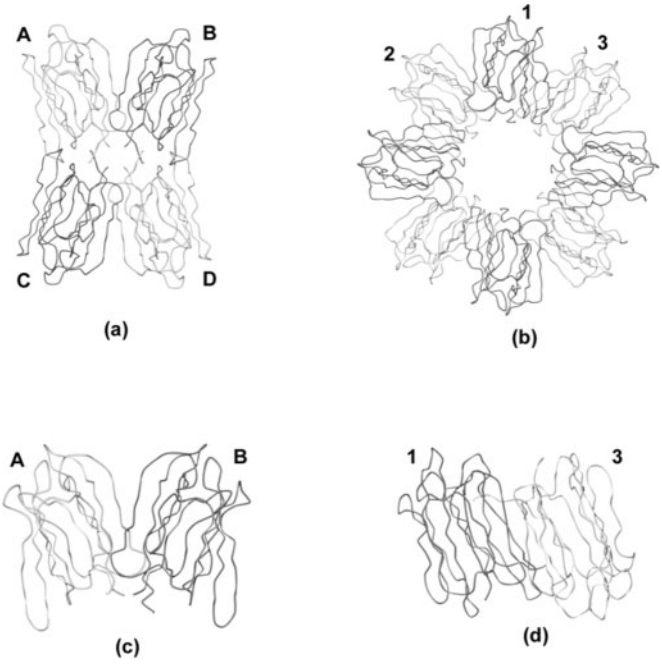


Fig. 2. Quaternary association in (**a**) artocarpin, (**b**) heltuba, (**c**) banana lectin, and (**d**) calsepa.

The N terminal strand is involved in interactions in all the four different types of inter-subunit interfaces. A few C terminal residues adjacent to this strand in Greek key I occur in three of the four interfaces, the 1–3 interface in heltuba being the exception. Residues 110–118 involving an outer strand of Greek key III occur only in the inter-subunit interface in banana lectin and the AB interface in artocarpin and the 1–2 interface in heltuba. On the contrary, the stretch 45–59 in Greek key II occurs in the interface in calsepa and to some extent in the AC interface of artocarpin and the 1–3 interface of heltuba. It does not occur in the inter-subunit interface in banana lectin. The same is true about the 16–24 stretch.

To further explore the possible effects of quaternary association on the tertiary structure, the subunits in tetrameric artocarpin, octameric heltuba, and dimeric calsepa were replaced by the banana lectin subunit. These models may be referred to as banana on artocarpin, banana on heltuba, and banana on calsepa respectively. Thus, for example, in banana on artocarpin, the arrangement of subunits in the tetramer is the same as in artocarpin, but each subunit was taken from the structure of banana lectin. Similarly, models of calsepa on the other three lectins, artocarpin on the other three lectins, and heltuba on the remaining three lectins were also constructed. Steric clashes in these models were then carefully examined, particularly in the regions involving stretches 16–24, 45–59, and 110–118, which occur in some interfaces but not in the others. All of them are involved in steric clashes. Those involving the 45–59 stretch are particularly extensive. This perhaps explains the substantial structural variation in this region among the four lectins (Figure 3).

Sugar binding sites and evolutionary implications

Two bound methyl-α-D-mannoside molecules could be clearly identified in each of the subunits (Figures 1 and 4). One more sugar molecule could be identified in one of the subunits (Figure 1); however density for it does not exist at the corresponding site in the other subunit. Thus, two sugar sites per subunit consistently occur in both the subunits. The third sugar associated with one of the subunits makes only two hydrogen bonds with the lectin, O1 with Asn106 ND2, and O4 with His63 NE2, as against 7–8 hydrogen bonds made by the other two sugars. Furthermore, the region where the third sugar binds is not characterized by normal features, such as cavity, associated with sugar binding sites in lectins. Thus, the binding of the third sugar appears to be an artifact resulting from high ligand concentration in the crystallization solution. Therefore, only the two sugar binding sites, which occur in both the subunits are considered in further discussion.

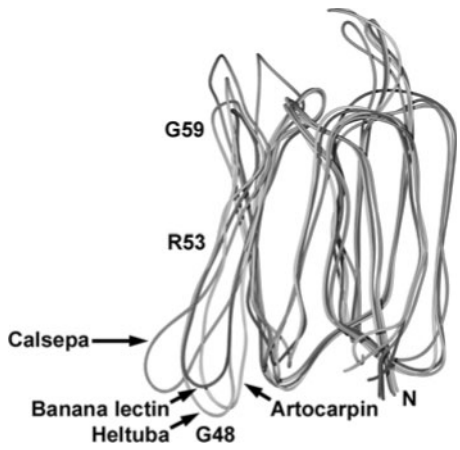


Fig. 3. Superposition of the main chain traces of banana lectin, artocarpin, heltuba, and calsepa.

A single sugar binding site per subunit exists in other β-prism I fold lectins whose sugar complexes have been X-ray analysed. Among the mannose binding lectins with this fold, the combining site has been well characterized in artocarpin and heltuba. Using the sequence numbering in banana lectin, this binding site is made up of loops 14–17, 129–133, and 83–86. The first two loops constitute the primary binding site for the monosaccharide, whereas loop 83–86, which is longer in artocarpin, is responsible for differences in the oligosaccharide specificity of artocarpin and heltuba (Jeyaprakash *et al.*, 2004). In banana lectin also, residue regions 14–17 and 129–133 are involved in binding methyl-α-D-mannoside (Figure 4, Table I) with interactions similar to those found in artocarpin and heltuba. The corresponding loops in the second binding site are 57–61 and 34–38 (Figure 4, Table I). Surprisingly, the geometries of the two binding sites and the nature of the interactions with sugar at them are almost identical.

The above observation appeared intriguing to start with. A close examination of the sequences of the concerned lectins however yielded a plausible explanation for it. In Figure 5, sequences corresponding to the three Greek keys in each of the four lectins are aligned on the basis of structural superposition among them. In the case of jacalin, it has been suggested that the lectin could have evolved through gene duplication and fusion from a common ancestral motif of about 40-residue length (Shankarnaryanan *et al.*, 1996). This suggestion appears to hold good for other lectins with β-prism I fold as well. Over a period of time, the sequences of the stretches have diverged substantially. There are only one and two residues common to all the three stretches in artocarpin and calsepa, respectively. The number is four in heltuba as well as in banana lectin. In artocarpin and calsepa, the sequence identity between Greek keys I and II, and II and III is low in the range of 2–4, whereas it is 9–11 in banana lectin and heltuba. The sequence identity between I and III is high in all cases and varies between 6 and 10. It is interesting that the primary carbohydrate binding site lies on Greek key I, whereas Greek key III carries the secondary site which determines the specificity at the oligosaccharide level in artocarpin and heltuba. The main component of the primary binding site and the secondary binding site reside at comparable regions in the sequences, but they do not have identical residues in them.

The most interesting observation in relation to carbohydrate binding is the relation between Greek keys I and II in banana lectin. The main carbohydrate binding stretch in Greek key I has a sequence GDXXD where X is a hydrophobic residue. Significantly this sequence is reproduced in the same position in Greek key II as well, only in banana lectin. Gly 15 in Greek key I, which also interacts with the

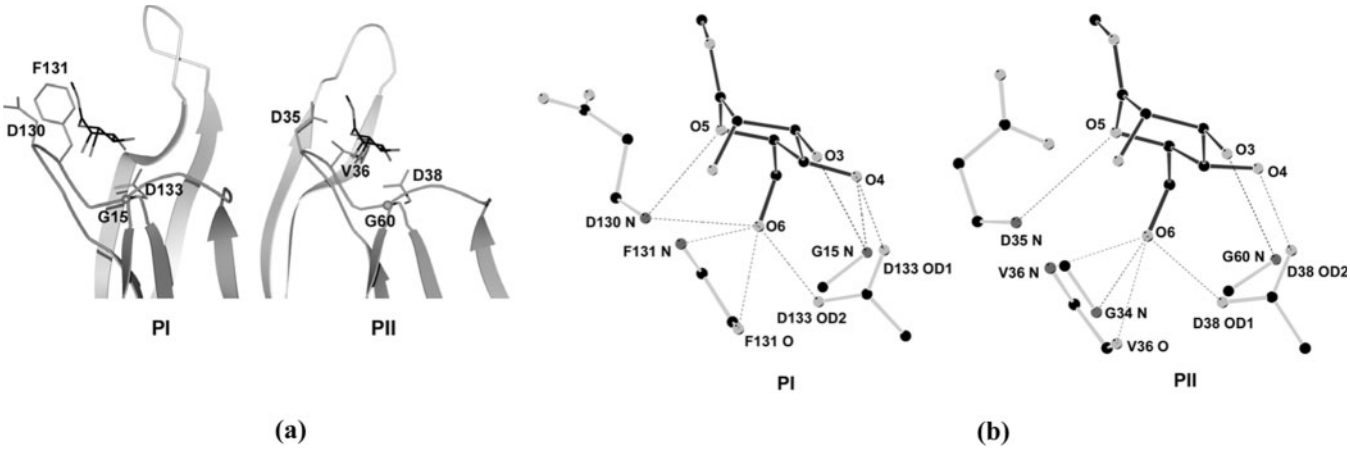


Fig. 4. (**a**) The primary binding sites (PI and PII) and the common secondary binding loop (long loop) in banana lectin. See text for details.(**b**) Lectin-sugar hydrogen bonds at PI and PII.

Table I. Hydrogen bonding between methyl-α-D-mannoside and banana lectin at the two primary sites (PI and PII)

Sugar site	O3	O4	O5	O6
PI	G15 N	G15 N	D130 N	D130 N
		D133 OD1		F131 N
				F131 O
				D133 OD2
PII	G60 N	D38 OD2	D35 N	G34 N
				V36 N
				V36 O
				D38 OD1

In addition to residues indicated above, V86 makes a hydrophobic contact with sugar at PI.

sugar at the primary site, also exists at an identical position in Greek key II. Thus, unlike in the other lectins, banana lectin has two identical primary binding sites, one situated on Greek key I and the other on Greek key II. In the other lectins, the second site disappeared primarily on account of divergent evolution. In this context, it is interesting to note that the banana plant is a monocot, whereas the other lectins are from dicots.

Unique oligosaccharide specificity of banana lectin

In artocarpin and heltuba, the primary binding site is constituted by loops 14–17 and 129–133, and they bind mannose in the same way. However their specificity for dimannosides and higher oligosaccharides differ on account of their differences in the length and composition of the third loop (residues 83–86 in banana lectin) on Greek key III, which constitute the secondary binding site in both the lectins. As indicated earlier, banana lectin has two primary binding sites; one the same as that in the other two lectins situated on Greek key I and the other made up of loops 57–61 and 34–38 situated on Greek key II.

Interactions of dimannosides with banana lectin were modelled separately with nonreducing ends situated in each of the two primary sites. In each case, the mannose residue at the primary site was assumed to have the same interactions with the protein as observed in the crystal structure. The sugars used were Man-α-12-Man, Man-α-13-Man, and Man-α-16-Man. Their conformations can be described by the following torsion angles: 1–2, 1–3 linkages, Φ = O5-C1-O-Cx′; ψ = C1-O-Cx′-Cx′-1, where x = 2 and 3 respectively; 1–6 linkage: Φ = O5-C1-O-C6′; ψ = C1-O-C6′-C5′; ω = O-C6′-C5′-C4.′

From a statistical analysis of conformations observed in crystal structures, it has been shown that two conformers can occur about the Man-α-12-Man linkage (conformer 1: Φ 62.2 ± 8.3°; ψ –175° ± 10.3°; conformer 2: Φ 71.9° ± 13.1°; ψ–104.4° ± 15.4°), one about the Man-α-13-Man linkage (Φ 72.5° ± 11°; ψ –112.3° ± 22.5°) and three about the Man-α-16-Man linkage. (conformer 1: Φ 65.4° ± 9.0°; ψ 182.6° ±22.5°; ω 66.4° ± 10.2°; conformer 2: Φ 66.5° ±10.8°; ψ 180.7° ± 15.1°; ω 185° ± 11.2°; conformer 3: Φ 67.4° ± 14.4°; ψ 109.1° ± 13.7°; ω 203° ± 22.7°) (Petrescu *et al.*, 1999). All the possible conformers were used in modelling with the nonreducing mannose at primary site I as well as primary site II. None of them led to any serious steric clash with the protein. The interactions of the second sugar residue in the energy-minimized models are listed in Table II. All of them exhibit additional interactions involving the second sugar residue, in consonance with the experimental results indicating that, unlike in the case of artocarpin and heltuba, banana lectin binds the three dimannosides equally.

All the models except one interact with loop 83–86, irrespective of whether the nonreducing mannose is at primary site I or primary site II. Thus the two primary sites have a common secondary site. A composite view of two typical models, one using primary site I and the other primary site II is shown in Figure 6. Although the secondary site is common to both, the disposition of the loop bearing the site is not the same with respect to the two primary sites. Therefore, the sugar binding site as a whole is asymmetric. The implication of this asymmetry becomes clear when the

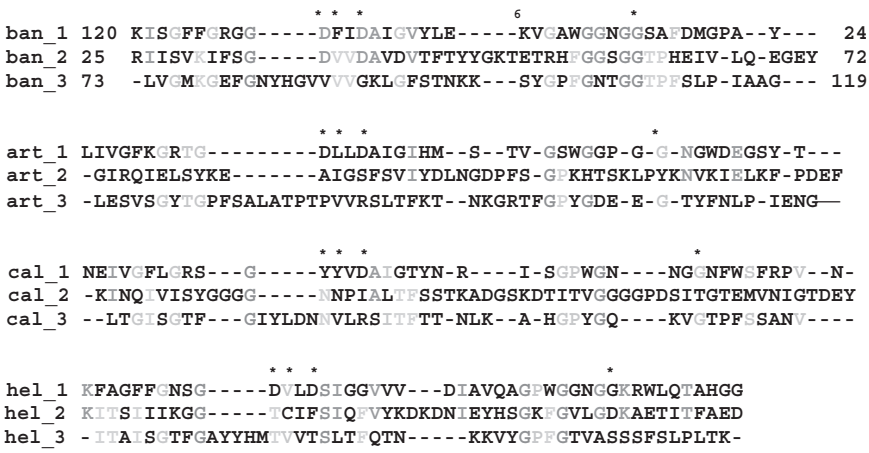


Fig. 5. Alignment of Greek keys I, II, and III in artocarpin, heltuba, calsepa, and banana lectin. Residues common in I and II, I and III, and II and III are in different shades of grey.

Table II. Interactions of the second sugar residue in the modelled dimannoside with banana lectin

Disaccharide	O1	O2	O3	O4	O5	O6
Man-α12-Man (conformer 1)						
PI			D130 OD1			
PII	Y83 OH					
Man-α12-Man (conformer 2)						
PI						H84 O
PII	D35 OD1 & OD2					
Man-α13-Man						
PI	D130 OD2					
PII	D35 OD1 & OD2	Y83 OH				
Man-α16-Man (conformer 1)						
PI			H84 O			
PII				Y83 OH		
Man-α16-Man (conformer 2)						
PI		H84 O				
PII		H84 ND1, Y83 OH			Y83 OH	
Man-α16-Man (conformer 3)						
PI				D130 OD1		
PII				D35 OD2		

In addition, several van der Waals contacts with the 83–86 stretch also exist.

binding of higher oligosaccharides to the lectin is considered below.

The second sugar residue in none of the disaccharides anchored at primary site I could be readily linked with that of any of the disaccharides at primary site II, although they interact with the same loop. An attempt was then made to explore the possibility of a fifth sugar residue bridging the two. In this exploration, a third residue was added using α1–2, α1–3, and α1–6 linkages to the reducing mannose residue in the disaccharide anchored at primary site I, to start with. Here again two possible conformations were used about the α1–2 linkage, one about the α1–3 linkage and three about α1–6 linkage. Thus, there were altogether mannotrioses with 36 distinct conformations anchored at site I. The third residue in some of them came close to the second residue of some of the disaccharides anchored at site II. In a similar fashion models of trisaccharides with 36 distinct conformations anchored at site II were constructed. In

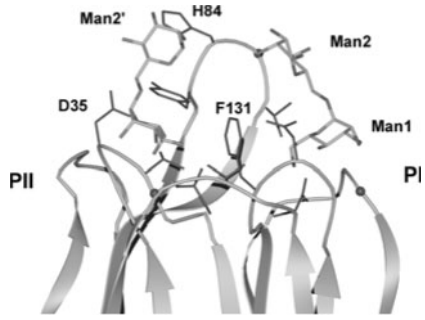


Fig. 6. A composite view of the two typical models of disaccharide complexed with banana lectin. Both have 1-6 linkages (Man- α16-Man [conformer 2] in Table II). See text for details.

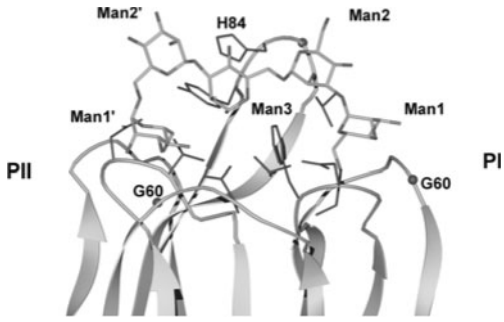
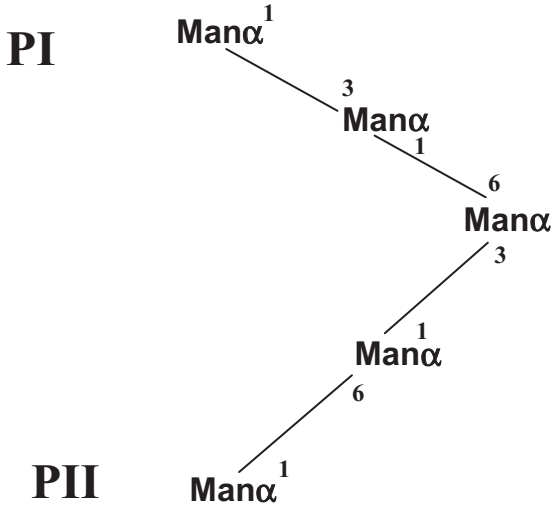


Fig. 7. Energy-minimized models of the branched pentasaccharide complexed with banana lectin. See text for details.



Scheme 1. Schematic representation of the modelled pentasaccharide.

no case did the third residue come close to the second residue of any of the disaccharides anchored at site I in this case.

In two of the 36 trisaccharides anchored at site I, the third residue was particularly close to the second residue in the disaccharides anchored at site II. In both the cases, the third residue of the trisaccharide and the second residue of the disaccharide could be readily linked with minor changes in the appropriate torsion angles leading to branched pentasaccharides schematically illustrated in Scheme 1. The only difference between the two was in the conformation about the 1–6 linkage between the second and third residue starting from primary site I. However, the two converged to essentially the same conformation after energy minimization. The energy-minimized pentasaccharide model along with the binding regions of the lectin is shown in Figure 7. In addition to the crystallographically defined interactions at the primary sites involving the terminal residues, the other three sugars also interact with Asp130, His84, and Tyr83.

Thus one readily obtains branched oligosaccharides of mannoses with the nonreducing ends of the two branches anchored at the two primary sites I and II. The residue at the junction (bridging residue) and those adjacent to it on either side interact primarily with the common secondary binding site. The ease with which a branched oligosaccharide could be modelled into the carbohydrate binding region of the lectin, as a direct consequence of the presence of two nearly equivalent primary binding sites, readily explains the unique specificity of banana lectin for branched mannans. The branched oligomannosides which have been shown to bind the lectin include the trisaccharide Man α1,6 (Manα1,3) Man and the pentasaccharide found in the core region of N-linked glycan chains (Mo *et al.*, 2001) as well as $Man_{8,9}$ $GlcNAcAc_2$ reported earlier (Koshte *et al.*, 1990). It is satisfying that all of them have branching involving 3 and 6 positions as in the model.

Materials and methods

Crystal structure determination

In our earlier communication, we reported the extraction, purification, crystallization, data collection at room temperature (298 K) to 3 Å resolution and finally structure solution using program PHASER, of banana lectin (Singh *et al.*, 2004). The structure was refined using CNS (Brunger *et al.*, 1998). In an effort to get better diffracting crystals, 5 mg/mL of protein and 100 mM of methyl-α-D-mannopyranoside solution in double distilled water was crystallized using the sitting drop method. A typical drop contained 40 µl of the above protein–sugar solution and 2 µl of reservoir buffer. It was equilibrated against 20 mL of the reservoir buffer containing 0.01 M zinc acetate dihydrate, 0.1 M sodium cacodylate pH 8.0, and 3 M 1,6-hexanediol. The crystallization trays were stored at 298 K. Crystals of size $0.4 \times 0.2 \times 0.1$ mm grew in a week's time. The crystals were directly flash cooled at 100 K in a stream of nitrogen gas. Data were collected using a Mar research MAR 300 imaging plate mounted on a Rigaku RU-200 X-ray generator. The data were processed using the HKL package (Otwinowski and Minor, 1997). Intensities were converted to structure factors using TRUNCATE in Collaborative Computational Project No. 4 (1994). Data collection statistics along with cell parameters are given in Table III.

Solvent content was estimated using the method of Matthews (1968). The coordinates of the refined room temperature structure solved earlier were used for structure solution using the program AMoRe (Navaza, 1994). The structure was refined using CNS in the early stages and

Table III. Data collection and refinement statistics

Space group		$P3_221$
a (Å)		80.82
b (Å)		80.82
c (Å)		148.0
α (degree)		90.0
β (degree)		90.0
γ (degree)		120.0
Z		6
Resolution (Å)	2.45	
Last shell (Å)	2.54–2.45	
Number of observations	1,24,621	
Number of unique reflections	21617 (2146)	
Completeness (%)	99.0(99.3)	
R_{merge}[a] (%)		7.0(53.3)
Multiplicity		5.8
Protein atoms	2050	
Sugar atoms		65
Water oxygen	498	
R-factor[b] (%)	22.4	
R_{free}[b] (%)		26.0
Resolution range (Å)	24.92–2.45	
RMS deviations from ideal value		
Bond length (Å)	0.009	
Bond angle (degree)	1.53	
Residue (%) in Ramachandran plot		
Core region		83.4
Additionally allowed region	15.1	
Generously allowed region	1.5	
Disallowed region	0.0	

Values within parentheses refer to the last resolution shell.
[a] $R_{merge} = \Sigma|I_i - \langle I \rangle| / \langle I \rangle$.
[b] $R = \Sigma||Fo| - |Fc|| / \Sigma|Fo|$; R_{free} is calculated in the same way but for a subset of reflections R_{free} that is not used in the refinement.

refinement was completed using the program REFMAC in Collaborative Computational Project No. 4 to a final *R* value of 22.4 and *R* free value of 26.0%. The final model had clear density for residues 4–141 in the first subunit, 2–141 in the second subunit, 498 water molecules, 3 zinc atoms, 6 molecules of 1,6-hexanediol which was present in the crystallization buffer. Clear densities for methyl-α-D-mannopyranoside were located at two different sites in both the subunits. A third sugar binding site was also located in the first subunit alone.

Analysis and modelling

Hydrogen bonds in the crystal structure were identified using the program hbplus (McDonald and Thornton, 1994). CONTACT in Collaborative Computational Project No. 4 was used for calculating nonbonded distances in models. Molecular superpositions were performed using the program ALIGN (Cohen, 1997). The dimannosides were modelled using the program SWEET (Bohne *et al.*, 1998) and the molecular manipulation in the models was done using INSIGHT II. Program NACCES was used for calculating the accessible surface area (http://wolf.bms.umist.ac.uk/naccess). X-PLOR (Brunger, 1992) was used for energy minimization. Distance-dependent dielectric constant was used throughout. During minimization, the anomeric oxygens of the nonreducing mannose at primary site were constrained. The observed lectin–sugar hydrogen bonds and the different torsion angles in the modelled sugars were restrained with a force constant of 10 KCal/mol. RIBBONS (Carson, 1997) and MolScript (Esnouf, 1997) were used for generating figures.

Protein data bank accession codes

The atomic coordinates and the structure factors of the complex were deposited in the RCSB Protein data bank (PDB accession code 1X1V).

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Note added in proof

The following paper [Meagher, J.L., Winter, H.C., Ezell, P., Goldstein, I.J., and Stuckey, J.A. (2005) Crystal structure of banana lectin reveals a novel second sugar binding site. *Glycobiology*, **15**, 1033–1042] describes the structure of banana lectin from a different species. The structure reported by us and that reported by Meagher et al. lead to the same basic results. Their results additionally indicate the preference of the primary binding sites for the reducing ends of the disaccharides used for complexation. Through modelling studies we have provided a rationale for the affinity of the protein for branched mannans. We have also dealt with the evolutionary origin of the two binding sites in the lectin.

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