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Mutational analysis of the sugar-binding site of pea lectin

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Comparison of x-ray crystal structures of several legume lectins, co-crystallized with sugar molecules, showed a strong conservation of amino acid residues directly involved in ligand binding. For pea (*Pisum sativum*) lectin (PSL), these conserved amino acids can be classified into three groups: (I) D81 and N125, present in all legume lectins studied so far; (II) G99 and G216, conserved in almost all legume lectins; and (III) A217 and E218, which are only found in Vicieae lectins and are possibly determinants of sugar-binding specificity. Each of these amino acids in PSL was changed by site-directed mutagenesis, resulting in PSL molecules with single substitutions: for group I D81A, D81N, N125A; for group II G99R, G216L; and for group III A217L, E218Q, respectively. PSL double mutant Y124R; A126S was included as a control. The modified PSL molecules appeared not to be affected in their ability to form dimeric proteins, whereas the sugar-binding activity of each of the PSL mutants, with the exception of the control mutant (as shown by haemagglutination assays), was completely eliminated. These results confirm the model of the sugar-binding site of Vicieae lectins as deduced from X-ray analysis.

Keywords: mutagenesis; pea lectin; sugar binding

Introduction

Legume lectins constitute a family of sugar-binding plant proteins that has been studied extensively over the past few decades, and now generally represent the best characterized group of lectins [1, 2]. Legume lectins are dimeric or tetrameric molecules, composed of identical monomers each containing one sugar-binding site [3]. Close to the sugar-binding site, each monomer contains a calcium-binding site in which one calcium ion pulls amino acid residues into the required positions for sugar-binding [3–6]. At short distance, binding of one transition-metal ion contributes to the organization of the calcium-binding site. A well-studied example within this group of lectins is PSL, the dimeric lectin from pea (*Pisum sativum*). PSL is abundantly present in pea seeds [7], and only small amounts can be found in other parts of the plant [8]. The protein is encoded by a single gene [9, 10], the promoter of which is an attractive tool for the study of seed-specific gene expression [11]. PSL is synthesized as a pro-lectin, and processing yields an α and a β chain in each monomer. In seeds two isolectins are found, representing different stages of C-terminal processing [12]. Because processing does not affect the sugar-binding site, the sugar-binding properties of both PSL isolectins are identical [7]. Mature PSL is not glycosylated, and the (dimeric) protein has a total molecular mass of about 50 kDa.

Alignment of the primary sequences of several legume lectins shows a high percentage of homology. This homology results in very similar three-dimensional structures, which are dominated by the presence of β-sheets [2, 3, 13–16]. In spite of this structural homology, there is a considerable variation in sugar-binding specificity among this group of lectins [3]. Several legume lectins have been co-crystallized with saccharides, and structural analyses of these crystals has yielded detailed information about the lectin-sugar interactions [4, 6, 17–23]. As an example, binding of a tri-mannoside in the sugar-binding site of PSL and binding of α-methyl-mannopyranoside in the sugar-binding site of LOL I (*Lathyrus ochrus* isolectin I) are depicted in Fig. 1 [6, 17, 18, 20, 21]. Both lectins are members of a group of very homologous mannoside/glucoside-binding lectins in the legume *Vicieae* (Table 1). In both lectins, a (terminal) mannose residue is directly bound in a network of hydrogen bonds, with the C-4 of the sugar deep in the sugar-binding cavity, yielding very similar sugar-binding patterns. Amino acids directly involved in sugar-binding can be classified into three groups. The most important group consists of residues N125 and D81. N125 is bonded to O-4 of the saccharide via NHD2 and directly to calcium via OD1. D81 is bonded to OH-4 and OH-6 of

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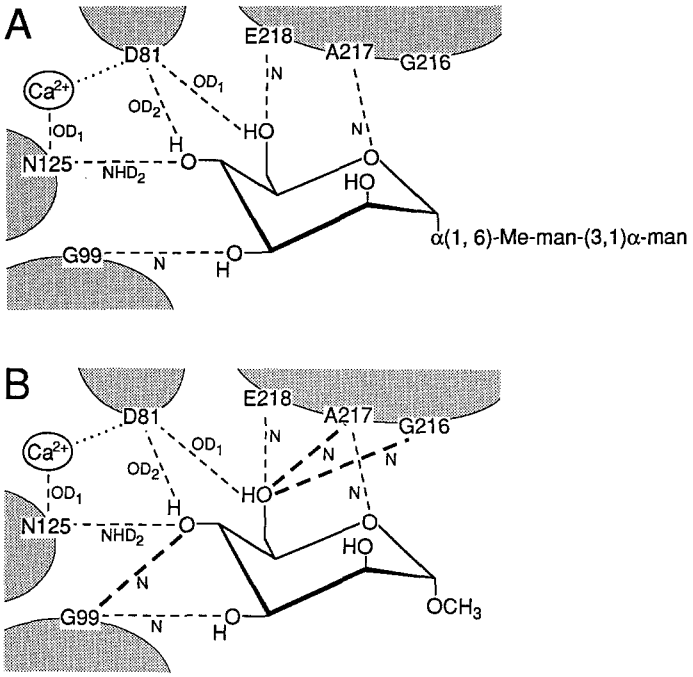

Figure 1. Binding of methyl (3,6)-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyrannoside by PSL (A) [6, 20] and α-methyl-mannopyranoside by LOL I (B) [17, 18, 21], respectively. Binding is the result of several direct interactions, i.e. hydrogen bond interactions, between amino acid residues of PSL and LOL I, and one of the terminal α-linked mannose residues. Direct interactions are represented by broken lines, amino acid residues by one letter characters and the positions within the protein by numbers. Sugar-peptide linkage interactions are marked with *N*, and sugar-amino acid side-chain interactions are marked with OD1, OD2 and NHD2, respectively. Comparison of both models shows three hydrogen-bonds in the LOL I-mannose interaction (B) (G99 to O-4, G216 to O-6 and A217 to O-6; indicated by thicker broken lines) which are replaced by Van der Waals interactions in the PSL-mannose interaction (A; not shown in this figure). In both cases the D81-Ca^{2+} interaction represents the only indirect interaction (stippled lines), because a water molecule is bound between the ion and the amino acid side-chain.

the saccharide via OD2 and OD1, respectively. Both amino acids are present at these positions in all legume lectins studied so far (Table 1), and their function accentuates the importance of OH-4 in sugar-binding by these lectins. D81 is indirectly bound to calcium via a water molecule, but this function is not conserved since in concanavalin A (ConA), the jack bean lectin, the calcium ion is indirectly bound by an arginine residue at a site corresponding to G99 in PSL [6, 20, 23]. The PSL-model is not only consistent with results obtained with LOL I, but also with results obtained with another *Vicieae* lectin, VFL (*Vicia faba* lectin) [19]. However, in case of VFL the molecular positions are numbered differently (D82, G100, N126, G211, A212, and E213).

A second class of sugar-binding amino acids consists of G99 and G216. In this class, there is a small difference between the binding patterns of the mannose residue by PSL and LOL I, respectively. In LOL I the G99 and G216 residues are involved in hydrogen-bonding to O-3, O-4, and O-6 of the sugar molecule, respectively, whereas in PSL direct interactions by hydrogen-bonds between G99 and O-4 of the mannose and between G216 and O-6 of the mannose are replaced by Van der Waals-interactions (Fig. 1A and B). Despite being highly conserved (Table 1), these residues are not essential for sugar-binding in some legume lectins other than PSL and LOL I. For instance, in ConA an arginine-NH replaces the G99-NH to bind O-3 of the mannose, whereas G216 does not contribute to binding of the saccharide in this lectin.

The third class of sugar-binding amino acids consists of A217 and E218. These residues are highly conserved only in lectins from the legume *Vicieae* (Table 1), and apparently contribute to the determination of sugar-binding specificity rather than being essential for sugar-binding in general. Comparison of the three-dimensional structures of PSL in the presence or absence of sugar molecules shows a shift in position of the 216–218 loop toward the sugar upon ligand binding, to maximize hydrogen bond interactions with the sugar molecule [20]. The importance of this loop in determining sugar-binding specificity is also suggested from results of Delbaere *et al.* [24] and Shaanan *et al.* [22].

This study aimed at testing the model of the sugar-binding site of PSL, as obtained by x-ray diffraction studies, by site-directed mutagenesis. The sugar-binding amino acid residues shown in Fig. 1 were substituted independently, and the modified PSL molecules were tested for their ability to agglutinate human erythrocytes, an assay known to be suitable for the testing of sugar binding.

Materials and methods

Bacterial strains

E. coli strains DH5, *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1, and DH5α, *sup*E44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1, were both used for production of PSL. Bacteria were grown in Luria Complete medium (LC) [25] at 37 °C.

Site directed mutagenesis of psl *cDNA*

Mutations were introduced by using the polymerase chain reaction (PCR) with mutagenic oligonucleotide primers. As a template pMP 2809 was used, containing the *psl* cDNA described previously by Van Eijsden *et al.* [26]. For the introduction of D81A and D81N, two independent PCR reactions were performed (Fig. 2). The first PCR reactions were carried out in 100 µl containing 100 pmol of each primer (molar concentrations were based on $M_r = 330$ for each dNTP) and 2.5 U AmpliTaq polymerase (Perkin-Elmer). Reactions were performed on a PREM^{TM}III apparatus from Biozym Nederland, and comprized 25

Table 1. Alignment of segments of the sugar-binding site of several legume lectins [*from 2 and 35, and references therein*].

	81	*99*	*125*	*216*
PSL	N V A **D** G F T .... T G – – G **G** Y L G V	T F Y **N** A A W	A T T **G** **A** **E** Y A A
LCL	N V A **D** G F T .... T G – – G **G** Y L G V	T F Y **N** A A W	A T T **G** **A** **E** F A A
VFL	N V A **D** G F T .... T G – – G **G** Y L G V	T F Y **N** A A W	A T T **G** **A** **E** Y A T
LOLI	N V A **D** G F T .... T G – – G **G** Y L G V	T F Y N T A W	A T T **G** **A** **E** F A A
LOLII	N V A **D** G F T .... T G – – G **G** Y L G V	T F Y N T A W	A T T **G** **A** **E** F A A
SBA	R L A **D** G L A .... T H – – A **G** Y L G L	T F R **N** S – W	A T – **G** – L D I P
ECorL	L P A **D** G L V .... Q G – – Y **G** Y L G I	T F S **N** P – W	A T – **G** A Q R D A
PHA-L	G P A **D** G L A .... D K – – G **G** F L G L	T L Y N K D W	A T T **G** I N K G N
PHA-E	G P A **D** G L A .... D K – – G **G** L L G L	T L Y N V H W	A T T **G** I T K G N
DBL	S F A **D** G I A .... R N – – G **G** Y L G V	T N S N S G W	A T T **G** L S E G Y
LBL	V T D **D** P A W .... K K – – G **R** L L G L	T C H N L D W	A T S **G** A – Y – –
SL	R G G **D** G I T .... S G – – G **G** Y L G I	T F S **N** R – W	A A T **G** – D L V E
ConA	H P A **D** G I A .... S G S T G **R** L L G L	T Y P N T D I	A S T **G** – L Y K E

Comparison of amino acid sequences of several legume lectins, harbouring the highly conserved residues involved in sugar-binding. Amino acid residues are given by one character code, and numbering is according to the PSL sequence. The highly conserved residues, which are directly involved in sugar-binding, are given in bold or outlined type (only in the case of two Arginine residues present in ConA and LBL), respectively. Gaps (–) are introduced to enable optimal matching. Parts of the sequences which are not given in detail are indicated by (....). PSL, *Pisum sativum* lectin; LCL, *Lens culinaris* lectin; VFL, *Vicia faba* lectin; LOLI, *Lathyrus ochrus* isolectin I; LOLII, *Lathyrus ochrus* isolectin II; SBA, *Glycine max* lectin; ECorL, *Erythrina corallodendron* lectin; PHA-L, *Phaseolus vulgaris* lectin, leucoagglutinin; PHA-E, *Phaseolus vulgaris* lectin, erythrocytes agglutinin; DBL, *Dolichos biflorus* lectin; LBL, *Phaseolus limensis* lectin; SL, *Onobrychis viciifolia* lectin; ConA, *Canavalia ensiformis* lectin.

cycles of 45 s denaturation at 95 °C, 1 min annealing at 56 °C, and 2 min elongation at 72 °C each. The amplified products were isolated from low melting agarose (SeaPlaque GTG, FMC BioProducts, USA) using Geneclean (Westburg, The Netherlands) and were used as double stranded primers in a second PCR reaction. The cycle times of the second PCR reactions were adapted to the use of double stranded DNA as a primer: 30 cycles of 2 min denaturation at 95 °C, 15 min annealing at 60 °C and 2 min elongation at 72 °C each. After the second amplification, the elongated product was digested with *Eco* RI and *Eco* RV, introduced at the corresponding position in pMP 2809 and sequenced according to Sanger *et al.* [27] (see Fig. 2). For the introduction of G216L, A217L and E218Q, the method described by Perrin *et al.* and Higuchi *et al.* was applied [28, 29], using overlap extension with two complementary mutant primers and two flanking primers. In these experiments we used the 3′-universal −40 primer and the 5′-universal reverse primer as non-mutagenic flanking primers, respectively. Amplified products were isolated, digested with *Bam* HI and *Hind* III, introduced at corresponding positions in pMP 2809, and sequenced according to Sanger *et al.* [27]. Mutagenic primers, with corresponding mutations, are listed in Table 2.

Islolation of PSL from E. coli

Isolation of PSL from *E. coli* was performed as described previously [26, 30]. Briefly, the procedure involves overexpression of PSL in *E. coli*, isolation of inclusion bodies, followed by denaturation and renaturation of proteins. Renatured proteins (an average yield of 20 mg l^{-1}) from these inclusion bodies which consisted mainly of PSL, were dialysed against deionized water, lypophilized, and dissolved in PBS (10 mM sodium phosphate, pH 7.4, 0.9% NaCl).

Isolation of renatured dimeric PSL molecules was done by gel filtration, using a fast performance liquid chromatography (FPLC) unit coupled to a Superdex 75 HR 10/30 column (Pharmacia LKB, Uppsala, Sweden). The samples were fractionated in TBS (10 mM Tris, pH 6.8, 150 mM NaCl) containing 0.2 M D-glucose at a flow rate of 0.5 ml min^{-1}. Fractions eluting at a molecular weight of 55 kDa (according to molecular weight standards) were pooled, dialysed against deionized H_2O, and lypophilized.

Detection of PSL by SDS-PAGE and Western-blotting

Protein fractions were loaded on SDS-polyacrylamide gels, consisting of a 15% separating and 3% stacking gel, according to Lugtenberg *et al.* [31]. The separated proteins were blotted onto nitrocellulose filters (0.45 μm pore size; Schleicher & Schuell, Dassel Germany) for 1 h, using a LKB NovaBlot Electrophoretic Transfer Unit, operating at 0.8 mA cm^{-2}. The blots were blocked in a solution of 2% (wt/vol) dried nonfat powdered milk in PBST (PBS, pH 7.4, 0.1% Tween-20) and incubated overnight with appropriate dilutions of polyclonal anti-PSL antibodies, raised against SDS-denatured seed PSL [8]. The filters were washed in PBST and incubated with a 2000-fold diluted anti-rabbit IgG alkaline phosphatase conjugate in PBST. After 1-h of incubation the filters were washed in PBST for 30 min and developed using nitroblue tetrazolium (NBT)

Table 2. Sequence of oligonucleotide primers.

Substitution	*Primer sequence*
D81N	5′ CGTAAACCCGTTGGCAAC 3′
D81A	5′ CGTAAACCCGGCGGCAAC 3′
G99R	5′ CCGAGATATCGACCGCC 3′
N125A	5′ GCTTGGATCCCATGCAGCAGCATAGAAAG 3′
G216L, sense	5′ CTACCACACTAGCAGAATATGC 3′
G216L, anti-sense	5′ GCATATTCTGCTAGTGTGGTAG 3′
A217L, sense	5′ CTACCACAGGACTAGAATATGC 3′
A217L, anti-sense	5′ GCATATTCTAGTCCTGTGGTAG 3′
E218Q, sense	5′ CCACAGGAGCACAATATGCAGC 3′
E218Q, anti-sense	5′ GCTGCATATTGTGCTCCTGTGG 3′

This table shows the sequences of the PCR primers used in site-directed mutagenesis of PSL. Amino acid residues are given by the one character code. All primers contained one or two mismatches, marked by bold characters. The extensions sense and anti-sense refer to the strand of DNA which will be synthesized using that particular primer in a PCR reaction.

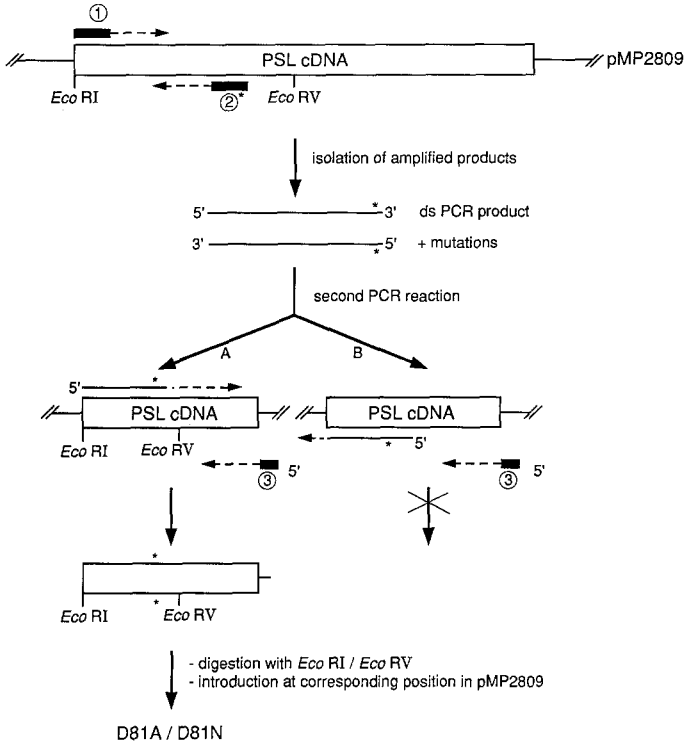

Figure 2. Introduction of the D81N and D81A mutations in the cDNA of PSL. For the introduction of these mutations, two independent PCR reactions were required. In the first reaction the mutation was introduced, using the universal 5′-reverse primer ① in combination with a mutagenic primer ②. Amplified products were purified and used as primers in a second PCR reaction, in combination witth the universal 3′-40 primer ③. Both strands of the former amplified product could anneal at the template in the second reaction, yielding two starting points, A and B. Only in situation A, will a double stranded product of the correct length be produced. This product was digested with *Eco* RI and *Eco* RV, introduced at the corresponding position in pMP 2809 and sequenced.

and 5-bromo-4-chloro-3-indolulylphosphate (BCIP) as substrates [32].

Determination of Ca^{2+} and Mn^{2+} concentrations in modified PSL

Lyophilized mutated PSL was dissolved in 50 ml twice-deionized H_2O, containing 1% (vol/vol) concentrated HNO_3, to a final concentration of 5 mg ml^{-1}. Aggregates were removed by centrifugation for 10 min at 7000 rpm. Measurements for the Ca^{2+} and Mn^{2+} contents were performed by Inductively Coupled argon Plasma Atomic Emission Spectroscopy (ICP-AES), with analysis lines for CaII and MnII of 393.366 and 257.610 nm, respectively. The number of measurements was four, with an average RSD of 1% for CaII and 1.1% for MnII.

Haemagglutination assays

The ability of wild type and mutated PSL to agglutinate a 2% (v/v) suspension of human A^+ erythrocytes in PBS was assayed as described in Van Eijsden *et al.* [26]. Agglutination was judged after 1 h of incubation at room temperature.

Results and discussion

Mutations in *psl* cDNA were introduced using PCR with mutagenic oligonucleotide primers (see also [26] and [30]). Expression of wild-type or mutagenized *psl* cDNA in *E. coli* resulted in production of unprocessed proteins, with an average yield of 20 mg PSL per litre of induced *E. coli* culture. After PSL purification, Western blots of the different PSL mutants showed in each case major bands with the expected molecular mass of about 28 kDa, corresponding with that of unprocessed PSL from pea plants (Fig. 3). As judged from gel filtration experiments, the introduced mutations did not have a major effect on

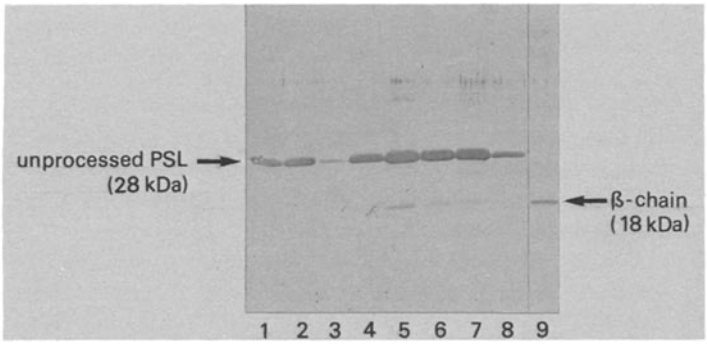

Figure 3. Immunoblot of purified PSL fractions. Mutated PSL, isolated by gel filtration and analysed by SDS-PAGE, was blotted onto nitrocellulose, and was incubated with polyclonal anti-PSL. Lane 1, wt-PSL; Lane 2, PSL G99R; Lane 3, PSL N125A; Lane 4, PSL D81A; Lane 5, PSL D81N; Lane 6, PSL G216L; Lane 7, PSL A217L; Lane 8, PSL E218Q; Lane 9, seed PSL. In the figure, unprocessed PSL molecules isolated from *E. coli*, and β-chains from seed PSL are indicated. Processed PSL α-chains are not visible due to low antigenity and low protein concentrations. Minor bands present in the lanes of the modified PSL molecules probably represent degradation products.

formation of PSL dimers. All PSL mutants were eluted corresponding to a molecular mass of about 55 kDa, with a minor fraction of monomers (results not shown). The 55 kDa fractions were isolated and used to test the sugar-binding activity by haemagglutination assays.

In a previous study, we showed that substitution of N125 for aspartate in the sugar-binding site of PSL eliminates sugar-binding activity without affecting binding of calcium [26]. This result demonstrated that the NHD2 group of N125 is essential in sugar-binding by PSL. Similar results have been found with PHA-L (*Phaseolus vulgaris* leucoagglutinin) in which N128, corresponding with PSL N125, was also replaced by aspartate [33]. Sugar-binding activity of modified PHA-L was completely eliminated, as well as the leucoagglutinating and mitogenic activities. In the present study, we removed both NHD2 and OD1 from N125 by replacing this residue by alanine (mutant N125A). As expected, N125A did not show haemagglutination activity. Surprisingly however, ICP-AES measurements showed that N125A contained the normal amount of both calcium and manganese, that is about 2 mol mol^{-1} dimeric PSL. Apparently, the other amino acid residues known to be involved in calcium binding, D81, D121, F123 and D129 [15], provide enough binding capacity. As a control, we substituted the two amino acids flanking N125, yielding the double mutant Y124R; A126S. These substitutions were rather drastic, since an aromatic residue was replaced by a positively charged one, and a moderately hydrophobic residue was replaced by a moderately hydrophilic one. Nevertheless, this double mutant showed normal haemagglutinating activity and sugar-binding specificity. This result accentuated the specific role of N125 in sugar binding.

The role of D81 was tested by its substitution for asparagine (mutant D81N; change of OD2 into NHD2) or for alanine (mutant D81A; removal of both OD1 and OD2). Both mutations yielded a complete loss of haemagglutinating activity. The amounts of calcium and manganese present were normal. These results corroborate the important position of D81 in the model for sugar-binding by PSL, and also suggest that calcium is sufficiently bound by D121, F123, N125 and D129.

The less strictly conserved glycine residues at positions 99 and 216 were substituted with arginine and leucine, respectively (mutants G99R and G216L). Mutant G99R appeared to be unable to agglutinate erythrocytes, in spite of the fact that in the related mannose-binding lectin ConA an arginine residue is present in the same position. However, in ConA the loop containing G216, opposite to the large side chain of arginine, is differently involved in sugar-binding than it is in LOL I and PSL. This is illustrated by absence of a role in sugar-binding for G216 in ConA, in contrast to the situation in LOL I and PSL, and by the presence of functional leucine and tyrosine residues instead of A217 and E218, respectively (Table 1), This adaptation may allow arginine to function at the position corresponding to G99 in PSL. Also in lima bean lectin, LBL, presence of an arginine at this position is correlated with changes in the loop containing G216 (Table 1). In the PSL mutant G99R the loop containing G216 has not been changed, and the charged side chain of arginine apparently prevents positioning of the saccharide in the binding pocket. Thus, the result of the mutation may be explained by steric hindrance. Also PSL mutant G216L did not show any haemagglutinating activity. This result may also be explained by steric hindrance by the introduced side chain of leucine, and is consistent with the involvement of G216 in sugar-binding by Van der Waals interactions.

Finally, the roles of A217 and E218 in sugar-binding by PSL were tested by substitution with leucine and glutamine, respectively (mutants A217L and E218Q). In wild-type PSL, the peptide NH of A217 is interacting with O-6 of the bound sugar by Van der Waals interactions, while it is hydrogen-bonded to the ring oxygen O-5. Introduction of the larger side chain of leucine apparently interferes with this binding pattern, since mutant A217L was unable to agglutinate erythrcytes. The peptide NH of E218 is bonded to O-6 of the sugar bound in wild-type PSL. Substitution of an OD2 group for NHD2 as realized in PSL mutant E218Q resulted in complete loss of sugar-binding activity.The introduced polar Q218 may interact with D81 due to the formation of a long hydrogen bridge, approximately 3.2 Å in length, between the NHD2-group of Q218 and the OD1-group of D81. As a result, the Q218 side chain is twisted in such a way that it penetrates the sugar-binding site, thereby preventing binding of sugar [B. Dijkstra, personal communication].

In all haemagglutination assays performed, seed PSL,

isolated from the seeds of *Pisum sativum* cv. Finale [34], and recombinant PSL expressed in *E. coli* showed normal agglutinating activity (see also [26]). Loss of haemagglutinating activity by PSL mutants most probably was not due: (i) to a significant change in sugar-binding specificity, since human A^+ erythrocytes have also been used for testing the haemagglutinating activity of galactose-, lactose, *N*-acetylglucosamine- and *N*-acetylgalactosamine-specific legume lectins [3]; or (ii) to a lack of dimerization, since all mutants have been isolated as 55 kDa proteins.

Taken together, the results of this study fully corroborate the model for sugar-binding by *Vicieae* lectins, as deduced from X-ray diffraction studies and as depicted in Fig. 1. Residues D81, G99, N125, G216, A217 and E218 each play an essential role in sugar-binding, and even relatively small changes such as in mutant A217L completely abolished haemagglutinating activity.

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