Mutational analysis of pea lectin: substitution of Asn125 for Asp in the monosaccharide-binding site eliminates mannose/glucose-binding activity

Eijsden, R.R. van; Hoedemaeker, F.J.; Diaz, C.L.; Lugtenberg, E.J.J.; Pater, B.S. de; Kijne, J.W.

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Mutational analysis of pea lectin. Substitution of Asn^{125} for Asp in the monosaccharide-binding site eliminates mannose/glucose-binding activity

Ron R. van Eijsden[1,2], Flip J. Hoedemaeker [2], Clara L. Díaz[2], Ben J. J. Lugtenberg[1,2], B. Sylvia de Pater[2] and Jan W. Kijne[1,2]
[1] *Institute of Molecular Plant Sciences and* [2] *Centre for Phytotechnology RUL/TNO, Leiden University, Nonnensteeg 3, 2311 VJ Leiden, Netherlands*

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Abstract

As part of a strategy to determine the precise role of pea *(Pisum sativum)* lectin, Psl, in nodulation of pea by *Rhizobium leguminosarum*, mutations were introduced into the genetic determinant for pea lectin by site-directed mutagenesis using PCR. Introduction of a specific mutation, N125D, into a central area of the sugar-binding site resulted in complete loss of binding of Psl to dextran as well as of mannose/glucose-sensitive haemagglutination activity. As a control, substitution of an adjacent residue, A126V, did not have any detectable influence on sugar-binding activity. Both mutants appeared to represent normal Psl dimers with a molecular mass of about 55 kDa, in which binding of Ca^{2+} and Mn^{2+} ions was not affected. These results demonstrate that the NHD2 group of Asn^{125} is essential in sugar binding by Psl. To our knowledge, Psl N125D is the first mutant legume lectin which is unable to bind sugar residues. This mutant could be useful in the identification of the potential role of the lectin in the recognition of homologous symbionts.

Introduction

Lectins are sugar-binding (glyco)proteins which bind reversibly and with high affinity and specificity to glycans of glycoproteins, glycolipids, or polysaccharides. Lectins are widely distributed in nature, being found in animals, plants and microorganisms [30]. Lectins can be grouped into families with sequence homologies and common structural properties. The best characterized family of lectins is present in leguminous plants [30, 31].

Primary structures of legume lectins are highly homologous, resulting in very similar three-dimensional conformations. These structures are dominated by β-sheets, whereas α-helices are virtually absent [27, 31, 36]. Another similarity is the presence of two metal ions, Ca^{2+} and Mn^{2+}, binding of which is necessary for sugar-binding activity [1, 27, 28]. Amino acids involved in metal binding are highly conserved among legume lectins. Differences between legume lectins are mainly based on differences in sugar-binding specificity. In spite of these differences, almost all lectins are able to agglutinate erythrocytes due to the large variety of sugar moieties at the red cell surface [36].

Lectins from pea (*Pisum sativum*), sweet pea

(*Lathyrus ochrus*), lentil (*Lens culinaris*) and vetch (*Vicia faba*), all belonging to the legume tribe Vicieae, show an identical binding specificity for *D*-mannose and *D*-glucose type residues [14, 19, 39]. Due to this binding property, these lectins are normally isolated and purified by affinity chromatography on dextran (e.g. Sephadex). Three-dimensional structures of Vicieae lectins have been determined [4, 11, 12, 26]. Lectins from *Pisum sativum* (Psl), *Vicia faba* (favin), and isolectin I from *Lathyrus ochrus* (LolI) have almost identical three-dimensional structures. Amino acids involved in sugar binding are highly conserved among these lectins and are present at comparable molecular positions, that is, in a cleft at the protein surface adjacent to the metal-binding site [1, 5, 6, 28]. Apparently, sugar binding sites of Vicieae lectins are very similar.

Psl, like other Vicieae lectins, is composed of two identical subunits, each containing one α and one β-chain, with a total molecular mass of about 50 kDa [33]. Each subunit is synthesized as a pre-pro-protein at the rough endoplasmatic reticulum, in a β-α direction [35, 36]. The signal peptide is co-translationally removed, and, along with further processing steps, Psl in seeds is transported to the protein storage vacuoles (protein bodies) [15, 16, 18]. In pea roots, Psl is located at the root surface, where it can be found on the top of growing root hairs and on trichoblasts [8].

Earlier studies in our laboratory demonstrated an involvement of Psl in host specificity in the *Rhizobium*-legume symbiosis. *Rhizobium leguminosarum* biovar. *viciae* (*Rl viciae*) is able to form nitrogen-fixing root nodules on *Pisum*, *Lathyrus*, *Lens* and *Vicia* roots, but not on clover (*Trifolium*) roots. Introduction of the *psl* gene into *Trifolium repens* L. (hairy) roots resulted in an extension of the host specificity range of *Trifolium repens*, allowing infection and delayed nodulation by *Rl viciae* [10]. Since legume lectins primarily differ in sugar-binding specificity and since *Rl viciae* exclusively nodulates Vicieae plants, sugar-binding activity of Psl might be involved in recognition of the homologous symbiont. As a part of a strategy to test this hypothesis, we aimed at production of a non-sugar-binding Psl mutant, to be followed by repetition of the experiments of Díaz *et al.* [9, 10].

This report concerns the first part of this approach, that is, the production of a non-sugar-binding Psl mutant. Substitution of a highly conserved amino acid residue in the sugar-binding site of Psl and its effect on sugar binding will be described. Asparagine^{125}, according to the Psl sequence, is the amino acid which possibly plays an important role in binding of saccharides as well as Ca^{2+} by Vicieae lectins [1, 5, 6, 28]. Crystallographical studies on Psl, favin and LolI suggest that the 04 atom of mannose interacts with the NHD2 part of the asparagine side chain. Figure 1 presents the model of the sugar binding site of LolI, based on co-crystallography with α-methyl-mannopyranoside [5, 6], whereas Table 1 demonstrates the homology of short segments of various legume lectins, including Psl and LolI, containing N125. The OD1 part of the N125 side chain is probably involved in direct binding of the Ca^{2+} ion (Fig. 1). Here we report that replacement in Psl of N125 by aspartate results in total loss of mannose/glucose binding activity with preservation of Ca^{2+} binding. To our knowledge, this is the first report on production of a mutant legume lectin which is unable to bind sugar residues. This mutant should be useful in the identification of the potential role of the lectin in the recognition of homologous symbionts.

Table 1. Homology of N125-containing segments of various legume lectins [from 4 and 36].

	115				120					125					130				
Psl	T	V	A	V	E	F	D	T	F	Y	N	A	A		W	D	P	S	N
LolI	T	V	A	V	E	F	D	T	F	Y	N	T	A		W	D	P	S	N
Lcl	T	V	A	V	E	F	D	T	F	Y	N	A	A		W	D	P	S	N
Vfl	T	V	A	V	E	F	D	T	F	Y	N	A	A		W	D	P	S	N
Sbl	V	V	A	V	E	F	D	T	F	R	N	S			W	D	P	P	N
PHA-E	T	V	A	V	E	F	D	T	L	Y	N	V	H		W	D	P	K	P
ConA	I	V	A	V	E	L	D	T	Y	P	N	T	D	I	G	D	P	S	Y

Psl, pea lectin; LolI, *Lathyrus ochrus* isolectin I; *Lcl*, lentil lectin; *Vfl*, favin; *Sbl*, soybean lectin; *PHA-E*, phytohaemagglutinin E; *ConA*, concanavalin A.

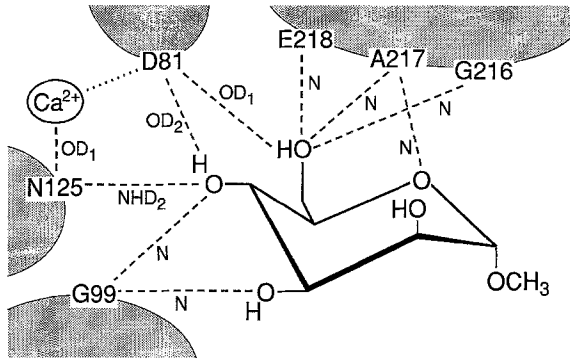

Fig. 1. Binding of α-methyl-mannopyranoside in the saccharide-binding pocket of LolI [from 5, 6]. Binding is the result of several direct interactions between amino acid residues and the sugar. Direct interactions are represented by the broken lines, amino acid residues by one letter characters and the 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. The D81-Ca^{2+} interaction is the only indirect interaction, because a water molecule is bound between the ion and the amino acid side-chain [from 5]. This model is consistent with results obtained with favin and Psl (see also [1] and [28], respectively). However, in case of favin the molecular positions are numbered differently, resulting in D82, G100, N126, A212 and E213, respectively.

Materials and methods

Bacterial strains

Escherichia coli strain DH5αF$^+$, *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1, was used for Psl production. Bacteria were grown in Luria Complete (LC) medium [21] at 37 °C.

Cloning and site-directed mutagenesis of Psl cDNA

Psl cDNA, derived from *Pisum sativum* seed RNA, was kindly provided by Dr M. Stubbs [32]. Almost the entire sequence encoding the signal peptide could be removed by introduction of an extra *Eco* RI restriction site, and subsequently the cDNA was cloned in frame with the *lac* promoter into pUC 18 [22, 40]. In this way, expression vector pMP 2809 was constructed (Fig. 2A), followed by its transformation to $CaCl_2$-competent *E. coli* DH5αF$^+$ cells.

Mutations were introduced by using the polymerase chain reaction (PCR) with mutagenic oligonucleotide primers. Reaction volumes were 100 μl containing 2.5 U of *Taq* polymerase (Biozym Nederland). Reactions were performed on a PREM III apparatus from Biozym Nederland, and comprised 25 cycles of 0.45 min/95 °C, 1 min/56 °C and 2 min/72 °C each. Molar concentrations were based on M_r 330 for each dNTP, and reactions were carried out using 100 pmol of each primer. Total amplified DNA was sequenced according to Sanger *et al.* [29], using sequenase version 2.0 enzyme (USB, Cleveland, Ohio). The extra *Eco* RI site was introduced by combination of primer 1 (5′-CAAGGTGAATTCA-ACTGAAAC-3′) and primer Psl N125D (5′-GCTTGGATCCCATGCAACATTATAGA-AAG-3′. In this reaction, primer 1 was used as the only mutagenic primer, because the amplified fragment was digested with *Eco* RI and *Eco* RV. This fragment was used to replace the corresponding fragment in the original cDNA (Fig. 2B). The same primer combination was used to introduce the mutation N125D, where primer Psl N125D was used as the mutagenic primer. In this case an *Eco* RV/*Bam* HI fragment was used to replace the corresponding fragment in pMP 2809. Combination of primer 1 and primer Psl A126V (5′-GCTTGGATCCCATGCAACAT-TATAGAAAG-3′) enabled the introduction of mutation A126V. The amplified fragment was digested, and the *Eco* RV/*Bam* HI fragment was used to replace the corresponding fragment in pMp 2809 (Fig. 2B).

Isolation of Psl from E. coli

The method for isolation of Psl from *E. coli* was a modified version of protocols described by Stubbs *et al.* [32] and Prasthofer *et al.* [24]. *E. coli* DH5αF$^+$ cells harbouring plasmid pMP 2809 were grown in 2 litres LC at 37 °C, containing 100 μg/ml carbenicillin, and were induced at mid-exponential phase by adding isopropyl-β-*D*-thiogalactopyranoside (IPTG, Boehringer Mannheim) to the medium to a final concentration of

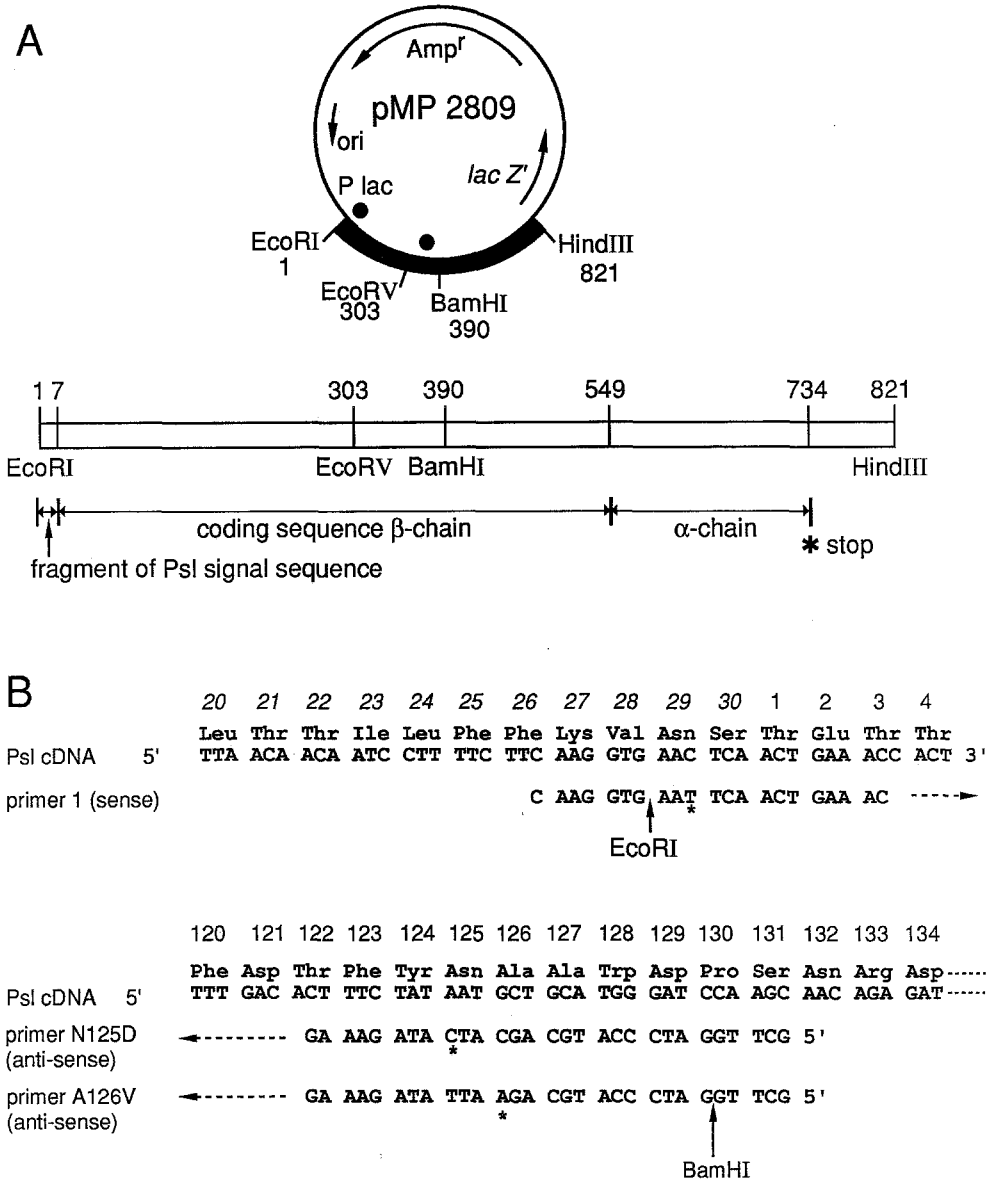

Fig. 2. Cloning and mutagenesis of Psl cDNA. A. Map of expression vector pMP 2809 from which high level expression of Psl was obtained. Closed circles show the binding regions of the mutagenic primers used. Transcription directions for the subcloned Psl cDNA, the truncated *lac Z'* gene and the β-lactamase gene encoding ampicillin resistance are marked by arrows. Ori shows the origin of replication. Given numbers correspond with nucleotide positions in the Psl cDNA. B. Sequence of the oligonucleotide primers used for the introduction of specific mutations into the Psl cDNA. Positions of nucleotide substitutions are marked with asterisks. Amino acids are given by three-letter codes and numbered from the first Thr residue at the N-terminus of the β-chain. Residues numbered in italics correspond with residues encoded by the Psl signal sequence.

0.5 mM. The cells were grown for 16 h, harvested, washed and resuspended in 20 ml TBS (10 mM Tris-HCl pH 6.8, containing 150 mM NaCl). Lysis was performed by French pressing (10.3–11.0 MPa) in the presence of 500 μM phenylmethylsulphonyl fluoride (PMSF), and inclusion bodies were collected by centrifugation for 30 min at 15 000 rpm. Inclusion bodies were denaturated

in 7 M guanidine-HCl pH 6.8, containing 10 mM $CaCl_2$ and 10 mM $MnCl_2$. Membranes and remaining aggregates were removed by ultracentrifugation for 45 min at 35000 rpm. The proteins were renaturated by a rapid 25-fold dilution of the protein suspension in ice-cold TBSM (TBS pH 6.8, containing 10 mM $CaCl_2$ and 10 mM $MnCl_2$), in the presence of 1.5 M urea. This solution was incubated overnight at 4 °C, and dialysed against deionized H_2O. Finally, the proteins were lyophilized and redissolved in PBS (10 mM sodium phosphate pH 7.4, 0.9% NaCl). Proteins were tested for their affinity to *D*-glucose type residues by affinity chromatography on Sephadex G75 [7].

Detection of Psl produced by E. coli

Protein fractions were loaded on SDS-polyacrylamide gels, consisting of a 15% separating and 3% stacking gel, according to Lugtenberg *et al.* [20]. After running, the gel was stained with $AgNO_3$ [2], or the proteins were blotted onto nitrocellulose filters (0.45 μm pore size; Schleicher & Schuell, Dassel, Germany). Transport of proteins was carried out using a LKB NovaBlot Electrophoretic Transfer Unit for 1 h, operating at 0.8 mA/cm^2. After transport, the filters were blocked in a solution of 2% (w/v) dried, defatted powdered milk in PBST (PBS pH 7.4, containing 0.1% Tween-20) and incubated with appropriate dilutions of polyclonal anti-Psl antibodies (raised against SDS-denaturated seed Psl) [8]. After washing in PBST, the filters were incubated for 1 h with a 2000-fold diluted anti-rabbit IgG alkaline phosphatase conjugate in PBST. After washing for 30 min in PBST, the filters were developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) as substrates [13].

Isolation of Psl by gel filtration

FPLC gel filtration, using a Superdex 75 HR 10/30 column (Pharmacia LKB, Uppsala, Sweden), was used to test dimer formation by Psl produced in *E. coli*. Separation was performed by running the sample in TBS containing 0.2 M *D*-glucose at a flow rate of 0.5 ml/min. Fractions containing peak I were pooled, dialysed against deionized H_2O, and lyophilized.

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

Lyophilized Psl was dissolved in 50 ml twice deionized H_2O, containing 1% (v/v) concentrated HNO_3, to a final concentration of 5 mg/ml. Aggregates were removed by centrifugation for 10 min at 7000 rpm. Measurements were performed by inductively coupled argon plasma atomic emission spectroscopy (ICP-AES), with analysis lines for CaII and MnII of 393366 and 257610 nm, respectively. The number of measurements was 4, with an average RSD of 1% for CaII and 1.1% for MnII.

Haemagglutination-inhibition assays

The ability of Psl to agglutinate a 2% (v/v) suspension of human A^+ erythrocytes in PBS was assayed as described in Kijne *et al.* [17]. with the following modifications: 25 μl lectin solution with a starting concentration of 1 mg/ml and 25 μl of 2% (v/v) human erythrocytes were used. In order to test sugar binding specificity of agglutination, Psl was incubated with *D*-glucose, *D*-mannose, and *D*-galactose, respectively. The starting concentration of haptens was 0.1 M, followed by serial dilution. Psl concentration remained at a value of 125 μg/ml. Agglutination and inhibition of agglutination were judged after 1 h of incubation at RT.

Results

Site-directed mutagenesis of Psl cDNA

Expression of the original Psl cDNA in *E. coli* resulted in the production of an unprocessed pro-

tein, containing 11 N-terminal amino acid residues from the signal peptide [32]. By introducing an extra *Eco* RI site, this number of residues could be reduced to two (Fig. 2B), yielding a molecule that is almost devoid of its signal peptide (designated wt Psl). These remaining signal peptide residues are preceded by three residues from the expression vector, Met, Ile and Thr, resulting in a Psl molecule with a total N-terminal extension of five amino acid residues. (A similar strategy to express and isolate recombinant Psl from *E. coli* was based on introduction of a *Bam* HI site, yielding a Psl molecule with three residual amino acids of the signal peptide at the amino terminus [24]. The shortened cDNA was cloned in frame with the *lac* promoter (= pMP 2809). Using pMP 2809 as a template, N125D and, as a control, A126V were the next mutations to be introduced (Fig. 2B). Sequencing of entire PCR products confirmed success of these mutations (without introduction of other nucleotide substitutions). The resulting lectins were designated Psl N125D and Psl A126V, respectively.

Expression and isolation of Psl from E. coli

Induction of the *lac* promoter resulted in expression of the different lectin constructs. Isolation of each of these lectins yielded 30–40 mg of crude lectin per 2 l of *E. coli* culture. Western blots of the different Psl forms showed in each case major bands with a molecular weight of about 28 kDa (Fig. 3A, lanes 2–4). This molecular weight corresponds with that of unprocessed Psl from pea plants (Fig. 3A, lane 1). Bands with a smaller molecular weight might partly result from *E. coli* protease activity. In the case of mature pea seed lectin, a minor band of unprocessed Psl could be detected, in addition to the major 18 kDa β-chains. Since the antiserum used mainly interacts with epitopes of the β-chain, the 6 kDa α-chain is hardly visible in a western blot.

After isolation, the ability of wt Psl, Psl N125D and Psl A126V to bind *D*-glucose-type residues was tested using affinity chromatography on dextran. Standard glucose elution patterns of wt Psl and Psl A126V were similar to that of seed Psl,

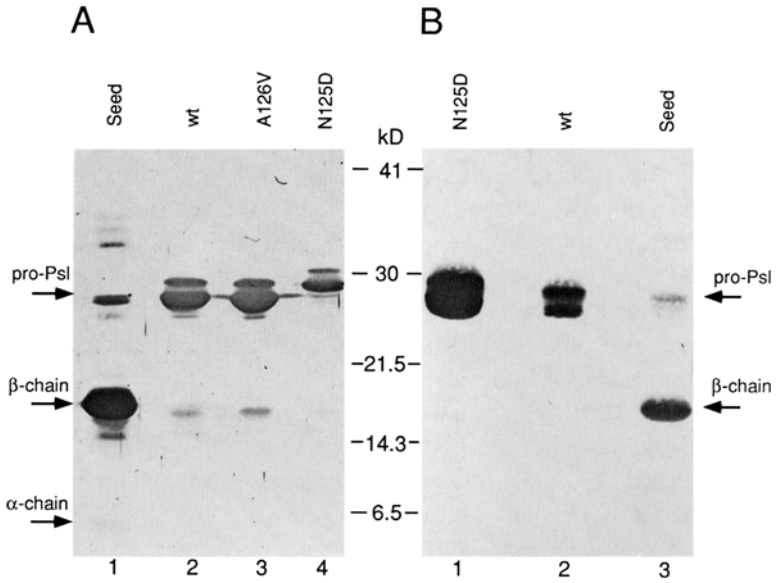

Fig. 3. Immunodetection of different Psl forms. A. Immunodetection was performed with wt Psl, Psl N125D and Psl A126V, directly after isolation from *E. coli*. Mature pea lectin from seed (seed Psl) was loaded as a reference. Lane 1, seed Psl; lane 2, wt Psl: lane 3, Psl A126V; lane 4, Psl N125D (150 ng of each). B. Immunodetection of peak I fractions, which were isolated by gel filtration (see Fig. 4). Lane 1, Psl N125D; lane 2, wt Psl; lane 3, seed Psl. In both figures, pro-Psl corresponds with unprocessed Psl molecules, β-chain with processed Psl β-chains, α-chain with processed Psl α-chains, whereas molecular weight standards are shown in between both figures.

isolated from *Pisum sativum* cv. Finale [7]. In contrast, Psl N125D did not show any affinity for Sephadex and eluted from the column in buffer (data not shown).

Purification of Psl dimers by gel filtration

Psl consists of two identical subunits, each containing one α and one β-chain contributing to one sugar-binding site. Because Psl N125D could not be purified using affinity chromatography, and in order to test the effect of the introduced mutations on association of the subunits and thus on haemagglutination activity of Psl, the different lectins were purified by gel filtration.

Elution patterns of seed Psl, wt Psl, Psl N125D, and Psl A126V appeared to be similar (Fig. 4, Psl A126V not shown), with each pattern containing a major peak I. Calibration with molecular weight standards showed that this fraction eluted corresponding to a molecular mass of ca. 55 kDa for wt Psl, Psl N125D as well as Psl A126V. A similar peak was present in the pattern of seed Psl, but, due to processing of this lectin, this peak eluted corresponding to a slightly smaller molecular weight. Lectins isolated from *E. coli* yielded an additional minor peak (peak II), corresponding to a molecular weight of about 30 kDa. SDS-PAGE and immunoblotting of the peak I fractions of each Psl form yielded bands with a molecular mass of ca. 28 kDa (Fig. 3B), corresponding with unprocessed Psl monomers.

From these results, it can be concluded that the introduced mutations did not have a major effect on formation of Psl dimers.

Determination of the Ca^{2+} and Mn^{2+} concentration in Psl

Like other legume lectins, Psl needs presence of both Ca^{2+} and Mn^{2+} to be able to bind sugar molecules. In theory, two moles of each have to be present in one mol of native mature protein [36]. Because N125 is involved in direct binding of both the Ca^{2+} ion and the sugar, we tested if Ca^{2+} binding was affected in Psl N125D.

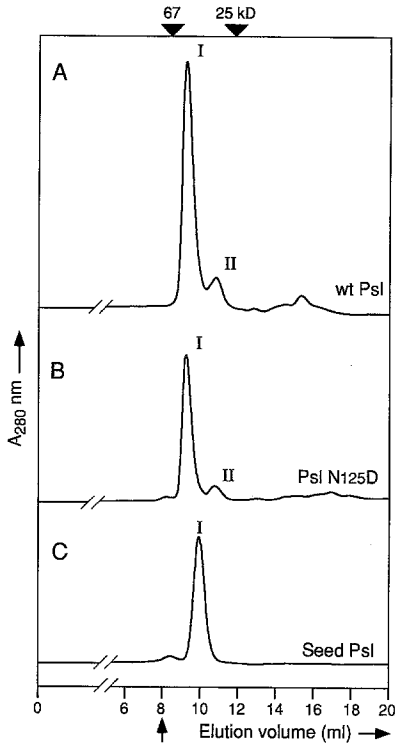

Fig. 4. Gel filtration of Psl after isolation from *E. coli*. Gel filtration was performed to test dimer formation by wt Psl, Psl N125D and seed Psl, shown in A, B and C, respectively. Large arrowheads at the top of the figure represent molecular markers, whereas the vertical arrow at the bottom indicates the void volume.

Ion-measurements were performed with wt Psl and Psl N125D, and the results are listed in Table 2. Psl N125D contained the same concentration of ions as did wt Psl, that is, 2.2 mol Ca^{2+} and 2.3 mol Mn^{2+}, both values being close to the

Table 2. Determination of the Ca^{2+} and Mn^{2+} content in wt Psl and Psl N125D.

Psl	Ca^{2+} concentration (mol/mol Psl)	Mn^{2+} concentration (mol/mol Psl)
Wild type	2.25	2.26
N125D	2.17	2.29

Ca^{2+} and Mn^{2+} concentrations were determined in wt Psl and Psl N125D, using ICP-AES (inductively coupled argon plasma atomic emission spectroscopy). Concentrations of both ions were calculated, using a molecular massof 55000 kDa for Psl N125D and wt Psl, respectively.

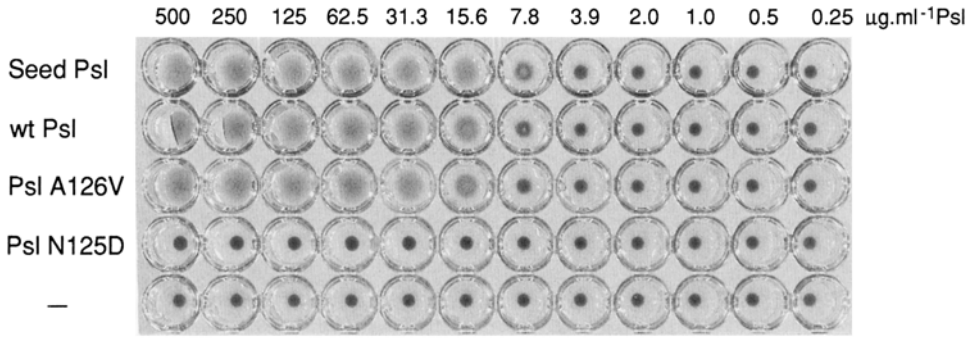

Fig. 5. Haemagglutination assay with wt and mutant Psl. The ability to agglutinate a 2% suspension of human A^+ erythrocytes was tested for seed Psl, wt Psl, Psl N125D and Psl A126V. The assay started from lectin concentrations of 0.5 mg/ml, which were serially diluted. The photograph was taken 1 h after addition of the erythrocytes and incubation at RT.

theoretical value. From these results it can be concluded, that Psl N125D bound the same amount of Ca^{2+} and Mn^{2+} as did wt Psl and that this mutation did not influence metal binding.

Haemagglutination and hapten inhibition assays with wt and mutant Psl

Haemagglutination activities of purified wt Psl, Psl A126V and Psl N125D were compared with the activity of seed Psl. With seed Psl, wt Psl and Psl A126V agglutination activity in a double-dilution assay could be observed down to a lectin concentration of 16 µg/ml or lower. In case of Psl N125D, no agglutination activity could be detected (Fig. 5). Inhibition of haemagglutination by monosaccharides was compared for seed Psl, wt Psl and Psl A126V, and in each case agglutination was inhibited after addition of 100 mM *D*-glucose or *D*-mannose (data not shown). Dilution of hapten to a critically low concentration restored agglutination. As expected, inhibition by mannose was stronger due to a higher affinity of Psl for this monosaccharide [34, 38]. *D*-galactose, a non-hapten, was not able to inhibit the agglutination activity of each Psl type tested. These results corroborate earlier findings that sugar binding activity of recombinant Psl from *E. coli* is similar to that of seed Psl [32] and demonstrate that loss of dextran binding ability for N125D is coupled with inability to agglutinate erythrocytes.

Discussion

Asparagine 125 is conserved in all legume lectins tested [36]. In two ways, it is thought to be involved in sugar binding: (1) by binding of calcium, the presence of which is necessary for structuring the sugar-binding site, and (2) by a direct interaction with the sugar present in the monosaccharide-binding pocket. These interactions were determined by high-resolution crystallographical studies with pea lectin, favin and the isolectin I from *Lathyrus ochrus* [1, 5, 6, 27, 28]. Our results corroborate this model for pea lectin by showing that replacement of N125 by aspartate (in other words, replacement of the NDH2 group by a hydroxyl group) eliminates mannose/glucose binding whereas calcium binding by the conserved OD1 group is still possible. Moreover, these results demonstrate that the conserved presence of other amino acids involved in sugar binding [1, 5, 6, 27, 28] (Fig. 1) apparently is insufficient for sugar binding and that N125 plays an essential role in this process. Since (1) human A erythrocytes are also used for testing of haemagglutination activity of galactose-, lactose-, N-acetyl-glucosamine- and *N*-acetyl-galactosamine-specific legume lectins [36], and (2) N125 is conserved in all legume lectins, PslN125D most probably is incapable of sugar binding in general. It is unlikely that the mutation yielded a drastic change in overall structure of the lectin. However, determination of the three-dimensional structure

of N125D will be necessary to test if the monosaccharide-binding pocket in the Psl mutant is still intact. Introduction of the mutation A126V did not detectably affect sugar-binding ability of Psl. These results demonstrate that the sugar residue is specifically interacting with N125, and not with the adjacent residue A126. The latter mutant lectin may function as a control in future experiments.

Production of recombinant pea prolectin by *E. coli* apparently does not influence the basic properties of the molecules (see also [24] and [32]), despite the inability of the bacterium to process the protein. The molecular weight has the expected value (Fig. 3B) and dimers are formed, enabling sugar-specific haemagglutination. Presence of a small amount of Psl monomers in recombinant Psl preparations, in contrast to the situation with mature seed Psl (Fig. 4), points at either a slight inhibition of dimerization in *E. coli* or, most probably, incomplete dimerization of Psl during renaturation. At present, we cannot explain why recombinant Psl appears as a doublet after SDS-PAGE. The slightly lower mobility of crude N125D in comparison with the other Psl preparations (Fig. 3A) might be correlated with the introduction of an extra charged residue [23].

To our knowledge, Psl N125D is the first non-sugar binding legume lectin produced. In view of the conserved state of N125, one can predict that the same mutation in other legume lectins will also result in loss of sugar binding ability. Such mutant lectins are excellent tools to test several current hypotheses on the role of sugar binding in the function of legume lectins, like recognition of *Rhizobium* symbionts ([10]; see Introduction), toxicity for insects [3] and vertebrates [25], and mitogenicity [33]. Furthermore, substitution of other amino acids involved in sugar binding will enable definition of their specific role in this process under non-crystallographical conditions.

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