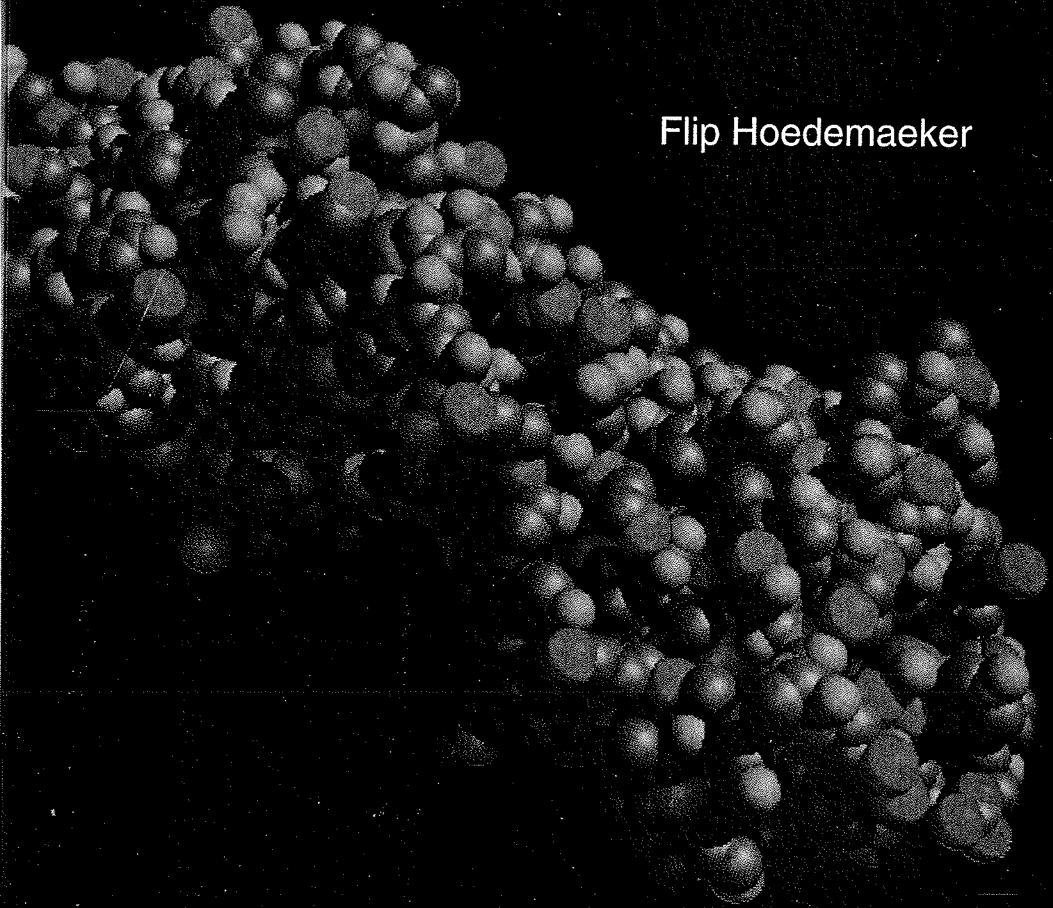
STRUCTURE AND STABILITY OF LEGUME LECTIN

Flip Hoedemaeker



STRUCTURE AND STABILITY
OF LEGUME LECTIN

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Co-promotor:	Dr. B.S. de Pater
Referent:	Prof. dr. B.W. Dijkstra (RUG)
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Aan mijn ouders

Voor Inge

CONTENTS

CHAPTER 1

INTRODUCTION

Lectins

Lectins are non-enzymatic carbohydrate-binding proteins of non-immune origin. Some authors include the ability to agglutinate cells or particles in the definition [67], with the implication that lectins must have at least two sugar-binding sites. Lectins have been found in over 1000 species of plants, in all classes and subclasses of vertebrate and invertebrate animals, in most (entero) bacteria, in protozoa, and even in viruses. Lectins occur in an enormous variation in structure, size and sugar-binding specificity.

History

The first report on a lectin was published by Dixson in 1887 [71]. This publication was followed shortly afterwards by the doctoral thesis of Hermann Stillmark in 1888 (University of Dorpat, now called Tartu, Estonia). He found that an extract of castor bean (*Ricinus communis*) could agglutinate erythrocytes. In addition he found that this haemagglutination was caused by a protein, which he named "ricin". Shortly after, a colleague of Stillmark, H. Hellin, discovered a similar protein in jequirity bean (*Abrus precatorius*) [67]. The sugar-binding specificity of lectins was discovered almost 50 years later by James B. Summer [71]. He showed that haemagglutination by concanavalin A (CON A, the seed lectin from *Canavalia ensiformis*) could be inhibited by addition of cane sugar. He suggested that haemagglutination was caused by an interaction between the lectin and carbohydrates present at the surface of the erythrocytes. At the end of the 1940s, William Boyd and Karl Renkonen independently reported that certain seeds contain agglutinins specific for certain human blood group antigens. It was on the basis of this research that William Boyd and Elizabeth Shapleigh proposed the name "lectin" in 1954, from the Latin *legere,* meaning to pick out or to choose [67]. A couple of years later it was reported that lectins also possess specific biological activities, apart from their ability to agglutinate erythrocytes. Mitogenic activity of phytohaemagglutinin (PHA, the lectin from *Phaseolus vulgaris*) was first discovered in 1960 by Peter Novell, causing a small revolution in immunology [49]. Soon after that, several other lectins were found to be mitogenic. The involvement of sugar moieties on the cell surface in lectin-mediated mitogenesis was shown by inhibition of mitogenic activity of CON A with low concentrations of mannose. The first 3D structure of a legume lectin was published in 1975 by Joseph Becker *et al* [4]. Today, lectins are used worldwide as powerful tools in cancer research, immunology and histochemistry [67].

Legume lectins

Until now, lectins from seeds of legumes are the best studied group of lectins. Legume lectins have been described for over 600 species. More than 70 lectins have been isolated, and approximately 40 lectin genes have been sequenced [68]. At this moment, the 3D structures of at least 8 legume lectins are known [4,5,8,18,19,54,66]. Twenty-nine well-characterized legume lectins, together with their aligned amino acid sequences and with their sugar-binding specificities, have been listed in Table 1.

Structure

All legume lectins are ß-barrel proteins [55], and are organised as dimers or tetramers with one sugar-binding site per monomer. As an example, the monomer of pea (*Pisum sativum*) lectin (PSL) is depicted in Figure 1. Basically, a monomer consists of two antiparallel ß-pleated sheets interconnected by a series of loops. The "back" sheet, which includes the C- and N- termini, consists of six strands with short interconnecting loops. The front sheet of each monomer consists of seven strands and is twisted, unlike the back sheet.

In every legume lectin one Ca^{2+}- and one Mn^{2+}-ion per subunit is present. The Mn^{2+} ion can be replaced *in vitro* by other transition metals such as Zn^{2+} [4]. These metals are required for sugar binding activity [68]. A large loop is found opposite to the C- and N-termini, which contains the metal-binding residues (Figure 2). Close to the metal binding site four loops contribute to the sugar-binding site

(Figure 3).

Another striking feature is the presence of a very large, twisted surface loop interconnecting two ß strands in the front sheet. This loop will be discussed in more detail in Chapters 3 and 4.

All lectins with known 3D structures contain a *cis*-peptide bond at position 80-81[1] between (in most cases) alanine and aspartic acid (Figure 4, Table 1). The *cis* peptide bond is a rare phenomenon in proteins, and the majority of these *cis* peptide bonds are imide bonds (between any amino acid and a proline). Steric hindrance between adjacent α-carbon substituents seems to be the principal reason for the preference for *trans* bonds [70]. This particular *cis* peptide bond, however, enables the aspartic acid sidechain to contribute to the sugar binding site, as well as to the calcium binding site, and this probably compensates for the unfavourable interaction with the sidechain of Ala 80.

Close to the C-terminus a large hydrophobic cavity is found. The entrance to this cavity is formed by a single α-helix turn. It has been shown that this cavity can bind non-polar substances, such as *o*-iodophenol [4]. The function of this cavity is unknown until now.

The monomers of many legume lectins are post-translationally processed. In the dimeric lectins of the Vicieae tribe the monomer is cleaved into a larger ß-chain and a smaller α-chain. In CON A circular permutation occurs: the monomer is cleaved and the original C- and N-termini are ligated together [11]. The three-dimensional structures of monomers of legume lectins are very much alike, in spite of considerable differences in processing and multimerisation.

All legume lectins characterised so far are dimers or tetramers. Dimers are usually formed by a "head-to-tail" alignment of two N-terminal ß-strands. In this way, two back sheets combine to form a 12-strand sheet (Figure 5). In tetrameric lectins, such as CON A, two ellipsoidal dimers interact back-to-back to form a tetrameric structure (Figure 6). Exceptions to this rule are peanut (*Arachis hypogea*) lectin (PNA), *Erythrina corallodendron* lectin (EcorL), and *Griffonia simplicifolia* lectin 4 (GS4), which have a different quaternary structure [2]. For GS4 and EcorL, this

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[1] Each amino acid in this chapter is numbered according to the PSL sequence.

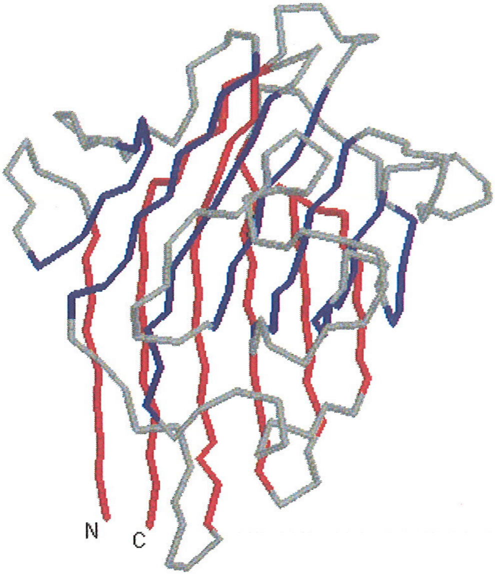


Figure 1. The monomeric structure of PSL. The N-terminus and C-terminus are indicated, the two ß-sheets are depicted in red and blue.

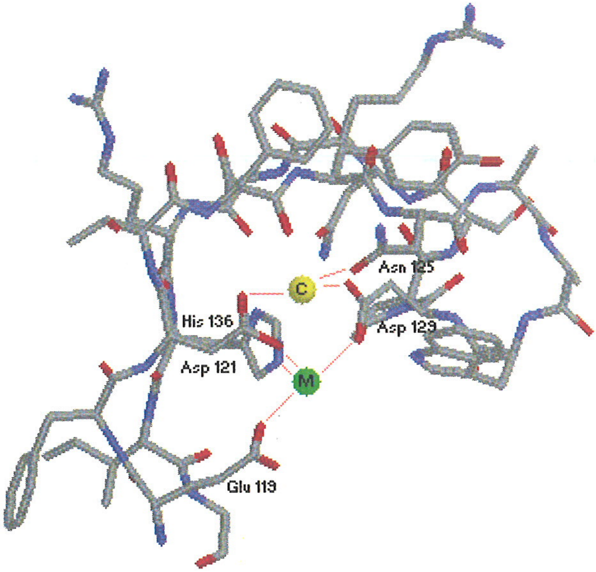


Figure 2. The PSL loop containing the amino acids involved in the binding of Ca^{2+} (yellow) and Mn^{2+} (green).

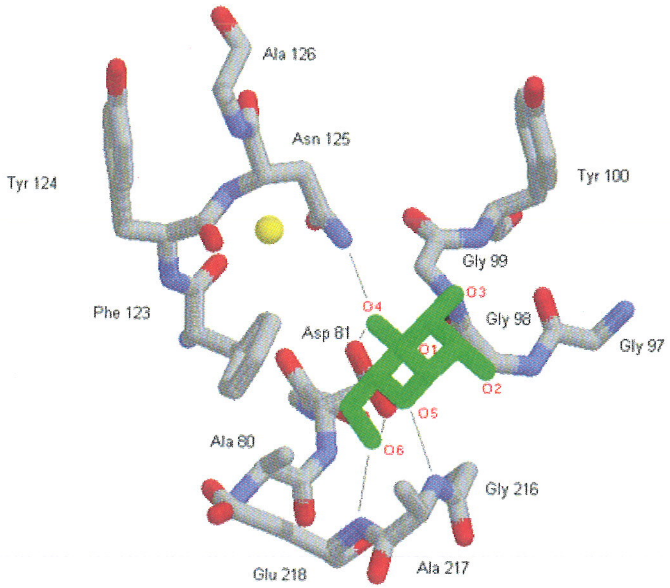


Figure 3. The monosaccharide-binding site of PSL. A mannose molecule (green) is bound. The hydroxyl-groups of the mannose are numbered in red. See also the model on page 90.

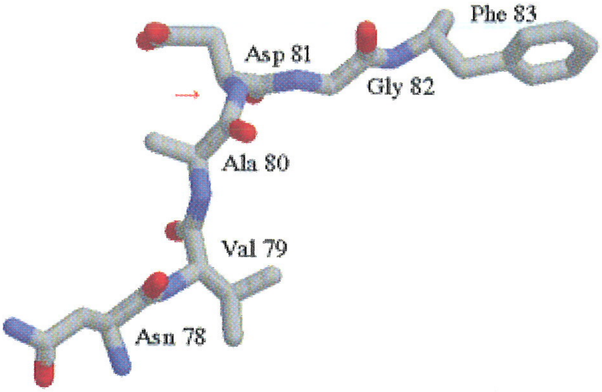


Figure 4. The *cis*-peptide bond (indicated by a red arrow) in PSL.

phenomenon can be due to physical interactions of bulky carbohydrate chains at the interface of the annealing site. However, such interactions are not present in PNA.

Expression of legume lectins

The expression of a few legume lectins has been studied. Pea plants contain only one functional lectin gene copy, psl [22,38]. This gene is highly expressed in seed and very poorly in other parts of the plant [15]. A 22-base pair element in the psl promoter seems to be responsible for the seed-specific expression pattern [13]. The amount of PSL reflects the steady-state levels of mRNA [38].

In seeds, lectins are present in specialised protein storage vacuoles (protein bodies) [15,31]. The N-terminal signal peptide required for this targeting is removed co-translationally upon entering of the protein into the lumen of the endoplasmic reticulum. Post-translational cleavage of the pro-protein into an α- and a ß-subunit occurs in the protein bodies [30,31]. The N-terminus of the ß-subunit and the complete α-subunit have been determined [56, 74]. PSL is correctly targeted to the vacuoles of tobacco and potato plants and is also processed correctly into α- and ß subunits [17,33]. Furthermore, glycinin, a major storage protein from soybean, is processed by a protease with a similar specificity, implicating that the proteases responsible for this kind of processing are conserved in various plant species [26,65].

In pea, two isolectins are formed, PSL1 and PSL2, which are usually present in roughly equal amounts in mature pea seeds. These isolectins differ in charge: PSL1 has a pI of 7.2 and PSL2 has a pI of 6.1 [15]. Upon germination, PSL1 rapidly disappears from the seeds. In the vegetative parts of the pea plant only one isolectin, with a pI of 6.1, is found [15,23]. The presence of only one functional copy of psl indicates that post-translational processing, probably of the C-terminus of the α-chain, is responsible for the difference in charge between the two isolectins [59]. The processing of PSL is discussed in more detail in Chapter 2.

Recently a transcript with homology to psl has been isolated from actively growing buds from pea plants, but it has not been established whether the encoded protein is a lectin [50]. Both genes seem to be members of a multi-gene family located on chromosome 7 [45], but sofar no other lectins than PSL have been purified from pea plants.

In *P. vulgaris*, at least two lectin genes are present which are highly homologous and are closely linked [32]. One gene, dlec1, encodes a lectin capable of agglutinating erythrocytes (PHA-E), while the other, dlec2, encodes a leucocyte-specific lectin (PHA-L). Together, five different tetrameric lectins can be formed, PHA-E_4, PHA-E_3L_1, etc. Each of these lectins is glycosylated and accumulates in protein bodies, like PSL [33]. A short stretch of amino acids containing a highly conserved glutamine residue (Gln 21, see Table 1) seems to be an important signal in the vacuolar targeting of legume lectins and of a number of related proteins [73]. In bean at least three proteins are present that are very similar to PHA, arcelin [37] and two *α*-amylase inhibitors (*α*-AI1 and *α*-AI2) [46,72], all non-lectins. The genes encoding these proteins are closely linked on the chromosome, pointing at evolutionary divergence from a common ancestor gene [32,46,72].

In a number of other legumes also more than one lectin gene is present. These genes may encode lectins with different expression patterns. For example, in *Dolichos biflorus* two similar lectins with a high sequence homology (92%) are encoded by different genes which are differentially expressed [27].

Apart from expression in specific tissues, lectins can be expressed during specific periods. In pea roots, PSL can be detected in very young roots, but the amount of lectin decreases rapidly seven days after germination [15]. In *Robinia pseudoacacia*, a lectin is present only from September till May (in Europe) [79].

Sugar specificity

Legume lectins come in a large variety of oligosaccharide specificity, but most of them can be classified into groups based on inhibition by simple monosaccharides. A large group of lectins is specific for D-mannose and (with lower affinity) D-glucose. Another group of lectins is specific for galactose and/or N-acetyl-D-galactosamine (see Table 1). Some lectins, like PHA, bind only to complex carbohydrates, and their activity can not be inhibited by monosaccharides [67]. PSL belongs to the D-man/D-glc group. The amino acids that form the monosaccharide binding site are Asp 81, Gly 99, Asn 125, Gly 216, Ala 217, and Glu 218 [60].

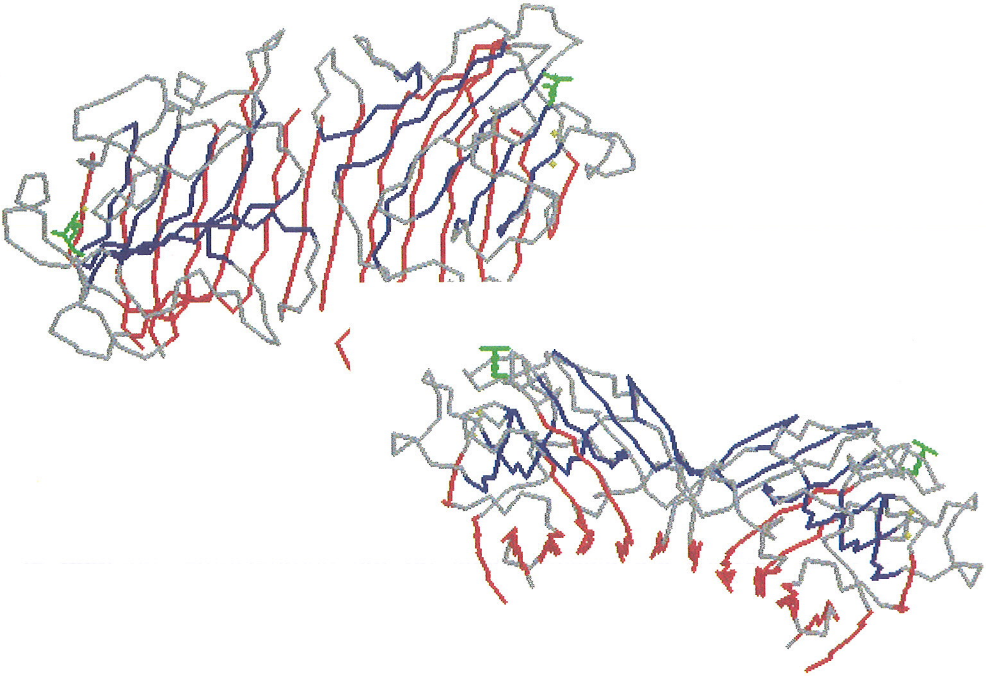


Figure 5. The dimeric structure of PSL, viewed from two different angles.

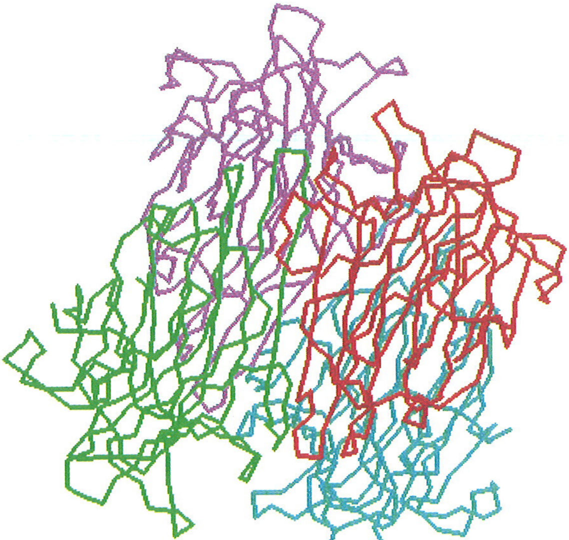
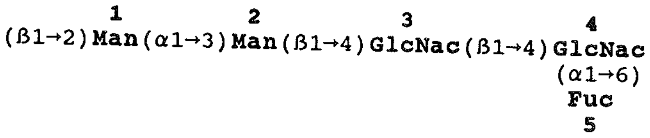


Figure 6. The tetrameric structure of CON A.

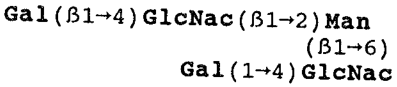
Each amino acid in the monosaccharide binding site is essential for sugar-binding activity [75].

Like the very similar *Lathyrus ochrus* lectin (LOL), PSL has a much higher affinity for oligosaccharides with the following core structure[2] [10,43]:



$$(\beta1\rightarrow2)\overset{1}{\mathbf{Man}}(\alpha1\rightarrow3)\overset{2}{\mathbf{Man}}(\beta1\rightarrow4)\overset{3}{\mathbf{GlcNac}}(\beta1\rightarrow4)\underset{\substack{(\alpha1\rightarrow6)\\ \mathbf{Fuc}\\ \mathbf{5}}}{\overset{4}{\mathbf{GlcNac}}}$$

In LOL, Man **1** is bound in the monosaccharide pocket, GlcNac **4** is bound by Asn 78, and Fuc **5** is bound by Glu 218 [10]. Lectins with different sugar specificities can occur in one legume species. In soybean (*Glycine max*) for instance, two lectins are present, one specific for N-acetyl-D-galactosamine (SBL), and one specific for 4-O-methylglucuronic acid [62,77]. PHA-L specifically binds the oligosaccharide



$$\mathbf{Gal}(\beta1\rightarrow4)\mathbf{GlcNac}(\beta1\rightarrow2)\underset{\substack{(\beta1\rightarrow6)\\ \mathbf{Gal}(1\rightarrow4)\mathbf{GlcNac}}}{\mathbf{Man}}$$

whereas PHA-E is specific for

NANA→Gal→GlcNac→Man

[25]. Heterotetramers of PHA-L and PHA-E combine both specificities. In some other legumes, such as pea, different isolectins appear to have the same sugar-binding specificity.

Lectin function

Little is known of the physiological function of legume lectins. It has been shown that lectins in roots can play a role in the N_2-fixing symbiosis of legumes with

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[2] Abbreviations used: Gal = galactose; Man = mannose; Fuc = Fucose; GlcNac = N-acetylglucosamine; NANA = N-acetylneuraminic acid.

NAME	SOURCE	SPECIFICITY	REF
BPL	Bauhinia purpurea	D-GalNac	81
GSL4	Griffonia simplicifolia	D-Gal	5
LAL1	Laburnum alpinum (scotch laburnum)	Di-N-acetyl-chitobiose	40
UEL1	Ulex europaeus (furze)	Di-N-acetyl-chitobiose	41,42
UEL2	Ulex europaeus (furze)	L-Fuc	41,42
LOL1	Lathyrus ochrus	D-Glc/D-Man	8,78
LOL2	Lathyrus ochrus	D-Glc/D-Man	57,78
LNL1	Lathyrus nissolia	D-Glc/D-Man	61
LNL2	Lathyrus nissolia	D-Glc/D-Man	61
PSL	Pisum sativum (pea)	D-Glc/D-Man	22,38
LCIL	Lathyrus cicera	D-Glc/D-Man	68
VFL	Vicia faba (faba bean)	D-Glc/D-Man	28,34
LCUL	Lens culinaris	D-Glc/D-Man	20
MTL1	Medicago truncatula	D-Glc/D-Man?	3
LSL	Lathyrus sphaericus	D-Glc/D-Man	58
DBL1	Dolichos biflorus	GalNac	63
DBL2	Dolichos biflorus	GalNac	64
PHAL	Phaseolus vulgaris	Complex sugar	31
PHAE	Phaseolus vulgaris	Complex sugar	31
LBL1	Phaseolus lunatus (lima bean)	GalNac	68
LBL2	Phaseolus lunatus (lima bean)	GalNac	68
SBL	Glycine max (soybean)	GalNac	77
CSL	Cytisus scoparius	D-Gal	80
ECORL	Erythrina corallodendron (Coral tree)	D-Gal, LacNac	1
OVL	Onobrychis vicifolia (sanfoin)	D-Glc/D-Man	43
LTA	Lotus tetragonolobus	L-Fuc	68
PNA	Arachis hypogea (peanut)	D-Gal	82
CONA	Canavalia ensiformis (jack bean)	D-Glc/D-Man	11

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         1                           20                             40                      60                  76
BPL    ...TLTGFTF PNFWSNTQE. ...NGTEIIF LGNA.TYTPG ALRLTRIGE. DGIPLKSNAG QASYSRPVFL W...DSTGHV ASFYTSFSFI VR...SIDVP
GSL4   ..ZNTVNFTY PDFWSYSL.K ...NGTEITF LGDA.TRIPG ALQLTKTDA. NGNPVRSSAG QASYSEPVFL W...DSTGKA ASFYTSFTFL LK...NYGAP
LAL1   ..LNELSFNF ........DK FVPNQNNILF QGVASVSTTG VLQVTKV... ..TNT..GIK RALYAAPIHA WDDDSETGKV ASFATSFSFV VKEPPIQSRK
UEL2   NLSDDLSFNF ........DK FVPNQKNIIF QGDASVSTKG VLEVTKVSK. P.TTR..SIG RALYAAPIQI W..DSITGKV ASFATSFSFV VKDEPDE..K
UEL1   ..SDDLSFKF ........KN FSQNGKDLSF QGDASVIETG VLQLNKVGN. NLPDE..TGG IARYIAPIHI W..NCNTGEV ASFITSFSFF METSANPK..
LOL1   ..TETTSFSI ........TK FGPDQQNLIF QGDGYTT.KE RLTLTKAVR. N......TVG RALYSSPIHI W..DSKTGNV ANFVTSFTFV I.DAPN.S..
LOL2   ..TETTSFSI ........TK FGPDQPNLIF QGDGYTT.KE RLTLTKAVR. N......TVG RALYSSPIHI W..DSKTGNV ANFVTSATFV I.DAPN.S..
LNL1   ..TETTSFLI ........TK FSADQQNLIF QGDGYTT.KD KLLLTKAVR. N......TVG RALYSSPIHI W..DSQTGNV ANFVTSFTFV I.NAPN.S..
LNL2   ..TETTSFLI ........TK FSADQQNLIF QGDGYTT.KD KLLLTKAVR. N......TVG RALYSSPIHI W..DSQTGNV ANFVTSFTFV I.NAPN.S..
LCIl   ..TETTSFLI ........TK FGPDQQNLIF QGDGYTT.KE RLTLTKAVR. N......TVG RALYSSPIHI W..DSKTGNV ANFVTSFTFV I.NAPN.S..
PSL    ..TETTSFLI ........TK FSPDQQNLIF QGDGYTT.KE KLTLTKAVK. N......TVG RALYSSPIHI W..DRETGNV ANFVTSFTFV I.NAPN.S..
VFL    .TDEITSFSI ........PK FRPDQPNLIF QGGGYTT.KE KLTLTKAVK. N......TVG RALYSLPIHI W..DSETGNV ADFTTTFIFV I.DAPN.G..
LCUL   ..TETTSFSI ........TK FSPDQQNLIF QGDGYTG.KE GLTLTKVSK. E......TGG RALYSTPIHI W..DRDTVNV ANFVTNGSQV FRESPN.G..
MTL1   ..TELTSFTI ........TK FSQDQKNLIF QGNAITTSTG KLQLTKAVK. N......SIG RALYSAPIHI W..DSKTGDV ANFETLFTFA I.TAPY.S..
MTL2   ..AETTSFSI ........TK FVPDQKNLIF QGDAKTASTG KLELSKAVK. N......SIG RALYSAPIHI W..DSKTGSV ANFQTTFTFT I.TAPN.T..
LSL    TETETTSFSI PKTDQPSSPK FVSGQPNLIF QGNAYST.DG KLILTEAKQ. N......TVG RALYSAPIHI W..DRKTGKV ADFTASFTFY IRPNSD.S..
DBL1   ..ANIQSFSF ........KN F..NSPSFIL QGDATVSS.G KLQLTKVKE. NGFPLRFPSG RAFYSSPIQI Y..DKFTGAV ASWATSFTVK ISAPSKAS..
DBL2   ..ADIQSFSF ........KN F..NSSSFIL QGDATVSS.S KLRLTKVKG. NGLPTLSSLG RAFYSSPIQI Y..DKSTGAV ASWATSFTAN IFAPNKSS..
PHAE   ..ASQTSFSF ........QR F..NETNLIL QRDATVSSKG QLRLTNVND. NGEPTLSSLG RAFYSAPIQI W..DNTTGAV AASPTSFTFN IDVPNNSG..
PHAL   ..SNDIYFNF ........QR F..NETNLIL QRDASVSSSG QLRLTNLNG. NGEPRVGSLG RAFYSAPIQI W..DNTTGTV ASFATSFTFN IQVPNNAG..
LBL1   ...AELFFNF ........QT F..NAANLIL QGNA.VSSKG HLLLTNVTH. NGEPSVASSG RALYSAPIQI R...DSTGNA SSTPTSHSYT LQQ.....IF
LBL2   .EFAELFFNF ........QT F..NAANLIL QGNASVSSSG QLRLTEVKS. NGEPKVASLG RAFYSAPIQI W..DSTTGKV ASFATAFTFN ILAPILSN..
SBL    ..AETVSFSW ........NK FVPKQPNMIL QGDAIVTSSG KLQLNKVDE. NGTPKPSSLG RALYSTPIHI W..DKETGSV ASFAASFNFT FYAPDTKR..
CSL2   ..SEELSFSF ........TK FKTDQKNLIL QRDALITPTG KLQLTTV.E. NGKPAAYSLG RALYSTPIHI W..DKSTGDE ASFATFFSFV ISDAPNPS..
ECORL  ..VETISFSF ........SE FEPGNDNLTL QGAALITQSG VLQLTKINQ. NGMPAWDSTG RTLYAKPVHI W..DMTTGTV ASFETRFSFS IEQPYTRP..
OVL    .AENTVSFDF ........SK FLSGQENLIL QGDTVTDDSN RCLVLTREN. NGRPVQDSVG RVLYQTPIHL W..DKQIDKE ASFETSFTFF IYRENINR..
LTA    .....VSFNY ........TE FKDDGSLI.L QGDAKIWTDG RLAMPTDPLV NNPKTTRSAG RALYATPVPI W..DSATGNV ASFVTSFNFL FVIRELK...
PNA    ..AETVSFNF ........NS FSEGNPAINF QGDVTVLSNG NIQLTNLNKV N......SVG RVLYAMPVRI W..SSATGNV ASFLTSFS.. FEMKDIKD..
CONA   ....ALHFMF ........NQ FSKDQKDLIL QGDATTGTEG NLRLTRVS.S NGSPQGSSVG RALFYAPVHI W..ESSAV.V ASFEATFTFL IKSPDS....
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             *                      *                                            *
           80                      100                                120                              140         151
BPL     HITADGFAFF LAPVDSSVKD Y...GGCLGL FRYKTATDP. .........S KNQVVAVEFD T....WPNTE WS.DLR.... .YPHIGINVN STVSVATTR
GSL4    ..TADGLAFF LAPVDSSVKD Y...GGFLGL FRHETAADP. .........S KNQVVAVEFD T....WINKD WN.DPP.... .YPHIGIDVN SIVSVATTR
LAL1    ADGVDGLAFF LAPANSQIPS GSS.AGMFGL FCSS.DYN.. .........S SNQIIAVEFD TYFGKAYNP. WD  PDF... ..KHIGVDVN SIKSIKTVK
UEL2    IDGVDGLAFF LAPANSQIPS GSS.AGMFGL FCSSNDSK.. .........S SNQIIAVEFD SYFGKTYNP. WD  PDF... ..KHIGIDVN SIKSIKTVK
UEL1    .AATDGLTFF LAPPDSPLRR A...GGYFGL FEDTKDND.. .........S SYQTVAVEFD TI.GSPVN.. FD DPGF... ..PHIGIDVN RVKSINAER
LOL1    YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSKDYD.. .........K TSQTVAVEFD T....FYNTA WDPSNGD... ..RHIGIDVN SIKSINTKS
LOL2    YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSKDYD.. .........K TSQTVAVEFD T....FYNTA WDPSNGD... ..RHIGIDVN SIKSINTKS
LNL1    YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSKDYD.. .........K TSKTVAVEFD T....FYNAA WDPSNGD... ..RHIGIDVN SIKSVNTKS
LNL2    YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSKDYD.. .........K TSKTVAVEFD T....FYNAA WDPSNGD... ..RHIGIDVN SIKSVNTKS
LCI1    YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSVDYD.. .........K TSQTVAVEFD T....FYNAA WDPSNRE... ..RHIGIDVN SIKSINTKS
PSL     YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NSAEYD.. .........K TTQTVAVEFD T....FYNAA WDPSNRD... ..RHIGIDVN SIKSVNTKS
VFL     YNVADGFTFF IAPVDTKPQT ...GGGYLGV F.NGKDYD.. .........K TAQTVAVEFD T....FYNAA WDPSNGK... ..RHIGIDVN TIKSISTKS
LCUL    YNVADGFTFF IAPVDTKPQT ...GGGYLGV FYNGKEYD.. .........K TSQTVAVEFD T....FYNAA WDPSNKE... ..RHIGIDVN SIKSVNTKS
MTL1    SNVADGLAFF IAPVDTQPQN IGR.AGFLGV F.NSETYN.. .........K SIQTVAVEID T....FHNT. WDPK.IN... ..RHIGINVN CIKSISTTS
MTL2    YNVADGLAFF IAPIDTKPKS IHH.GGYLGV F.DSKTYK.. .........K SIQTVAVEID T....FYNAQ WDPNPGNISS TGRHIGIDVN SIKSISTVP
LSL     QVVADGFTFF IAPVDTQPRG ..D.GGLLGV F.NREEYD.. .........P TIHTVAVEFD T....FHNQP WDPDYI.... ...HIGVDIN SIKSRITRP
DBL1    ..FADGIAFA LVPVGSEPRR ..N.GGYLGV FDSD.VYN.. .........N SAQTVAVEFD T....LSNSG WDP..SM... ..KHIGIDVN SIKSIATVS
DBL2    .S.ADGIAFA LVPVGSEPKS ..N.SGFLGV FDSD.VYD.. .........N SAQTVAVEFD T....FSNTD WDP..TS... ..RHIGIDVN SIKSIRTAS
PHAE    ..PADGLAFV LLPVGSQPKD ..K.GGLLGL FNNY.KYD.. .........S NAHTVAVEFD T....LYNVH WDP..KP... ..RHIGIDVN SIKSIKTTT
PHAL    ..PADGLAFA LVPVGSQPKD ..K.GGFLGL FDG...SN.. .........S NFHTVAVEFD T....LYNKD WDP..TE... ..RHIGIDVN SIRSIKTTR
LBL1    QNVTDDPAWL FALVPVDSQP KKK.GRLLGL FNKSEN.... ........DI NALTVAVEFD T....CHNLD WD...KNS.. ...IAVNLGG SV......P
LBL2    .SP.DGLAFA LVPVGSQPKF ..N.GGFLGL FQNV.TYD.. .........P TAQTVAVEFD T....CHNLD WDP..KGS.. ...HIGIDVN SIKSIKTVP
SBL     ..LADGLAFF LAPIDTKPQT ..H.AGYLGL FNEN...E.. .........S GDQVVAVEFD T....FRNS. WDP..PN... ..PHIGINVN SIRSIKTTS
CSL2    TAATDGLAFF LAPADTQPQS ..  AGGYLGL FEKDSSYN.. .........S SNQIVAVEFD T....YYNSA WDP.QTN... ..PHIGIDVN TIKSKKVSS
ECORL   .LPADGLVFF MGPTKSKPA. ..QGYGYLGI FNNSKQ.D.. .........N SYQTLGVEFD T....FSN.Q WDP.PQV... ..PHIGIDVN SIRSIKTQP
OVL     ..GGDGITFF LAPTDTQPKS ...GGGYLGI F...KDAE.. .........S NETVVAVEFD T....FSNR. WDP..ANS.. ...HIGINVN SVKSKITTP
LTA     YTPTDGLVFF LAPVGTEIPS GST.GGFLGI FDGSN..... .........G FNQFVAVEFD S....YHNI. WDPKSLRSS. ...HVGIDVN SIMSLKAVN
PNA     YDPADGIIFF IAPEDTQIPA GSIGGGTLGV SDTK...... . .......G AGHFVGVEFD T....YSNS. .EYNDPPTD. ...HVGIDVN SVDSVKTVP
CONA    .HPADGIAFF ISNIDSSIPS GST.GRLLGL FPDANVIRNS TTIDFNAAYN/ADTIVAVELD T....YPNT. .DIGDPSY . ..PHIGIDIK SVRSKKTAK

                                               ↓                                *     **
                   160                   180                 200                      220                               241
BPL     WDNDDAYVTK .STAHITYDA TSKIITVLLT Y.....DNGR HYQLSHVVDL PKILPERVRI GFSGGTGFNE TQY....ILS WSFTSTLNST KISALTQKLR SSASYSSM
GSL4    WENDDAYGSS IATAHITYDA RSKILTVLLS Y.....EHGR DYILSHVVDL AKVLPQKVRI GFSAGVGYDE VTY....ILS WHFFSTLDGT NK........ ........
LAL1    WDWRNG...D VANVVITYRA PTKSLTVSLS YPS...DQTS NIVTA.SVDL KAILPEWVSV GFSAGVG..N AAKFNHDILS WYFTSNLEPN NPAVNQAQ.. ........
UEL2    DDWRNG...E VADVVITYRA PTKSLTVSLS YPS...DGTS NIVTASSVDL KAILPEWVSV GFSGGVG..N AAKFDHDVLS WYFTSNLEAN QSQT...... ........
UEL1    WNKRYGLN.N VANVEIIYEA SSKTLTASLT YPS...DQTS ISVT.SIVDL KEILPEWVSV GFSGGTY..I GRQATHEVLN WYFTSNLINT NS........ ........
LOL1    WKLQNG...K EANVVIAFNA ATNVLTVSLT YP....NET. SYTLNEVVPL KEFVPEWVRI GFSATTG... AEFAAHEVLS WFFHSEL... ......AGTS SSN.....
LOL2    WKLQNG...K EANVVIAFNG ATNVLTVSLT YP....NET. SYTLNEVVPL KEFVPEWVRI GFSATTG... AEFAAHEVLS WYFNSEL... ......SVTS SSN.....
LNL1    WKLQNG...K EANVVIAFNA ATNVLTVSLT YP....NSV. SYTLNEVVPL KDVVPEWVRV GFSATTG... AEFAAHEVLS WSFHSEL... ......AGTS ASKQ....
LNL2    WKLQNG...K EANVVIAFNA ATNVLTVSLT YP....NSV. SYTLNEVVAL KDVVPEWVRI GFSATTG... AEFAAHEVLS WSFHSEL... ......DGTS SSN.....
LCI1    WKLQNG...V EANVVIAFNA ATNVLTVSLT YP....NVT. SYTLNEVVPL KDVVPEWVRI GFSATTG... AEFAAHEVLS WSFHSEL... ......GETS ASKQ....
PSL     WKLQNG...E EANVVIAFNA ATNVLTVSLT YP....NVT. SYTLSDVVSL KDVVPEWVRI GFSATTG... AEYAAHEVLS WSFHSEL... ......SGTS SSKQ....
VFL     WNLQNG...E EAHVAISFNA TTNVLSVTLL YP....NLT. GYTLSEVVPL KDVVPEWVRI GFSATTG... AEYATHEVLS WTFLSEL... ......TGPS N.......
LCUL    WNLQNG.... .......... .......... .......VT. SYTLNEVVPL KDVVPEWVRI GFSATTG... AEFAAQEVHS WSFNSQL... ......GHTS KS......
MTL1    WVLENG...R EANVLVRFDA HTNVLSVVLS YP....GLPD SYILSDVVPL KDIVPEWVRI GFSAATG... AEFAEHDIRY WSFHSELSLV FNNN.NANVS SSVQSA..
MTL2    WSLENN...K KANVAIGFNG ATNVLSVDVE YP.....LIR HYTLSHVVPL KDVVPEWVRI GFSSSTG... AEYSAHDILS WSFDSKLNLG FENNINANVS SSTQAA..
LSL     WNPHYD...T YSIAYIAYKA ATNELDVTVT YP....NSRD YATLREVVDL KQIVPEWVRV GLSASTA... TYYSAHEVYS WSFHSEL... ......GGTS SSN.....
DBL1    WDLANG...E NAEILITYNA ATSLLVVSLV HP....SRRT SYILSERVDI TNELPEYVGV GFSATTGLSE GYIETHDVLS WSFASRLPDD STA.EPLDLA RYLVRNVL
DBL2    WGLANG...Q NAEILITYNA ATSLLVASLV HP....SRRT SYIVSERVDI TNELPEYVSI GFSATTGFFE GYTETHDVLS WSFASKLPDD STT.EPLDIA SYLVRNVL
PHAE    WDFVKG...E NAEVLITYDS STKLLVASLV YP....SLKT SFIVSDTVDL KSVLPEWVIV GFTATTGITK GNVETNDILS WSFASKLSDG TTS.EALNLA NFALNQIL
PHAL    WDFVNG...E NAEVLITYDS STNLLVASLV YP....SQKT SFIVSDTVDL KSVLPEWVSV GFSATTGINK GNVETNDVLS WSFASKLSDG TTS.EGLNLA NLVLNKIL
LBL1    WNFR.DYDGQ NADVLITYDS STKFLAVSLF YPI....TGK RNNVSANVEL EKVLDDWVSV GFSATSG... .AYETHDVLS SSFASKLSSL DECPTGEN.. ..LLNKIL
LBL2    WSLLNG...H NAKVLITYDS STKLLVASLV YP....SGST SYIISEKVEL KSVLPEWVNI GFSATSGLNK GNVEAHDVLS WSFASKLSDG TPC.EDLSLA NIVLNKIL
SBL     WDLANN...K VAKVLITYDA STSLLVASLV YP....SQRT SNILSDVVDL KTSLPEWVRI GFSAATGLDI P.GESHDVLS WSFASNLPHA SSNIDPLDLT SFVLHEAI
CSL2    WGFKNG...N VATVLITYQP SSKSLVASLV YPSGQTSDKT SYIISANVDL KATVPEWVRI GFSATTGQTD NYIETHDILS WSFKSKLPAT KN........ ........
ECORL   FQLDNG...Q VANVVIKYDA SSKILHAVLV YP....SSGA IYTIAEIVDV KQVLPEWVDV GLSGATGAQR DAAETHDVYS WSFQASLPET NDAVIPTSNH NTFAI...
OVL     WGLKNDYF.. ..TVTITYDA TRSLSVSSF. ...YRNKPDD IFTVKASVHL RDALPQWVRI GLSAATG... DLVEQHRLYS WSFKSVLPLD SST....... ........
LTA     WNRVSGSL.E KAT.II.YDS QTNILSVVMT SQ.....NGQ ITTIYGTIDL KTVLPEKVSV GFSATTGN.. PEREKHDIYS WSFTSTLKEP EEQA...... ........
PNA     WNSVSGAV.V KVT.VI.YDS STKTLSVAVT ND.....NGD ITTIAQVVDL KAKLPERVKF GFSASGSL.. GGRQIHLIRS WSFTSTLITT TRRS...... ........
CONA    WNMQNGKV.G TAH.II.YNS VDKRLSAVVS YP.....NAD SATVSYDVDL DNVLPEWVRV GLSASTGL.. .YKETNTILS WSFTSKLKSN STHQTD.... ........
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Table 1. Name, source, specificity and sequence alignment of twenty-nine legume lectins. Numbering is according to the PSL sequence. Amino acids known to be involved in the monosaccharide binding site in PSL and LOL are indicated by an asterisk. An arrow indicates the processing site between β- and α chains in the two-chain lectins, and a / indicates the site of protein ligation in processing of CON A. The Z in GSL4 stands for hydroxyproline.

Rhizobium bacteria [6,15,16]. Sugar binding activity is required for the improvement of nodulation by PSL [76]. A specific role for lectin in determination of host specificity in the *Rhizobium*-legume symbiosis was proposed earlier [6,39]. Bohlool and Schmidt reported that soybean lectin (SBL) binds to 22 of the 25 *Bradyrhizobium japonicum* strains that can infect soybean, and that this lectin does not bind to rhizobia from other cross-inoculation groups [6]. Dazzo *et al.* showed that clover plants produce a lectin that can agglutinate *Rhizobium leguminosarum* bv. *trifolii* [14]. Until now, the exact mechanism of action of lectins in this symbiosis remains unclear. It could be that coating and/or agglutination of specific rhizobia with lectins is one of the steps required for establishing infection, but this hypothesis has to be backed by more experimental evidence.

It has further been hypothesized that lectins can protect seeds and leaves against herbivorous animals [12]. Some lectins are toxic to rats, pigs, or insects [7,48,52]. It has been shown by Fountain *et al.* that lectins are released upon imbibition of dry seeds [21]. They are therefore present in the vicinity of germinating seeds, and can contribute to protection of the young seedling [12,21]. Induction of lectin genes upon predation of legumes has not been reported sofar.

Lectins as anti-nutritional factors

In the feeding industry, legume lectins are considered to be anti-nutritional factors (ANF) [24,35,36]. Lectins have been shown to pass through the stomach largely unchanged and to subsequently bind to gut epithelial cells of animals feeding on meal from raw legume seeds, causing damage to the intestine [24,29,53]. This is especially true for PHA, whereas other lectins, such as PSL, seem to be less toxic [24]. The adhesion of lectins to epithelial cells [29] suggests that the sugar-binding activity of lectin is required for toxicity, but this has not been conclusively demonstrated.

Legume seed meal has to be heat-treated for a considerable period of time, in order to eliminate the adverse activity of lectins, and to improve its nutritional value. A standard heat treatment is steam heating for 40 minutes at 104°C and 19% moisture, so-called toasting [35]. Apparently, lectin is toxic in its native state, and toxicity is lost upon irreversible denaturation of the protein. Toasting requires energy and may reduce the nutritional value of legume seeds and seed meal, making the use of legume seed meal as a fodder for pigs economically unfavourable.

Outline of this thesis

This thesis concerns the structure and function of legume lectin. The major goal of the research discussed in this thesis was to find a way to eliminate the anti-nutritional properties of these lectins without interfering with any of the alleged biological functions of lectins in plants. As a model lectin we have chosen PSL, because:

(1) it is a well studied legume lectin [13-19,22,23,30,31,38,39,75,76]

(2) only one functional copy of the lectin gene is present in pea plants [23,38]

(3) PSL is not a glycoprotein; therefore, PSL isolated from *E.coli* is very similar to the plant lectin, and it has the same structure and specificity [51,69].

(4) an assay for testing the role of PSL in nodulation is available [16].

Chapter 2 describes the post-translational processing of PSL as determined by protein sequencing, and the exact differences between PSL 1 and 2. In this chapter we present further evidence that PSL in roots is identical to seed PSL2, and that a carboxypeptidase-like processing activity is responsible for the difference in charge between PSL1 and PSL2. Chapter 3 deals with destabilization of PSL by a single mutation in a surface loop, and describes the possibility of producing lectins which are active *in planta*, but are easily inactivated *in vitro*. This could be a way to circumvent the anti-nutritional activities of lectins without impairing their proposed role in symbiosis. Chapter 4 demonstrates that the mutation described in Chapter 3 can be introduced in PHA-L and that this mutation has a similar effect in PHA-L, showing that this method can be used to destabilize other legume lectins, even if their exact three-dimensional structure is not known. Chapter 5 concerns the biological activity of the unstable PSL mutants described in Chapter **3**, their role in symbiosis, their insect toxicity and their stimulation of lymphocyte activity. Chapter **6** describes the elimination of the sugar-binding activity of PSL by introducing a minor change in the sidechain of a single residue, corroborating the crystallographic data on the sugar-binding site. Finally, Chapter **7** is a summarizing discussion.

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CHAPTER 2

PEA (*PISUM SATIVUM* L.) SEED ISOLECTINS 1 AND 2 AND PEA ROOT LECTIN RESULT FROM CARBOXYPEPTIDASE-LIKE PROCESSING OF A SINGLE GENE PRODUCT

Flip J. Hoedemaeker, Michael Richardson, Clara L. Díaz, B. Sylvia de Pater and Jan W. Kijne.

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ABSTRACT

The complete amino acid sequences of the α-subunits of pea (*P. sativum* L.) seed and root lectin, the C-terminal amino acids of the ß-subunits of pea seed lectin, and most of the sequence of the ß-subunit of pea root lectin have been determined. In contrast to earlier reports it was shown that the ß-subunits of both seed isolectins end at Asn 181. The α1 subunits end at Gln 241 (major fraction) or Lys 240 (minor fraction), whereas the α2 subunits end at Ser 239, Ser 238, Ser 237, or Thr 236. psl cDNA clones from seed are identical with psl cDNA clones from root, and root PSL is identical with seed PSL2, ending at Ser 239, Ser 238 or Ser 237. It seems that the presence of Lys 240 is the sole determinant of the charge difference between pea isolectins. PSL1 can be converted to PSL2 by carboxypeptidase P from *Penicillium janthinellum.* These results confirm that PSL from roots is encoded by the same gene as PSL from seeds. Thus, it seems that, next to an Asn-X specific protease responsible for the processing at positions 181/182 and 187/188, a carboxypeptidase is responsible for the conversion of PSL1 into PSL2, which is probably the final processing product.

INTRODUCTION

Lectins from seeds of legumes are among the best studied non-enzymatic carbohydrate-binding proteins [25,26]. In these seeds, lectins are present in high amounts in specialised protein storage vacuoles (protein bodies) [3,14,26]. Legume lectins are also present in other parts of the plant, usually in low amounts. Little is known with regard to the function of legume lectins. However, it has been demonstrated that pea (*Pisum sativum* L.) lectin plays a role in the N-fixing symbiosis with *Rhizobium* bacteria [4]. It has also been hypothesized that legume lectins play a role in plant defence against herbivores and plant pathogens [2].

The primary and three-dimensional structures of a variety of legume lectins have been established [26]. These lectins are ß-barrel proteins [19], and are organised as dimers or tetramers with one sugar binding-site per subunit. After folding, each subunit can be processed post-translationally and in some cases circular permutation occurs [25,26]. The subunits of legume lectins basically have a similar structure, despite the differences in processing and multimerisation. Pea plants apparently contain only one functional copy of a lectin gene, psl [8,15]. Recently a different transcript with homology to legume lectins has been isolated from actively growing buds from pea plants, but it has not been established whether or not this transcript encodes an active lectin [17].

Pea lectin, PSL, is a dimer consisting of two identical subunits, which are post-translationally cleaved into a ß-chain and a smaller α-chain. PSL is synthesised as a pre-pro-protein in a ß → α direction (see Figure 1). The N-terminal signal peptide is removed co-translationally upon entering of the protein into the lumen of the ER. In the seeds, post-translational cleavage of the pro-protein into an α- and a ß-subunit occurs in the protein bodies [13,14]. The N-termini of the ß- and α-subunits have been determined [27]. As judged from the N-terminal sequence of the α-subunit, an Asn-X specific protease apparently is responsible for the processing into ß- and α-subunits. Recently, it has been shown that a number of proteins, present in protein bodies of legumes, can be processed into their mature forms by an Asn-X specific protease [10,22,24]. It has also been shown that transgenic potato plants, carrying the psl gene, correctly process PSL into α- and ß-subunits [6].

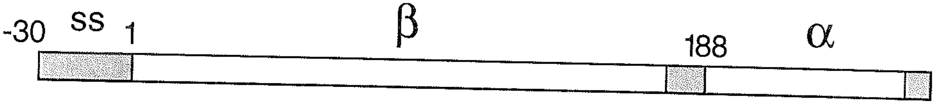

Figure 1. The synthesis of PSL as a pre-pro-protein. SS = the N-terminal signal sequence, ß = the ß-subunit, *α* = the *α*-subunit. The positions of the N-terminal amino acids are indicated.

In pea, two isolectins are formed, PSL1 and PSL2, which are usually present in roughly equal amounts in mature pea seeds. These isolectins differ in charge: PSL1 has a pI of 7.2 and PSL2 has a pI of 6.1 [3]. Upon germination, PSL1 rapidly disappears from the seeds. In the vegetative parts of the pea plant only one isolectin, with a pI of 6.1, is found [3,9].

Conflicting results have been published concerning the exact processing sites of PSL [7,18,21,27]. Furthermore, until now it has not been established that PSL isolectins from seeds and PSL from roots are derived from one functional PSL gene. We report the sequence of cDNA clones from psl mRNA isolated from both seed and root. We also report the complete amino acid sequence of the *α*-chains of PSL1 and PSL2 from seeds and PSL from roots, as well as the sequence of the C-terminal part of the ß-chains of both isolectins. We show that PSL1 and PSL2 from seeds and PSL from roots are encoded by one gene, and that root PSL most probably is identical to seed PSL2. Finally we report that PSL1 can be converted into PSL2 by processing of the *α*-chain C-terminus with a carboxypeptidase, strongly suggesting the involvement of such an enzyme *in vivo*.

MATERIALS AND METHODS

Cloning and sequencing of psl cDNA from seed and roots

Total RNA from both pea seeds and roots (cv. Feltham First) was isolated. cDNA was made using M-MuLV reverse transcriptase (BRL, Bethesda, MD, USA) and the anti-sense primer SP4 (5'-GGC CAA GCT TGC ATG CAA TTA TAA CCA ATA ATG GCT TC-3'). The cDNA was amplified with PCR, using the same anti-sense primer and the sense primer SP7 (5'-GGC CGA ATT CTG GAT CTG TCA CAC ATG ACT TGA C-3'), and was cloned as an EcoRI/HindIII restriction fragment into pBluescript II SK+ (Stratagene, La Jolla, CA, USA). Five random cDNA clones from seed RNA and five from root RNA were sequenced using Sequenase$^{2.0}$ (USB).

Purification of isolectins and isolectin subunits.

Pea seeds (*Pisum sativum* cv. Finale) were purchased from Cebeco, Rotterdam, The Netherlands. PSL was isolated from dry seed meal or from 4-day-old roots, purified using affinity chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden), and isolectins were separated using chromatofocusing as described before [3]. After purification, the isolectins were dissolved in 6M guanidine-HCl. The *α*- and ß-subunits were separated on a Biogel P-30 (Biorad, Richmond, CA, USA) column of 1 x 100 cm in 6M guanidine-HCl, using gravity flow. After purification, the subunits were dialysed extensively against demineralized H_2O and lyophilised.

Protein sequencing

Purified *α*-subunits of both isolectins were digested with *Staphylococcus aureus* V8 protease (Endoprotease Glu-C, Sigma, St. Louis, MO, USA) in 0.2 M ammonium bicarbonate, pH 8.2, for 24 hrs at 37°C. Purified ß-subunits were digested with chymotrypsin (Sigma, St. Louis, MO, USA) or trypsin (Boehringer, Mannheim, Germany) in the same buffer. In every case a protease:protein ratio of 1:50 (w/w) was used. After digestion, peptides were separated on a C18 reverse phase HPLC column (Vydac, Hesperia, CA, USA), using a gradient of 0-40% acetonitrile containing 0.1% TFA, in 120 min. Peak fractions were collected manually and lyophilised.

HPLC-purified peptides were sequenced manually using the DABITC-PITC double coupling method [29]. If appropriate, sequence analysis was confirmed using automated Edman degradation on a Applied Biosystems 477A Protein Sequencer.

Digestion of PSL1 with carboxypeptidase and IEF analysis

Carboxypeptidase P from *Penicillium janthinellum* was purchased from Boehringer, Mannheim, Germany. PSL1 was digested with this carboxypeptidase according to the instructions of the supplier. Aliquots were taken at T=0, 10, 20 and 30 min. These fractions were analysed by isoelectric focusing gel electrophoresis, as described before [3].

RESULTS

Cloning and sequencing of psl cDNA from seed and roots

To test whether PSL from seed and from root are derived from one gene, five random psl cDNA clones from seed and five clones from roots were sequenced. No consistent sequence differences between seed and root cDNA were found. A few point mutations were observed, but these could be attributed to the Taq polymerase used in the PCR reaction. The sequence found is identical with that published by Gatehouse *et al* [8]. Since the psl transcripts in seed and root seem to be identical, it is very likely that they are derived from the same gene. In addition, Southern analysis of genomic pea DNA only showed one major band hybridizing to the psl cDNA (data not shown), which supports this conclusion.

Isolation, purification and sequencing of PSL subunits

The ß- and *α*-subunits of PSL1 and PSL2 from seeds, as well as those from root PSL were purified. Each ß-subunit was digested with chymotrypsin. In addition, the ß-subunit of root PSL was digested with trypsin. Each *α*-subunit was digested with *Staphylococcus aureus* V8 protease (Endoprotease Glu-C). The isolated ß- and *α*-subunits are insoluble in aqueous buffer, but they dissolve upon digestion. After digestion, the resulting peptides were separated with reverse phase HPLC.

Each root lectin ß-subunit peptide isolated was sequenced to test sequence identity with the seed PSL sequence and that deduced from the cDNA sequence. The peptides represented about 80% of the total ß-subunit sequence. In these peptides no differences were found with the seed PSL sequence and the amino acid sequence as deduced from cDNA.

In the chymotrypsin digests of seed lectin ß-subunits, the C-terminal peptide was identified with the help of the HPLC elution pattern of *Lathyrus ochrus* L. lectin ß-subunit digested with chymotrypsin (M. Richardson, unpublished results), and only this peptide was sequenced. The PSL ß-subunits all ended at asparagine 181, in contrast with the results published by Rini *et al* [21], but in accordance with the structural data from Einspahr *et al* [7]. Figure **2** shows the cDNA deduced sequence compared with the sequence of the peptides that were found.

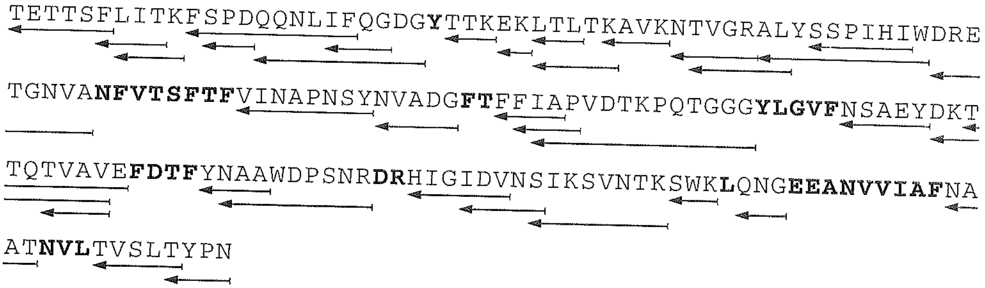

Figure 2. The ß-subunit peptides from PSL from roots that were sequenced compared to the cDNA derived PSL sequence. The peptides that were found and sequenced are indicated by arrows. They represent about 80% of the total ß-subunit sequence.

The sequences of the C-terminal *α*-subunit peptides are shown in Figure 3. The sequence of the *α*-subunits is in accordance with the cDNA sequence. The last digestion site for V8 protease is present at glutamic acid 232, resulting in C-terminal peptides starting at leucine 233. In the digest of the *α*-subunit of PSL1 two C-terminal peptides were found, one ending at glutamine 241 and one ending at lysine 240. The peptides containing the glutamine represented the major fraction. The psl gene also contains codons for alanine 242, alanine 243, aspartate 244 and alanine 245, but these amino acids were not found in the protein sequence.

In the digest of the *α*-subunit of PSL2 four different C-terminal peptides were found, ending at serine 239, serine 238, serine 237 and threonine 236. The peptide ending at serine 239 was the predominant one.

These results suggest that a carboxypeptidase activity is responsible for the shortening of the *α*-subunits by one residue at a time, and that the major difference between PSL 1 and 2 is the presence of lysine 240. Shorter C-terminal peptides were not found, but it is possible that carboxypeptidase activity continues until serine 234, which is the last amino acid in a ß-pleated sheet, and is also the last amino acid identified in the crystal structure of pea lectin [7]. Di- and tri-peptides can be lost or overlooked during HPLC purification.

In the *α*-subunit of root PSL three C-terminal peptides were found, ending at serine 239, serine 238 and serine 237. Apart from the absence of shorter C-

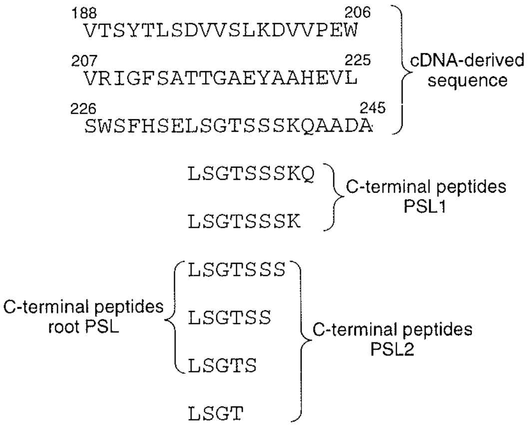

Figure 3. The sequence of the C-terminal peptides of the α-subunits from PSL1 and PSL2 from seeds and PSL from roots compared to the cDNA derived sequence.

terminal peptides, no differences were found between the α-subunits of root PSL and PSL2 from seeds. The HPLC elution pattern of the C-terminal peptides from seed and root PSL is shown in Figure **4**.

Digestion of seed PSL1 with carboxypeptidase

To determine whether digestion by a carboxypeptidase could be responsible for the charge difference of both isolectins, a solution of PSL1 was treated with a serine type carboxypeptidase. This carboxypeptidase readily cleaves off C-terminal amino acids, but the enzyme has a low affinity for serine residues. As judged from isoelectric focusing gel electrophoresis, PSL1 disappears from the solution upon incubation with carboxypeptidase, and PSL2 appears (Figure **5**). Since lysine 240 is the only charged residue in the sequence after glutamic acid 232, it has to be responsible for the charge difference upon carboxypeptidase treatment. This is in agreement with the protein sequencing data.

Apart from PSL2 a second band appears. This has been described as a "hybrid" band [**3**], and probably consist of dimers with one PSL1 and one PSL2 monomer, as a result of partial processing. This hybrid PSL molecule can also be isolated from seeds by chromatofocusing (data not shown), and is therefore not an artefact of iso-electric focusing.

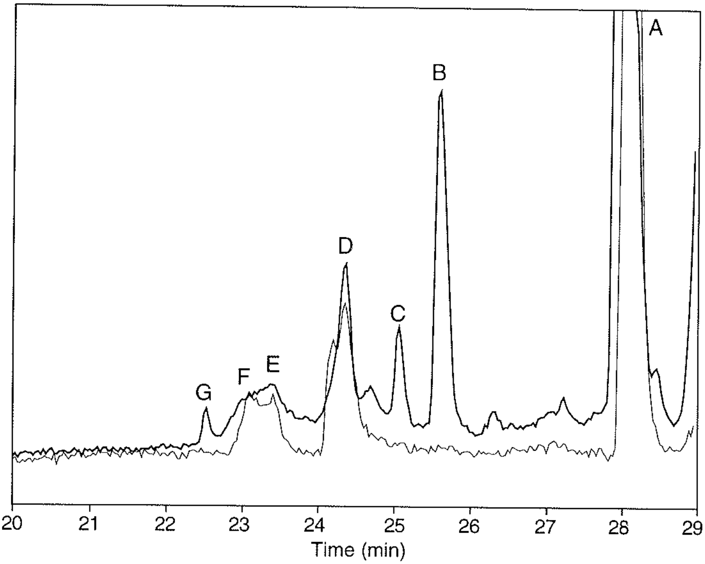

Figure 4. HPLC elution pattern of the C-terminal peptides of the α-subunits of PSL from seeds (indicated by a thick line) and roots. The seed peptides represent a mixture of PSL1- and PSL2-derived peptides. The sequences of the peptides are: **A** = **YAAHE** (an internal α-subunit fragment); **B** = **LSGTSSSKQ**; **C** = **LSGTSSSK**; **D** = **LSGTSSS**; **E** = **LSGTSS**; **F** = **LSGTS**; **G** = **LSGT**

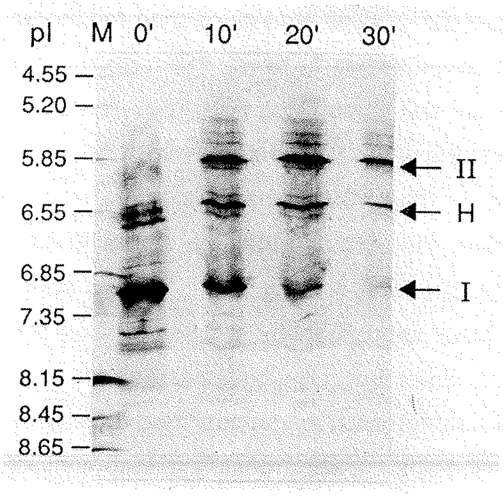

Figure 5. IEF gel, showing conversion of PSL1 into PSL2 by carboxypeptidase treatment. Aliquots are taken at T=0, 10, 20 and 30 minutes. The pI of the marker proteins (M) is indicated. **I** = PSL1; **H** = Hybrid PSL (see text for further explanation); **II** = PSL2.

DISCUSSION

From our results, it can be concluded that root PSL is encoded by the same gene as both seed isolectins, and that processing of PSL in seeds and roots is similar, if not identical. The presence of different C-termini is an indication that a carboxypeptidase is responsible for the post-translational processing of PSL, resulting in two different isolectins. The isolectins only differ in charge because of the presence or absence of lysine 240, a positively charged amino acid. The carboxypeptidase activity probably occurs after cleaving off of the C-terminal tetrapeptide Ala-Ala-Asp-Ala. It is possible that the protease responsible for the processing at Asn 181 and Asn 187 also cleaves the protein at Gln 241, since Asn and Gln are similar amino acids. Serine-type carboxypeptidases have been described in a large number of plant species, including legumes [16]. Their expression has been shown to be induced upon germination, and they play an important role in the processing of seed storage proteins [5,16]. It also has been shown that a similar processing activity is present in potato leaves [6]. The appearance of carboxypeptidase activity in protein bodies of germinating seeds may coincide with disappearance of PSL1 from these seeds. Conversion of PSL1 into PSL2 may be interrupted as the seeds reach maturation, after which the processing of PSL is completed upon germination.

Root PSL is identical with seed PSL2, and processing in the roots apparently is identical to that in seeds. The carboxypeptidase activity needed for processing is therefore not only confined to protein bodies in seed. PSL present in other vegetative organs of the pea plant has the same pI as root PSL. The transcript found in buds from pea plants [17] shows only 38% homology with psl, therefore it definitively encodes another protein than PSL. It has yet to be established whether or not it represents an active, that is sugar-binding, lectin. In *Lathyrus ochrus* L. (sweet pea), a species closely related to pea, two isolectins have been identified, LOL I and LOL II. They have approximately the same pI as the PSL isolectins (7.2 and 6.0) [20]. These isolectins, however, are encoded by two different genes, and they differ from each other at seven positions throughout the sequence [20,28]. Therefore, a post-translational processing step can not be responsible for the observed charge difference, but nevertheless the observed charge difference is exactly the same. Because the same isolectin pattern occurs in sweet pea, maybe the charges of the two isolectins are important and somehow play a role in their function, even if both isolectins have the same sugar specificity. On the other hand, in seeds of *Lens culinaris*, another closely related species, three isolectins with pI's of 8.65, 8.45, and 8,15 are found [3]. The above described findings support the idea that the introduction of the psl gene in transgenic clover roots results in the production of the correct PSL molecule, which is involved in the *Rhizobium*-legume symbiosis [4].

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CHAPTER 3

DESTABILISATION OF PEA LECTIN BY SUBSTITUTION OF A SINGLE AMINO ACID IN A SURFACE LOOP

Flip J. Hoedemaeker, Ron R. van Eijsden, Clara L. Díaz, B. Sylvia de Pater and Jan W. Kijne.

Appeared in: Plant Molecular Biology, vol 22, pp 1039-1046 (1993).

ABSTRACT

Legume lectins are considered to be anti-nutritional factors (ANF) in the feeding industry. Inactivation of ANF is an important element in processing of food. In our study on stability of *Pisum sativum* L. lectin (PSL), a conserved hydrophobic amino acid (Val^{103}) in a surface loop was replaced by alanine. The mutant lectin, PSL V103A, showed a decrease in the unfolding temperature (Tm) of approximately 10°C in comparison with wild type (wt) PSL, and the denaturation energy (ΔH) is only about 55% of that of wt PSL. Replacement of an adjacent amino acid (Phe^{104}) by alanine did not result in a significant difference in stability in comparison with wt PSL. Both mutations did not change the sugar-binding properties of the lectin, as compared with wt PSL and with PSL from pea seeds, at ambient temperatures. The double mutant, PSL V103A/F104A, was produced in *Escherichia coli,* but could not be isolated in an active, that is sugar-binding, form. Interestingly, the mutation in PSL V103A reversibly affected sugar-binding at 37°C, as judged from haemagglutination assays. These results open the possibility of production of lectins that are active *in planta* at ambient temperatures, but are inactive and possibly non-toxic at 37°C in the intestine of mammals.

INTRODUCTION

Lectins of leguminous plants are among the best studied carbohydrate-binding proteins [29,30]. The structures of a number of legume lectins are known [1,9,10,26,28,30]. The lectin of *Pisum sativum* L. (PSL) is a ß-barrel protein [27] and is organised as a dimer, like the other lectins from the Vicieae tribe of the Leguminosae family [30]. Both monomers are post-translationally processed into a small α- and a larger ß-chain. The mature protein therefore has an $\alpha_2ß_2$ configuration [15]. The overall three-dimensional structure of monomers of other legume lectins is very similar to that of the PSL monomer, in spite of differences in processing and multimerisation.

PSL is present in high amounts in seeds, whereas pea roots contain a very low amount of PSL [5]. In roots, PSL is involved in the host-specific symbiosis with *Rhizobium bacteria* [6]. The function of lectin in seeds is not known, but it has been hypothesized that lectin can protect seeds against herbivorous animals [2, 4, 25]. In the feeding industry, leguminous lectins are considered to be anti-nutritional factors (ANF). Lectins have been shown to bind to gut epithelial cells of pigs feeding on meal from raw legume seeds, and to cause damage to the intestine [14, 16, 17, 25]. This is especially true for *Phaseolus vulgaris lectin* (PHA), whereas other lectins, like PSL, seem to be less toxic [17]. The adhesion of lectins to epithelial cells [14] suggests that the sugar-binding activity of lectin is required for toxicity, but this has not been conclusively demonstrated.

In order to eliminate this adverse activity of lectins, legume seeds have to be heat-treated for a considerable period of time. Apparently, lectin is toxic in its native state, and toxicity is lost upon irreversible denaturation of the protein. A standard heat treatment is steam heating for 40 min at 104°C and 19% moisture, so-called toasting [17]. Toasting requires energy and reduces the nutritional value of legume seeds and seed meal, making the use of legume seed meal as a fodder for pigs economically unfavourable.

We have started site-directed mutagenesis experiments aimed at destabilisation of legume lectins, in order to produce lectins which are active *in vivo*, but are easily inactivated *in vitro*, thereby eliminating their anti-nutritional effects. As a model lectin, we have chosen PSL, because (1) it is a well studied legume lectin, (2) only one functional copy of the lectin gene is present in pea plants [**13, 19**] (see also chapter **2**), and (3) an assay for testing the role of PSL in nodulation is available [**6**]. We have chosen to substitute amino acids that are conserved throughout most other legume lectins, to enable extrapolation of our results to other lectins.

Our primary attention was drawn to a large surface loop of PSL (see Figure **1A** and Table **1**). This loop consists of the amino acids 87-115 of the ß-chain [**9**]. The -NH group of Glycine 99 forms an essential part of the monosaccharide-binding site [**3, 32**]. The loop is twisted, and the amino acids at the position of this twist are conserved in all legume lectins (amino acids 101-104 in PSL). The same loop is present in jackbean lectin (CONA), but it is discontinuous due to a circular permutation (Figure **1B**). In spite of this processing, the overall configuration of the loop including the conserved box is the same as that in PSL. A closer examination

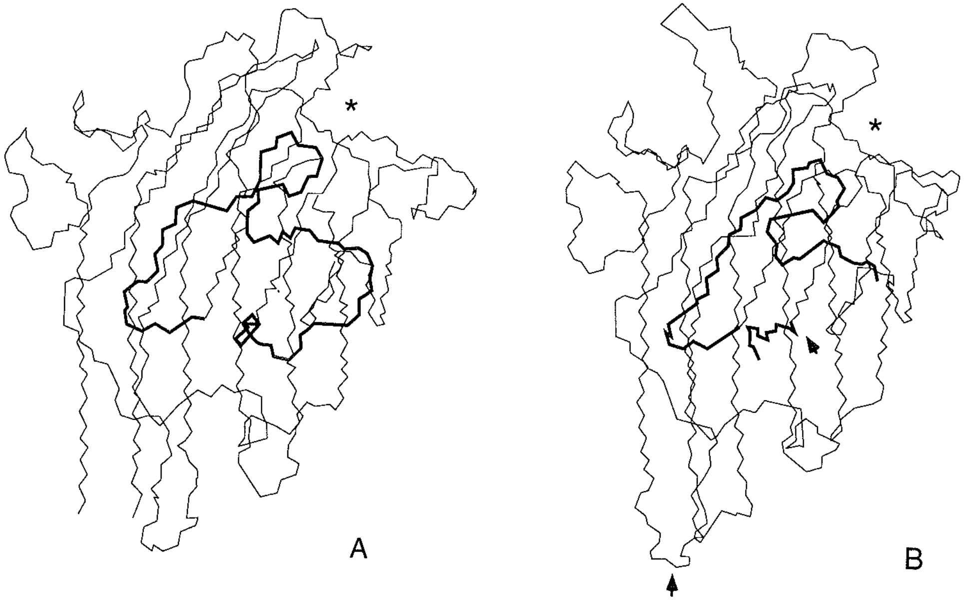

Figure 1. Three-dimensional structures of monomers of PSL (A) and CON A (B). The surface loop of both molecules is indicated with a thick line. Arrows indicate the processing sites involved in the circular permutation of CON A. An asterisk indicates the location of the sugar-binding site in both molecules [**1,10**].

of the amino acids in the conserved box reveals that their hydrophobic sidechains point downwards, contacting residues in the upper ß-pleated sheet (Figure **2**). Val^{103} has contacts with Phe^{86}, Thr^{84}, and Ser^{212}. Phe^{104}, apart from interactions with other residues in the loop itself, has contacts with Thr^{117}. The amino acids in this conserved box can therefore be important for the conformation and stability of the surface loop.

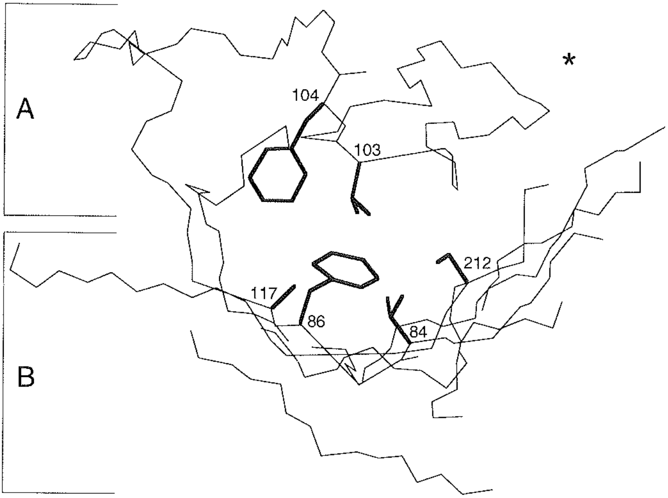

Figure 2. The positions of Val^{103} and Phe^{104} in detail. The C-α tracing of the surface loop (A) and the upper ß-pleated sheet (B) is shown. The approximate position of the sugar-binding site is indicated with an asterisk. Relevant amino acids are numbered, and their sidechains are depicted with thick lines. Val^{103} in the loop interacts with Phe^{86}, Thr^{84}, and Ser^{212} in the ß-pleated sheet. Phe^{104} only interacts with Ala^{117} in the ß-pleated sheet, but has additional interactions with other residues in the loop.

During the last five years, much progress has been made in correctly predicting the effect of point mutations in proteins [**12**]. It has been possible to draw a few general conclusions from earlier site-directed mutagenesis experiments. Protein stability depends on differences in entropy and enthalpy between the folded and unfolded state [**18**], and it has been shown that (1) the removal of bulky apolar sidechains from the hydrophobic core of the protein usually results in destabilization, because of loss of van der Waals contacts and of the possibility of water molecules to enter hydrophobic cavities [**11**]; (2) introduction of rigid structures, such as prolines or disulphide bridges, at suitable sites usually stabilizes proteins, because the entropy of the unfolded protein is decreased [**22, 23**]; (3) the net difference in the total number of hydrogen bonds between amino acids in the folded and in the unfolded molecule is an important factor determining protein stability [**8, 33**].

We hypothesized that removal of the hydrophobic sidechains of amino acids in the conserved box, by replacing the amino acids by alanine residues, would result in a loss of the hydrophobic contacts described above and/or in an increase in the flexibility of this loop. Both effects would destabilize the lectin molecule, possibly interfering with the sugar-binding activity of the lectin at elevated temperatures.

	87															100																115
PSL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y	**L**	**G**	**V**	**F**		N	S	A	E	Y	D	K	T	T	Q	T
LOL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y	**L**	**G**	**V**	**F**		N	S	K	D	Y	D	K	T	S	Q	T
LCL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y	**L**	**G**	**V**	**F**	Y	N	G	K	E	Y	D	K	T	S	Q	T
SBA	L	A	P	I	D	T	K		P	Q	T	H		A	G	Y	**L**	**G**	**L**	**F**		N	E	N	E			S	G	D	Q	V
DBA	L	V	P	V	G	S	E		P	R	R	N			G	Y	**L**	**G**	**V**	**F**	D	S	D	V	Y	N	N	S	A	G	Q	T
LTA	L	A	P	V	G	T	E	I	P	D	D	S	T	G	G	F	**L**	**G**	**I**	**F**	D	G	S				N	G	F	N	Q	F
PHA-L	L	V	P	V	G	S	Q		P	K	D	K		G	G	F	**L**	**G**	**L**	**F**	D	G	S				N	S	N	F	H	T
PHA-E	L	L	P	V	G	S	Q		P	K	D	K		G	G	L	**L**	**G**	**L**	**F**	N	N	Y	K		Y	D	S	N	A	H	T
SL	L	A	P	T	D	T	Q		P	K	S		G	G	G	Y	**L**	**G**	**I**	**F**		K	D	A	E	S	N	E	T			V
LBL	L	V	P	V	D	S	Q		P	K	K	K		G	R	L	**L**	**G**	**L**	**F**	N	K	S		E	N	D	I	N	A	L	T
DL	I	A	N	T	D	T	S	I	P	S	G	S	G	G	R	L	**L**	**G**	**L**	**F**	P		D	A	N	A	D		T			I
CONA	I	S	N	I	D	S	S	I	P	S	G	S	T	G	R	L	**L**	**G**	**L**	**F**	P		D	A	N	A	D		T			I
ECORL	M	G	P	T	K	S	K		P	A	Q		G	Y	G	Y	**L**	**G**	**I**	**F**	F	N	S	K	Q		D	N	S	Y	Q	T

Table 1. Comparison of the primary sequences of the surface loop of 14 different legume lectins [32]. Numbering is according to the PSL sequence. The conserved box is printed in bold, and the residue involved in sugar-binding is underlined. Abbreviations used: PSL = *Pisum sativum* lectin; LOL = *Lathyrus ochrus* lectin; LCL = *Lens culinaris* lectin; VFL = *Vicia faba* lectin (favin); SBA = *Glycine max* (soybean) lectin; DBA = *Dolichos biflorus* lectin; LTA = *Lotus tetragonolobus* lectin; PHA = *Phaseolus vulgaris* lectin (Phytohaemagglutinin); SL = *Onobrychis viciifolia* (sainfoin) lectin; LBL = *Phaseolus limensis* (lima bean) lectin; DL = *Dioclea grandiflora* lectin; CONA = *Canavalia ensiformis* lectin (Concanavalin A); ECORL = *Erythrina corallodendron lectin*

MATERIALS AND METHODS

Bacterial strains

E.coli strain DH5αF$^+$ (supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used for expression of wild type (wt) and mutant lectin genes and for the production of PSL. Bacteria were grown in Luria Complete (LC) medium at 37°C (Chapter **6**).

Cloning and side-directed mutagenesis of PSL

psl cDNA [**31**] was modified as described before (See chapter **6**) by introduction of an extra EcoRI restriction site, resulting in an almost complete removal of the signal sequence, and was cloned as an EcoRI/HindIII fragment into pUC 18 [**34**], to produce the expression vector pMP 2809. This resulted in the production of unprocessed PSL containing 5 extra amino acids at the N-terminus, i.e. three residues from the pUC 18 sequence and two from the original signal peptide. The N-terminus of the produced lectin was checked using protein sequencing by automated Edman degradation on a Applied Biosystems 477A Protein Sequencer. This molecule was designated wt PSL (Chapter **6**).

Mutations were introduced by use of the polymerase chain reaction (PCR), as described in chapter **6**. For every mutation, an 89 bp EcoRV/BamH1 fragment from pMP 2809 was amplified, using one mutagenic and one non-mutagenic primer. The codon for Val^{103}, GTT, was changed into GCT (coding for alanine) to produce V103A. The codon for Phe^{104}, TTC, was changed into GCC (also coding for alanine), to produce F104A. The double mutant was made by combining both mutations in a single mutagenic primer. PCR products were cloned and sequenced using sequenase 2.0^{tm} (USB, Cleveland, Ohio, USA). After DNA sequence analysis, the wt EcoRV/BamH1 fragment from pMP 2809 was replaced by the fragments containing the mutations, yielding pMP 3203, pMP 3204, and pMP 3211 respectively.

Isolation of PSL from E.coli

Isolation of PSL from *E.coli* was performed as described before [**24**], with some modifications: *E.coli* DH5αF$^+$ cells, harbouring pMP 2809 or one of its derivatives, were grown in 2L LC at 37°C, containing 100 μg/ml carbenicillin, and were induced at mid-exponential phase by adding IPTG (isopropyl-ß-D-thiogalactopyranoside; Boehringer, Mannheim) to the medium to a final concentration of 0.5 mM. After induction, the cells were grown for an additional 16 h at 37°C, harvested and washed in TBS (10 mM Tris-HCl, pH 6.8, containing 150 mM NaCl). All further steps were performed at 4°C, unless stated otherwise. The cells were resuspended in 25 ml TBS containing 0.5 mM PMSF (phenylmethylsulphonylfluoride) and lysed in a French pressure cell (American Instrument Company, Silver Spring, MD, USA), at a pressure of 20,000 PSI. Inclusion bodies containing PSL were collected by centrifugation for 30 min at 15,000 rpm. The protein was denatured overnight in TBSm (TBS containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$) in the presence of 7M guanidine-HCl. Membranes and remaining aggregates were removed by ultracentrifugation for 1 h at 175,000 x G. The supernatant was quickly diluted 25-fold in TBSm containing 1.5 M urea at 0°C. The proteins were allowed to refold during at least 24 h, after which the solution was dialysed extensively against deionised H_2O and lyophilized. Finally, the lyophilized proteins were redissolved in TBSm and purified by affinity chromatography at room temperature on Sephadex G-75 (Pharmacia, Uppsala, Sweden) in TBSm [5].

SDS-PAGE of PSL fractions was performed with a 15% running gel according to Lugtenberg *et al* [21]. After running, the gels were blotted onto PVDF membrane (Millipore, Bedford, MA, USA). Subsequently, immunochemical staining with the use of polyclonal anti-PSL antibodies was performed according to Díaz *et al* [5].

Haemagglutination assays

The haemagglutination assay used to test the sugar-binding ability and -specificity of PSL was described before [20]. In order to test stability, a PSL solution of 250 $\mu g.ml^{-1}$ in TBSm was incubated at 70°C. Aliquots were taken at 5 min intervals and placed on ice. Subsequently, these samples were tested in the haemagglutination assay for residual activity. In order to try to distinguish between reversible and irreversible inactivation, haemagglutination assays were performed at different temperatures (28°C, 37°C, 45°C and 55°C) instead of 20°C.

Differential Scanning Calorimetry (DSC)

In order to determine the denaturation temperature (Tm) and -energy (ΔH) of PSL, a Mettler TA-300 DSC apparatus, coupled to a TC-10 detector, was used. The temperature scan was carried out over a range from 5 to 100°C, at a scan rate of 10°C.min^{-1}. The samples contained 80% (w/w) H_2O.

RESULTS

Production of wt and mutant PSL in E.coli.

Plasmid pMP 2809, containing psl cDNA, was used as a template for site-directed mutagenesis. Introduction of the mutations V103A, F104A, and V103A/F104A in pMP 2809 yielded plasmids pMP 3203, pMP3204, and pMP 3211, respectively. Each of these plasmids was expressed in *E.coli*. We could typically isolate 10-20 mg of affinity-purified wt PSL from a 2L *E.coli* culture. The yield of PSL F104A was similar to that of wt PSL. However, PSL V103A was much more difficult to obtain, since only about 2 mg could be isolated from a 2L culture. The double mutant, PSL V103A/F104A, was also produced in *E.coli*, but could not be isolated in an active form by affinity chromatography. Wt and mutant PSL monomers all have an apparent molecular weight of about 28 kD as judged from SDS-PAGE (Figure **3**),

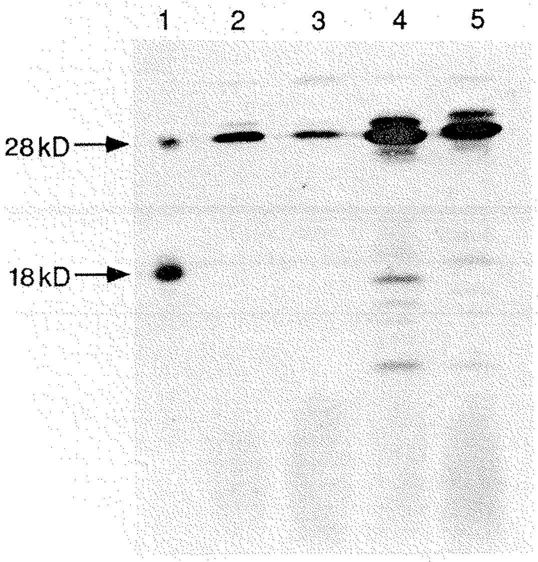

Figure 3. Immunoblot of crude *E.coli* extracts containing PSL. Lane 1: Marker (Pea seed lectin).Lane 2: wt PSL. Lane 3: PSL V103A. Lane 4: PSL F104A. Lane 5: PSL V103A/F104A. The polyclonal antiserum used does not react with the 6 kD α-subunit present in lane 1.

corresponding with that from unprocessed PSL from pea seeds. Unprocessed PSL isolated from *E.coli* has similar properties compared to the processed seed PSL [**24,31**] (see also chapter **6**). The molecular weights of native wt and mutant PSL, as judged from gel filtration experiments, appeared to be about 55 kD (data not shown). From this result it can be concluded that the introduced mutations did not affect PSL dimerisation.

Haemagglutination assays.

Wt PSL, PSL V103A, and PSL F104A all agglutinated a 2% suspension of human A^+ erythrocytes down to a concentration of about 16 μg/ml (data not shown). No significant differences in sugar-binding properties of wt and mutant PSL could be observed: mannose inhibits haemagglutination at a minimal concentration of 3 mM, glucose inhibits haemagglutination at a minimal concentration of 12.5 mM, and galactose does not inhibit haemagglutination at a concentration as high as 250 mM. (data not shown). Since four loops contribute to the sugar-binding activity of PSL [**3,32**], the haemagglutination results indirectly demonstrate that wt and mutant PSL are properly folded.

After incubation of PSL for various periods at 70°C, residual agglutination activity was determined. Wt PSL and PSL F104A lost their activity after incubation for 20-25 min at 70°C, whereas PSL V103A was already inactivated after incubation for 5 min at 70°C. In all cases, inactivation by prolonged incubation at 70°C appeared to be irreversible, since activity was not restored by subsequent incubation on ice for up to 30 min (Figure **4a**).

By assaying haemagglutination at elevated temperatures, it could be demonstrated that PSL V103A remains active at 28°C, but that the activity of this mutant is lost at 37°C (Figure **4b**). PSL F104A was still active at 37°C, but activity diminished at 45°C. Wt PSL was completely active at 45°C, but activity was lost at 55°C. Inactivation at these temperatures is reversible upon cooling on ice (data not shown). Assaying haemagglutination at these temperatures did not have a visible effect on the erythrocytes used.

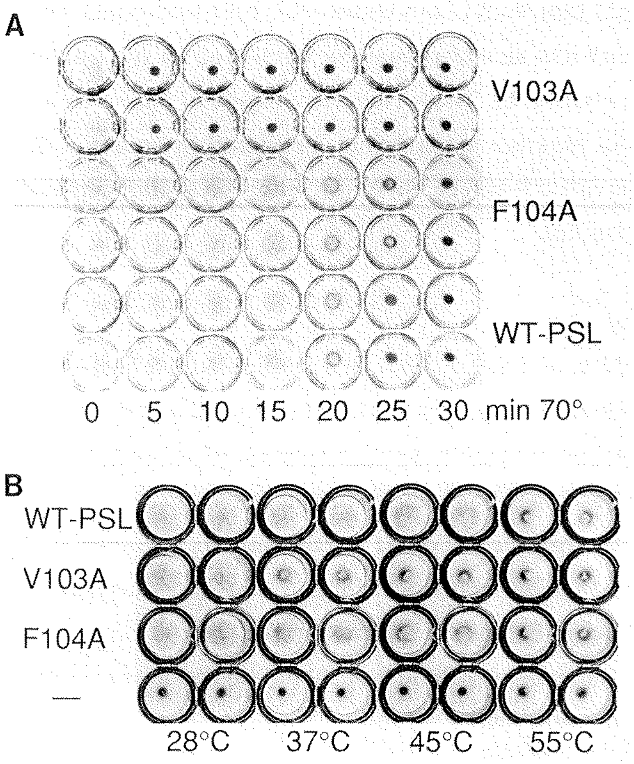

Figure 4. Haemagglutination assays: PSL agglutinates erythrocytes. When PSL is absent or inactive, the erythrocytes precipitate on the bottom of the well. **A)** Inactivation at 70°C of wt and mutant PSL. Measurements were done in duplo. **B)** Haemagglutination assay at elevated temperatures. Incubation temperatures are indicated, the measurements are done in duplo. The PSL concentration is 250 $\mu g.ml^{-1}$, in the last row no PSL is added.

Differential Scanning Calorimetry (DSC).

The denaturation temperature (Tm) and denaturation energy (ΔH) of wt PSL and the two mutants were determined using DSC, as summarized in Table 2. The DSC curves are shown in Figure 5. Tm and ΔH of seed PSL are not significantly different from the Tm and ΔH of wt PSL isolated from *E.coli*. The Tm of PSL V103A is approximately 11°C lower than that of wt PSL, and ΔH is only about 55% of ΔH of wt PSL. PSL F104A does not show a significant difference in Tm or ΔH when compared with wt PSL. These results corroborate the results of the haemagglutination experiments.

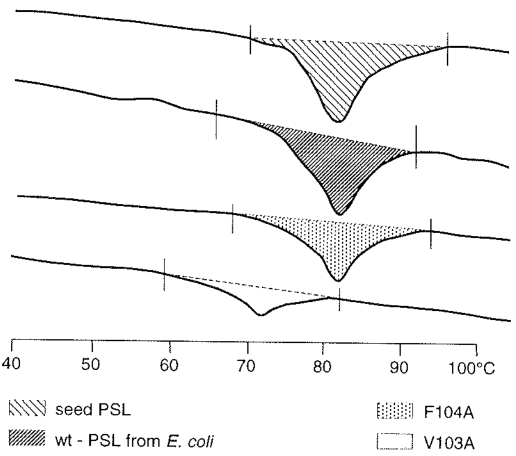

Figure 5. DSC patterns of wt and mutant PSL. The peaks indicate the denaturation temperature (Tm), the area under the curves represents the total denaturation energy (ΔH). See also Table 2.

	seed PSL	wt PSL *E.coli*	F104A	V103A
Tm (°C)	83.1 ± 0,6	82.3 ± 0.7	81.8 ± 0.5	71.4 ± 1.0
ΔH (J/g)	12,1 ± 0.2	11.9 ± 0.4	10.2 ± 1.1	6.2 ± 0.3

Table 2. Comparison of the stability of wt and mutant PSL.

DISCUSSION

Val^{103} and Phe^{104} are hydrophobic amino acids in a surface loop of PSL. The sidechains of both amino acids point inwards, making contacts with residues in the underlying ß-pleated sheet. Substitution of Val^{103} by Ala dramatically decreases the stability of PSL. Apparently, the loss of hydrophobic contacts between this residue and residues in the underlying ß-sheet is responsible for this decrease in stability. The properties of this mutant lectin at ambient temperatures are not changed, despite the fact that this residue is located close to the sugar-binding site.

The DSC patterns presented in Figure 5 show single denaturation peaks in wt and both mutant lectins. This implies that the mutation in PSL V103A facilitates total denaturation of the protein. A local effect on the conformation of the surface loop could not be detected in this experiment.

Performing the different haemagglutination experiments described above, one can make a distinction between reversible and irreversible inactivation of PSL. Reversible inactivation of a PSL solution in TBSm occurs at temperatures between 28 °C and 55 °C, and irreversible inactivation occurs between 60 °C and 80 °C. PSL V103A is less stable than wt PSL or PSL F104A. PSL F104A is slightly less stable than wt PSL at 45 °C, but at higher temperatures this difference is not found. Irreversible inactivation is likely to coincide with complete denaturation of the protein, whereas reversible inactivation probably can be attributed to local conformational changes in the surface loop, either causing local denaturation of this loop, or causing an decrease in sugar-binding affinity.

Replacing Phe^{104} by alanine does not have a large affect on the stability of PSL. This finding demonstrates that the hydrophobic contacts of Val^{103} with amino acids in the underlying ß-sheet are more important for the stability of PSL than those of Phe^{104}. It remains unclear why Phe^{104} is also highly conserved among legume lectins.

The refolding of denaturated PSL seems to require very specific circumstances. This phenomenon is especially encountered upon renaturation of PSL from *E.coli* inclusion bodies, and it would also explain why PSL V103A is more difficult to isolate than wt lectin, and why the double mutant, V103A/F104A, can not be isolated in an active form at all.

We have succeeded in producing a mutant PSL that retains its activity at ambient temperatures, but seems to lose its activity at 37 °C. This mutant therefore should still be toxic at ambient temperatures [2,4], but will probably be inactivated at the elevated temperatures in the intestine of pigs, and maybe will have lost its toxicity to these animals.

Our study confirms the feasibility of using the simple rules mentioned above to change the stability of proteins in a wide variety of research areas. This mutation can be introduced into other legume lectins and will probably have a similar effect. The results may be applicable for inactivation of other anti-nutritional proteins in which a loop, containing a conserved box of hydrophobic amino acids, forms part of the active site.

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CHAPTER 4

DESTABILISATION OF *PHASEOLUS VULGARIS* LECTIN (PHA-L) BY SUBSTITUTION OF A CONSERVED HYDROPHOBIC AMINO ACID IN A SURFACE LOOP

Flip J. Hoedemaeker, B. Sylvia de Pater, Jan W. Kijne.

Abstract

In *Pisum sativum* (pea) lectin, PSL, a large surface loop contributing to the sugar-binding site is anchored to the core of the protein by a conserved box of amino acid residues, including valine 103. Substitution of this residue with alanine results in a decrease in denaturation temperature as well as in denaturation energy compared to wild type PSL [Chapter 3]. Introduction of the corresponding mutation, L108A, in the leucocyte-specific *Phaseolus vulgaris* (common bean) lectin, PHA-L, has a similar effect on stability without affecting sugar-binding activity. Our results suggest that this particular mutation can be made in every legume lectin with a predictable destabilizing effect.

Introduction

In the feeding industry, lectins from leguminous plants are considered to be anti-nutritional factors (ANF) [6,11,17]. Lectins have been shown to bind to gut epithelial cells of rats and pigs feeding on meal from raw legume seeds, and to cause unwanted effects in the intestine [1,8,11,14,17]. Toxicity of legume lectins for insects has been reported as well [2,16]. The characteristics of adhesion of lectins to epithelial cells [14,17] suggest that sugar-binding activity of lectin is required for its toxicity. It has been shown that loss of sugar-binding activity in *Phaseolus vulgaris* (common bean) lectin, PHA-L, results in a loss of mitogenic activity towards human lymphocytes [15].

In order to eliminate the adverse activity of lectins, and other anti-nutritional factors, legume seed meal has to be heat-treated for a certain period of time [1,11]. Heat treatment requires energy and may reduce the nutritional value of legume seeds and seed meal. Previously, we have described the destabilisation of the lectin from *Pisum sativum* (pea), PSL, by introducing a mutation in a surface loop contributing to the sugar-binding site [Chapter 3]. As a result, PSL mutants were produced which retain their sugar-binding activity *in vivo*, but which are easily inactivated *in vitro*. PSL was used as a model lectin, because (i) it is encoded by a single gene [5,12, Chapter 2], (ii) it is not glycosylated, and (iii) the three-dimensional (3D) structure of the protein has been established [3,4]. Since common bean lectin, PHA, is much more toxic than PSL, destabilisation of PHA is more appropriate for the improvement of the nutritional value of legumes.

In bean, two lectin genes are present which are highly homologous and are closely linked [9]. One of these genes, dlec1, encodes a lectin capable of agglutinating erythrocytes (PHA-E), while the other, dlec2, encodes a leucocyte-specific lectin (PHA-L). Together, five different tetrameric lectins can be formed, PHA-E_4, PHA-E_3L_1, etc. Each of these lectins is glycosylated. Sugar-binding activity of PHA can not be inhibited by addition of simple mono- or disaccharides, unlike that of PSL [7]. The 3D structure of PHA has not been established yet. However, comparison of known structures of various legume lectins shows that the overall 3D structure of their monomers is very similar, in spite of differences in processing and multimerisation. Legume lectins are so-called ß-barrels [18], and are organised as dimers or tetramers [**19,20**]. It is most probable that general properties of the sugar-binding sites and contributing loops in PSL and PHA are similar.

Here we report on introduction in PHA-L of the mutation corresponding to the destabilizing mutation V103A in PSL. This mutation, L108A, appeared to have a similar effect on protein stability. We selected PHA-L rather than PHA-E because (i) the corresponding gene was available, and (ii) it has been shown previously that the structural information obtained by site-directed mutagenesis of the sugar-binding site of PSL can be extrapolated to PHA-L [Chapter **6**, ref **15**].

Site-directed mutagenesis of PHA.

The coding region of dlec2, encoding PHA-L, was modified by PCR as described before for the psl gene [Chapters **3,6**], and was cloned into pUC 18, to produce the expression vector pMP 3302. From this construct, PHA was produced in *E.coli*, most probably in a non-glycosylated form and containing 5 extra amino acids at the N-terminus, that is three residues from the pUC 18 sequence and two from the original signal peptide. This recombinant protein was designated wild type (wt) PHA.

The mutation leucine 108 to alanine (see Table 1 in Chapter **3**) was introduced with a two-step PCR procedure. A 150 bp PpuMI fragment containing the desired mutation was used to reconstruct the dlec2 gene. This mutant PHA will be referred to as PHA L108A.

Isolation and purification of PHA from *E.coli*

Crude wt PHA and PHA L108A were isolated from *E.coli* as described for PSL in Chapters **3** and **6**. The production levels of wt PHA and PHA L108A in *E.coli* are similar to those described earlier for wt and mutant PSL. For preparation of purified wt PHA and PHA L108A, the isolation procedure was changed: guanidine in the denaturation buffer was replaced by the same amount of urea. Next, PHA was purified using a porcine thyroglobulin column for sugar-binding affinity chromatography [**15**](Figure 3).

Figure 1 shows a Western blot with crude wt and mutant PHA isolated from *E.coli*, in comparison with a commercial PHA-L preparation. For this blot, polyclonal antibodies directed against PSL have been used (kindly supplied by Clara Díaz) [Chapter 6]. Monomers of wt PHA and PHA L108A have an apparent molecular weight of about 28 kD, which is in agreement with the calculated MW of 27,9 kD. Commercial PHA-L is slightly larger (33 kD), because it is glycosylated [13]. By gel filtration analysis we were able to show that the introduced mutation did not affect PHA-L multimerisation: both wt PHA and PHA L108A appeared to refold as tetramers after denaturation in 7M guanidine (data not shown). Isolation of tetrameric PHA from *E.coli* has been described before [10]. Purified wt PHA and PHA L108A each show a single band on SDS-PAGE with the correct molecular weight (data not shown).

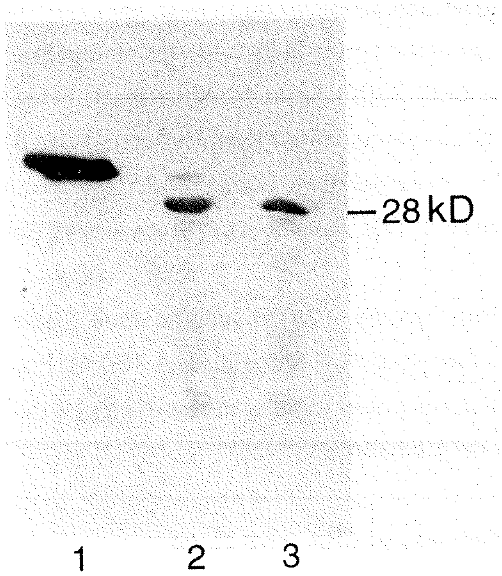

Figure 1. Western blot showing crude wt and mutant PHA, isolated from *E. coli*, compared with commercial PHA-L. Lane 1: Commercial PHA-L; Lane 2: wt PHA; Lane 3: PHA L108A.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetric studies have been performed on crude as well as on affinity-purified preparations of wt PHA and PHA L108A from *E.coli*. The calorimetric scans of crude wt PHA and PHA L108A are shown in Figure 2a. The denaturation temperatures and -energy values are listed in Table 1. Substitution in

PHA of leucine 108 for alanine causes a decrease in denaturation temperature of about 10°C. The energy required to denature PHA L108A is also considerably lower, approximately 60% of the energy needed to denature wt PHA.

	wt PHA		PHA L108A		comm. PHA-L
	crude	pure	crude	pure	
denaturation temp. (°C)	103.1	102.1	94.3	89.7	94.7
denaturation energy (J/g)	2.3	11.5	1.4	7.1	26.5

Table 1. Denaturation temperatures and -energies of crude and purified wt and mutant PHA, as deduced from DSC scans.

These results corroborate earlier findings with PSL, described in Chapter 3. The denaturation energy values for the purified proteins are very similar: 11.5 J/g for wt PHA and 11.9 J/g for wt PSL; 7.1 J/g for PHA L108A and 6.2 J/g for PSL V103A. However, the denaturation temperatures for PHA are significantly higher than those for PSL. Wt PSL denatures at about 83°C, whereas wt PHA does not denature until the temperature reaches 103°C. At present, this large difference is not yet understood, but it has to be noted that the amino acid identity of the two lectins is only 49.8%. Furthermore, up to now the three-dimensional structure of PHA has not been determined. Study of a structural model of PHA is required to find an explanation for the differences in stability between the two lectins. Nonetheless, the effect of the mutation in both purified lectins is the same: a decrease in denaturation temperature of about 12%. The observed denaturation energies for crude wt PHA and PHA L108A are much lower than those recorded for the purified proteins. A possible explanation is that crude PHA samples from *E.coli* consist for a major part of improperly folded lectin, which is removed by affinity chromatography.

The DSC scan of commercially obtained PHA-L (purchased from Boehringer, Mannheim, Germany), as shown in Figure 2b, yielded a denaturation temperature

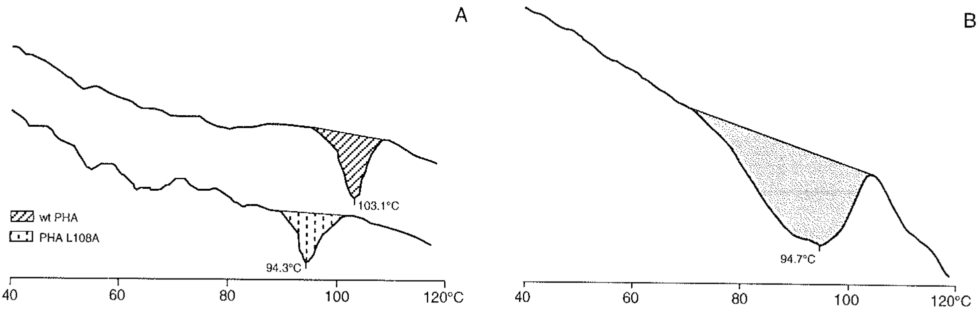

Figure 2. Differential Scanning Calorimetry (DSC). **A)** Comparison of the DSC scans from crude wt PHA and PHA L108A preparations. A TA-Instruments 2910 DSC apparatus was used. The temperature scan was carried out over a range from 10 to 120°C, at a scan rate of 5°C.min^{-1}. The samples contained 80% (w/w) H_2O. **B)** DSC scan of commercial PHA-L.

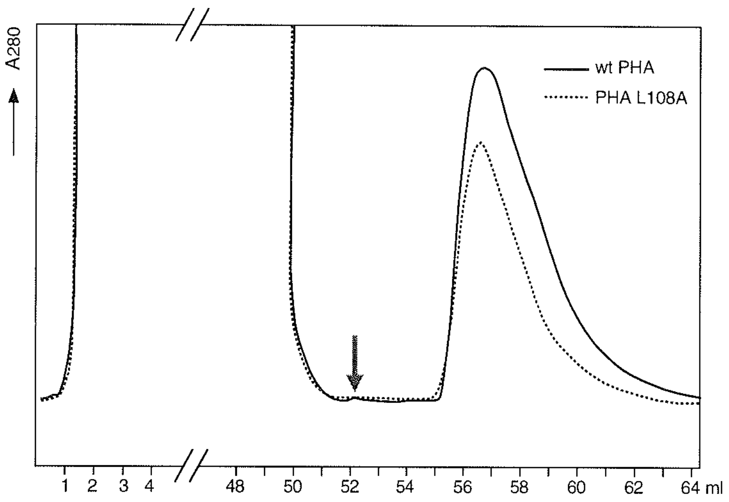

Figure 3. Affinity chromatography of wt PHA and PHA L108A on a column of porcine thyroglobulin coupled to Sepharose (Pharmacia, Uppsala, Sweden). Bound protein is eluted from the column by lowering the pH of the buffer from pH 7.3 to pH 2.7, at the point indicated by the arrow. Collected fractions were neutralized immediately by adding 10% (v/v) 1M Tris-HCl pH 9.0.

of 94.7°C. This value is closer to the denaturation temperature of the mutant than to that of wt PHA. The denaturation energy value however is much higher, more than twice the value for PSL from pea seeds [Chapter 3]. Since PHA from bean seeds is glycosylated with a high-mannose glycan and a complex glycan at N32 and N80, respectively [13], this glycosylation may cause the need for more energy to denature PHA in comparison with the non-glycosylated seed PSL and with the non-glycosylated lectins produced in *E.coli*. It is unclear whether glycosylation is responsible for a slight decrease in denaturation temperature.

In summary, we have demonstrated that introduction of the mutation L108A, in the leucocyte-specific common bean lectin PHA-L, has an effect on protein stability similar to that of the corresponding mutation V103A in PSL, in both cases without affecting sugar-binding activity. Our results suggest that this particular mutation can be made in every legume lectin with a predictable destabilizing effect, also in the absence of a 3D model.

Reversible denaturation of PHA

PSL V103A showed a reversible loss of hemagglutinating activity at 37°C in Tris-buffered saline [Chapter 3]. PHA L108A has not yet been tested for reversible inactivation yet, but we predict that this will occur at a higher temperature, in view of the apparent larger stability of PHA. This would imply that PHA L108A is still active at 37°C in the intestine of pigs, and that further destabilization of PHA will be necessary to eliminate its toxic effects. A target amino acid residue for additional destabilization of PHA might be F109, which is also involved in anchoring the large surface loop contributing to the sugar-binding site to the core of the protein [Chapter 3].

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CHAPTER 5

DESTABILISATION OF PEA LECTIN: EFFECTS ON BIOLOGICAL ACTIVITIES

Flip J. Hoedemaeker, Clara L. Díaz, Harry de Wit, Ben K. Stulp, B. Sylvia de Pater, Jan W. Kijne

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ABSTRACT

Previously, two *Pisum sativum* L. lectin (PSL) mutants with decreased stability, PSL V103A and PSL V103A/F104A, have been constructed [Chapter **3**]. PSL V103A, isolated from *E.coli*, showed a decrease in denaturation temperature of about 10°C compared to wild type (wt) PSL. PSL V103A/F104A could not be properly characterized, because it was impossible to isolate this mutant in an active form from *E.coli* inclusion bodies. Here, we show that the introduced mutations do not interfere with production and processing of PSL in transgenic tobacco. Three biological activities were tested, (i) toxicity to insect larvae, (ii) stimulation of root nodule formation in the *Rhizobium*-legume symbiosis, and (iii) mitogenic activity towards lymphocytes. In tobacco, PSL and both PSL mutants were found to be essentially non-toxic for larvae of *Manduca sexta* (tobacco hornworm). Nodulation experiments done with transgenic white clover (*Trifolium repens* L.) hairy roots, expressing either wt or mutant psl, demonstrated that destabilisation of PSL does not affect the legume-*Rhizobium* symbiosis. Wt PSL as well as both PSL mutants appeared to be able to activate human T-lymphocytes, with sugar-binding activity of PSL being necessary for this effect.

These experiments did not reveal functional differences between normal and destabilized forms of PSL. The mitogenic activity of the unstable PSL mutants at 37°C contrasts with the inability of the mutants to agglutinate erythrocytes at the same temperature [Chapter **3**]. This difference may be caused by an influence of the growth medium used for culturing lymphocytes on the stability of PSL.

INTRODUCTION

Lectins of leguminous plants are among the best studied carbohydrate-binding proteins [4,21,22]. The three-dimensional structures of a number of legume lectins have been established [1,3,10,11,23,25,26]. The lectin of *Pisum sativum* L. (PSL) is a ß-barrel protein [24] and is organised as a dimer, like the other lectins from the Vicieae tribe of the Leguminosae family [27,28]. Both monomers are post-translationally processed into a small α- and a larger ß-chain [Chapter 2]. Consequently, the mature protein has an $\alpha_2\beta_2$ configuration [21]. The overall three-dimensional structures of monomers of legume lectins are very similar, in spite of differences in processing and multimerisation.

PSL is present in high amounts in pea seeds, whereas pea roots contain a very low amount of PSL [5]. Two PSL isolectins are produced from a single copy gene, psl [17, Chapter 2]. Root PSL is involved in determination of host-specificity in the symbiosis of pea with nitrogen-fixing *Rhizobium* bacteria, as reported by Díaz *et al* [6]. These authors showed that white clover (*Trifolium repens* L.) hairy roots, which are normally only nodulated by *R.leguminosarum* bv. *trifolii*, can also be nodulated by *R.leguminosarum* bv. *viciae* when expressing the psl gene or cDNA. However, the function of lectin in seeds is not known. It has been hypothesized that lectins can protect plants and seeds against insects and other herbivorous animals [2,4]. A number of legume lectins, including PSL, have been claimed to be toxic to insects [2,20], and the use of legume lectin as a pest control agent in transgenic plants has been patented [13]. Lectin toxicity has also been found with rats [22] and pigs [16]. Therefore, legume lectins are considered anti-nutritional factors (ANF) in the animal feeding industry [16]. The interaction of legume lectins with intestinal epithelium cells can be studied *in vitro*, as described by Koninkx *et al* [18]. In addition, well-known *in vitro* assays of lectin activity are agglutination of erythrocytes and mitogenic stimulation of lymphocytes [27].

Elimination of toxic lectins from legume seed meal, to be used as animal fodder, may be facilitated by destabilisation of the proteins by site-directed mutagenesis [16]. However, the beneficial properties of these lectins *in planta* have to be preserved, most notably their function in the legume-*Rhizobium* symbiosis [6]. In Chapter 3, we described the production of two unstable PSL mutants. In both mutants, anchoring of a surface loop, which is involved in sugar binding, to the body of the protein has been weakened by substitution of conserved hydrophobic residues for alanine. PSL V103A differs from wild type (wt) PSL in that its denaturation temperature is reduced by about 10°C. The second mutant, V103A/F104A, could not be properly characterised, because it has not been possible to isolate this mutant in an active from *E.coli* inclusion bodies.

In this chapter, we report upon biological relevant properties of these destabilized PSL mutants. We examined expression and processing of these lectins *in planta*, their functionality in symbiosis, and their toxicity for *Manduca sexta*, the tobacco hornworm. Since PSL has been shown not to affect cultured intestinal epithelium cells [18], we used mitogenic stimulation of human lymphocytes by wt and mutant PSL as a model system for studying the interaction of the lectins with eukaryotic cells at 37°C. These experiments did not reveal functional differences between normal and destabilized forms of PSL.

MATERIALS AND METHODS

Mutagenesis, production and purification of lectins from E.coli, *and construction of wt and mutant psl for plant transformation.*

Site-directed mutagenesis of PSL and the production by and purification from *E.coli* of wild type (wt) and mutant PSL has been previously described [Chapter 3]. These psl constructs lack the original signal sequence. For plant transformation, the signal sequence of a genomic psl clone was amplified by PCR using the sense primer SP5 (5′-GGC CAA GCT TGC ATG CAA TTA ATA ACC AAT GGC TTC-3′) and the anti-sense primer SP35 (5′-GGC CGA ATT CAC CTT GAA AAG GAT TG-3′). The resulting DNA fragment was used to reconstitute wt and mutant psl cDNA. The psl genes were subsequently cloned into the binary vector pAGS HB 35S [6] under control of the CAMV 35S promoter, and conjugated into *A. tumefaciens* strain MOG 101 [14] for tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) leaf disk transformation according to Horsch *et al.* [15], and into *A. rhizogenes* strain 1334 for induction of hairy roots on white clover.

Isolation of PSL from transgenic tobacco

PSL content of leaves of *in vitro* grown transgenic tobacco plants was assayed using an ELISA with polyclonal rabbit anti-PSL antibodies conjugated with bovine alkaline phosphatase. A number of plants, showing PSL expression levels of 1-3% of total soluble protein, were selected, transferred to soil and grown under standard greenhouse conditions.

Leaves of transgenic tobacco plants containing PSL were ground in extraction buffer (50 mM Tris-ascorbate, pH 8.0, containing 500 mM NaCl and 2.5 mM thiourea) supplemented with 1% (w/v) PVP (Polyvinylpyrrolidone K30, Aldrich, Steinheim, Germany) at 0°C, using an omnimixer. PSL was further purified by affinity chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) as described previously [Chapter 2]. Since ascorbic acid absorbs at 280 nm, the column was washed with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) before elution of PSL with 0.2 M glucose in TBS. Processing of PSL in tobacco was tested by SDS-PAGE and Western blot analysis.

Insect toxicity tests

Manduca sexta (tobacco hornworm) L109A eggs were purchased from Carolina Biological Supply, Burlington NC, USA. Eggs were allowed to hatch at 28°C with a 12h daylight period on standard diet, which contains 160 $g.L^{-1}$ maize meal, 80 $g. L^{-1}$ wheat meal, 80 $g.L^{-1}$ dried yeast, 8 $g.L^{-1}$ ascorbic acid, 2 $g.L^{-1}$ sorbic acid, 100 $mg.L^{-1}$ streptomycin, and 3% agar in demineralized H_2O. Maize meal, wheat meal and dried yeast were purchased in a local reform shop.

Transgenic tobacco plants, containing wt or mutant PSL at approximately 1% of total soluble leaf protein, as well as untransformed control plants, were allowed to self-fertilize. For each construct, seven kanamycin-resistant seedlings were grown up to about 70 cm high. Single leaves were cut from these plants. The leaves were numbered downwards, the youngest fully extended leaf being number 1. Upon cutting, the petioles were put in Eppendorf vials containing a 0.4% (w/w) agar solution to prevent drying out of the leaves. Eight *M. sexta* larvae (first instar) were put on each leaf, and the leaves were incubated in large Petri dishes at 28°C, 12h light. The leaves were substituted every day for seven days by leaves of the same age from another seedling. After seven days, the larvae were frozen and weighed. Toxicity was measured as the difference in survival rate and individual weight between larvae fed on leaves with or without (wt or mutant) PSL, and the total biomass on each leaf was calculated.

Toxicity of PSL isolated from pea seeds was tested by transferring single first instar *M.sexta* larvae from standard diet to Petri dishes containing 10 gr. standard diet or standard diet containing PSL at 1.6 $mg.mL^{-1}$ (approximately 1-2% of the total protein content). After 7 days at 28°C, 12h light, the larvae were frozen and weighed, and toxicity was determined as described above.

Nodulation assay on Trifolium repens *L. (white clover) hairy roots.*

The nodulation assay on white clover hairy roots has been previously described [6]. Briefly, hairy roots were induced on 6 to 8-days-old seedlings using *A. rhizogenes* strain 1334, carrying a binary plasmid with or without (mutant) psl. Emerging hairy roots were inoculated with *R. leguminosarum* bv. *trifolii* strain ANU 843 or bv. *viciae* strain 248. Nodules and nodule-like structures were counted 40 days after inoculation.

Activation of T-lymphocytes

Human T-lymphocytes were isolated from blood of a healthy donor as described by Dokter *et al* [8], and subsequently cultured at a density of 1-2.10^6 in RPMI 1640 medium (Flow, Rockville, MD USA), supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 6 ng/ml colistin. The cells were left unstimulated, or were stimulated by adding CON A, wt or mutant PSL in a concentration of 25 μg.ml^{-1}. CON A was purchased from Calbiochem (La Jolla, CA, USA), wt PSL was isolated from pea seeds, the unstable PSL mutant proteins were isolated from transgenic tobacco, and a non-sugar-binding PSL mutant PSL G99R was isolated from *E.coli* [30]. After 24 h, the cells were labelled with fluorescent monoclonal antibodies against the T-cell activation marker CD25, and subsequently counted in a Fluorescence Activated Cell Sorter (FACS).

RESULTS

Production of wt and mutant PSL in tobacco

Since a routine method for the transformation of pea plants was not available, we transformed tobacco using binary vectors containing wt or (unstable) mutant psl. The production of PSL in tobacco was confirmed by ELISA and affinity chromatography of leaf extracts. Western blot analysis revealed that PSL was correctly processed into α- and ß chains as in pea plants, in contrast with PSL produced in *E.coli* (Figure 1).

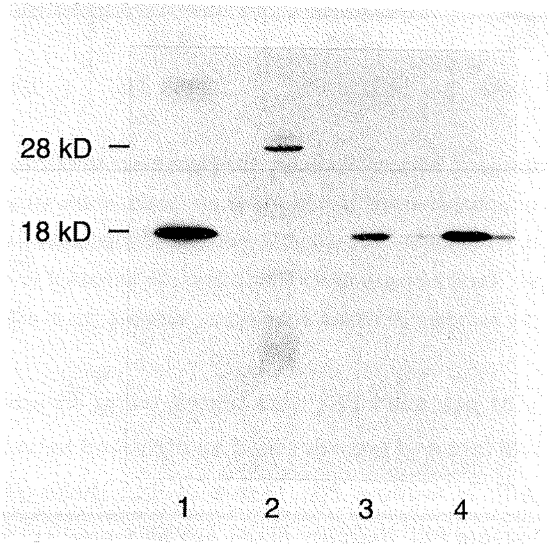

Figure 1. Western blot of PSL mutants produced in transgenic tobacco, compared with PSL from pea seed and PSL produced in *E.coli*. Lane 1: pea seed PSL; Lane 2: PSL from *E.coli*; Lane 3: PSL V103A; Lane 4: PSL V103A/F104A

Insect toxicity tests

Toxicity tests were performed with leaves from transgenic tobacco containing wt or mutant PSL at approximately 1% of total soluble leaf protein. The survival and growth rates of *M. sexta* larvae grown on tobacco leaves with or without (destabilized) PSL are listed in Table 1. Growth and survival rate was significantly higher on younger tobacco leaves, indicating that the nutritional value of tobacco leaves is very much dependent upon the age of the leaves. Differences could not be correlated with lectin content of the plants.

leaf #	1	3	5	7
SR1	**1301** 163 ±155	**1679** 210 ±104	**1150** 144 ±104	**366** 46 ±29
wt PSL	**1443** 180 ±120	**1412** 202 ±42	**1402** 175 ±69	**186** 37 ±18
3203	**2297** 328 ±117	**1140** 163 ±59	**1226** 204 ±99	**59** 30 ±11
3211	**1786** 223 ±95	**1450** 181 ±49	**1035** 129 ±76	**427** 85 ±55

Table 1. Toxicity of transgenic tobacco leaves for *Manduca sexta* larvae. In the first horizontal row the values for leaves of untransformed control plants are given, in the other rows the results with leaves containing wt or mutant PSL. The values are displayed separately per leaf number (see text for further explanation). Total biomass of surviving larvae is indicated in bold, and their average weight is given with the standard deviation in brackets. Weights are in milligrams.

The toxicity of pea seed PSL was tested using an artificial diet. A slight difference in survival rate and growth could be observed between *M. sexta* larvae grown on a diet with or without PSL (Table 2), but these differences were not significant. The unstable PSL mutants could not be tested in this assay due to the low yield of affinity-purified lectin from tobacco leaves. Taken together, these results strongly that PSL essentially is non-toxic for larvae of *M.sexta*.

	diet with PSL	diet without PSL
Total biomass (mg)	**1197**	**1755**
Survival rate (N=20)	65%	75%
Average weight (mg)	92.1 ±58.0	109.7 ±61.0

Table 2. Total biomass, survival rate, and the average weight of surviving *Manduca sexta* larvae after growth for 7 days on artificial diets with or without PSL.

Nodulation of white clover hairy roots

We tested whether the destabilizing mutations influenced the role of PSL in improving the nodulation of white clover (*Trifolium repens* L.) hairy roots by *R. leguminosarum* bv. *viciae* and bv. *trifolii*. Nodulation frequencies and the average number of root nodules per positive plant as measured 40 days after inoculation are summarized in Table 3. Virtually all plants inoculated with *R.leguminosarum* bv *trifolii* are nodulated after 20 days (data not shown). An increase in the average number of nodules is visible when wt or mutant PSL is expressed. About 50% of the plants inoculated with *R.leguminosarum* bv *viciae* show (delayed) nodulation on hairy roots transformed with wt or mutant psl. Apparently, no differences between wt and mutant PSL are noticeable in this assay. This experiment shows that neither the single nor the double destabilizing mutation affects the role of PSL in the legume-*Rhizobium* symbiosis.

Mitogenicity tests on human T-lymphocytes

We tested mitogenic activity of wt and mutant PSL on human T-lymphocytes to study the interaction of wt PSL and unstable mutants with eukaryotic cells at

	R.leguminosarum bv *viciae*		*R.leguminosarum* bv *trifolii*	
	% nodulated plants	# nodules per plant	% nodulated plants	# nodules per plant
control	9%	1.3	100%	3.5
wt PSL	48%	2.0	93%	6.4
PSL V103A	43%	2.2	93%	7.9
PSL V103A/ F104A	53%	1.4	100%	5.5

Table 3. Nodulation assay with white clover hairy roots, as measured 40 days after inoculation with *R. leguminosarum* biovars viciae (N = 30) and trifolii (N = 15), respectively. Plants with hairy roots transformed with an empty binary vector are used as controls.

37°C. As a control, we compared these activities with that of another PSL mutant, G99R. This mutant is impaired in binding of sugars due to an amino acid substitution at the tip of the same loop that is modified in both unstable mutants [30]. The results of the mitogenicity tests are shown in Figure 2. PSL stimulates T-lymphocytes at a concentration of 25 μg.ml^{-1}. Wt PSL and both unstable mutants approximately have the same activity. Since these tests were performed at 37°C, it seems that both instable mutants are still active at that temperature in the particular culture medium used for lymphocytes. PSL G99R did not have mitogenic activity at a concentration of 25 μg.ml^{-1} (see Figure 2), or 50 μg.ml^{-1} (data not shown). Therefore, we conclude that this activity of PSL is dependent on sugar binding.

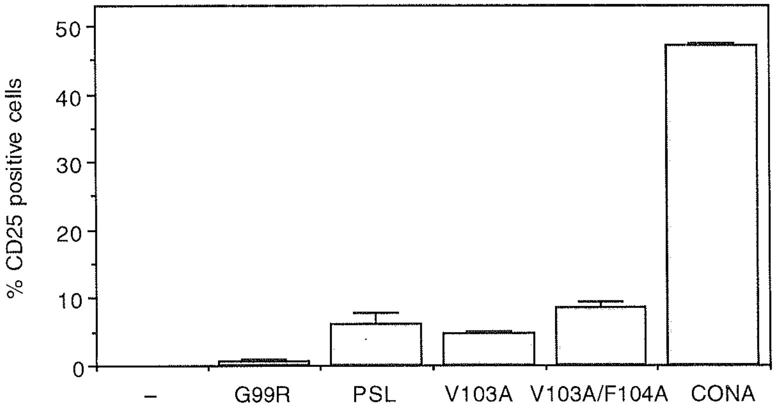

Figure 2. Induction of CD 25 on human lymphocytes by PSL and CON A. The standard deviation is indicated on top of each bar.

DISCUSSION

The cDNA's encoding wt PSL and two instable mutants were expressed in transgenic tobacco. In tobacco, PSL is correctly processed and it can be isolated in an active form. Apparently, an aspariginyl endopeptidase, responsible for the correct processing of PSL [Chapter 2], is present in tobacco like it is in pea. This specific processing activity has also been detected in potato [9], and has recently been isolated from *Canavalia ensiformis* seeds [29]. Therefore, it seems to concern a common processing enzyme in plants. The double mutant, PSL V103A/F104A, which could not be isolated in an active form from *E.coli* inclusion bodies, is normally produced in tobacco and can be purified using affinity chromatography. Apparently, these mutations do not interfere with sugar-binding activity of PSL.

The functionality of the unstable PSL mutants *in planta* could be demonstrated with use of the nodulation assay described earlier [6]. Both PSL mutants enabled nodulation of white clover hairy roots by *R.leguminosarum* bv *viciae*, like wt PSL does. With *R.leguminosarum* bv. *trifolii*, an increase in the number of nodules per plant was observed when wt or mutant *psl* was present. This increase has been noticed before [7]. No difference was observed between the effects of wt PSL and the unstable PSL mutants.

PSL appeared to be non-toxic for *M. sexta* larvae. The difference in growth rate and survival on artificial diet was not significant. If there is any effect of PSL on these insects, it would be a slight influence on the growth rate. Toxicity of PSL for *Callosobruchus maculatus* and *Heliothis virescens* has been shown by Boulter *et al* [2], and the use of psl in transgenic plants as a pest control agent has been patented [13]. It is possible that *M.sexta* reacts differently to PSL than *C.maculatus* or *H.virescens* do. On the other hand, toxicity of PSL for *C.maculatus* could not be demonstrated by Murdock *et al* [20]. The toxicity of PSL therefore remains questionable. In order to test the effect of destabilizing mutations on the toxicity of legume lectins, other more toxic lectins have to be mutagenized. In general, tobacco leaves do not seem to be an ideal choice for studying the expression of toxic proteins, because age-related endogenous factors strongly influence the nutritional value of these leaves (Table 1).

Pea seed PSL and both instable PSL mutants isolated from tobacco leaves were able to activate human T-lymphocytes. Mitogenicity of PSL has been reported previously [27]. Both wt and mutant PSL were less active than CON A at a concentration of 25 μg.mL^{-1}. CON A induced the T-cell activation marker CD 25 on 50% of the lymphocytes, and PSL on only 5%. However, this low percentage of activated lymphocytes represents a reproducible result. Therefore, the difference in stimulation between sugar-binding and non-sugar-binding PSL is significant. PSL possibly stimulates a specific type of T-lymphocyte, but this hypothesis has not been tested yet.

Interestingly, both wt and mutant PSL were active in this assay at 37°C. This is not consistent with our earlier finding that PSL V103A is impaired in agglutination of erythrocytes at 37°C [Chapter 3]. Probably, the stability of PSL is influenced by the buffer or medium used in such assays, and/or by binding to specific ligands. It can be concluded that the sugar-binding activity of PSL is necessary for mitogenic stimulation, since PSL G99R, a PSL mutant with no sugar-binding activity [30], is hardly able to activate lymphocytes. This has also been shown for leucocyte-specific *Phaseolus vulgaris* lectin (PHA-L) by Mirkov *et al* [19]. However, we have to note that PSL G99R is not processed in *E.coli*, and obviously has not been affinity-purified.

In an earlier report we showed the possibility to produce mutant PSL which is still active at ambient temperatures, but is easily inactivated with a heat treatment [Chapter 3]. We now show that these mutations do not interfere with at least two biological activities of PSL: stimulation of root nodule formation in the legume-*Rhizobium* symbiosis (at ambient temperatures) and mitogenicity towards lymphocytes (at 37°C). Because of the conflicting data on the activity of destabilized PSL at 37°C, it is still unclear whether a heat treatment will remain necessary to inactivate destabilized PSL, when present in legume seed meal. The stability of proteins apparently can be influenced by environmental factors. A possible difference in resistance of wt and destabilized PSL against acid treatment can be tested, since fodder passes the stomach and destabilized PSL may show reduced residual activity against intestinal epithelium cells. Eventually, the properties of destabilized PSL will have to be studied in meal from transgenic pea seeds to ascertain whether site-directed mutagenesis in general can be used to economically and safely improve the nutritional value of legumes.

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CHAPTER 6

MUTATIONAL ANALYSIS OF PEA LECTIN. REPLACEMENT OF ASN 125 BY ASP IN THE MONOSACCHARIDE BINDING SITE ELIMINATES MANNOSE/GLUCOSE BINDING ACTIVITY

Ron R. van Eijsden, Flip J. Hoedemaeker, Clara L. Díaz, Ben J. J. Lugtenberg, B. Sylvia de Pater and Jan W. Kijne

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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, PSL 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 weight of about 55 kD, in which binding of both 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 should 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 ß-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 ß-chain, with a total molecular weight of about 50 kD [33]. Each subunit is synthesized as a pre-pro-protein at the rough endoplasmatic reticulum, in a ß-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 determination of 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**].

	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

Table 1. Homology of N125-containing segments of various legume lectins (from ref. 4 and 36). PSL = pea lectin; LOLI = *Lathyrus ochrus* isolectin I; LCL = lentil lectin; VFL = favin; SBL = soybean lectin; PHA-E = phytohemagglutinin E; CONA = concanavalin A.

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 O4 atom of mannose interacts with the NHD2 part of the asparagine sidechain. 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 Asn 125. The OD1 part of the Asn 125 sidechain is probably involved in direct binding of the Ca^{2+}-ion (Figure 1). Here we report that replacement in PSL of Asn 125 by aspartate results in total loss of

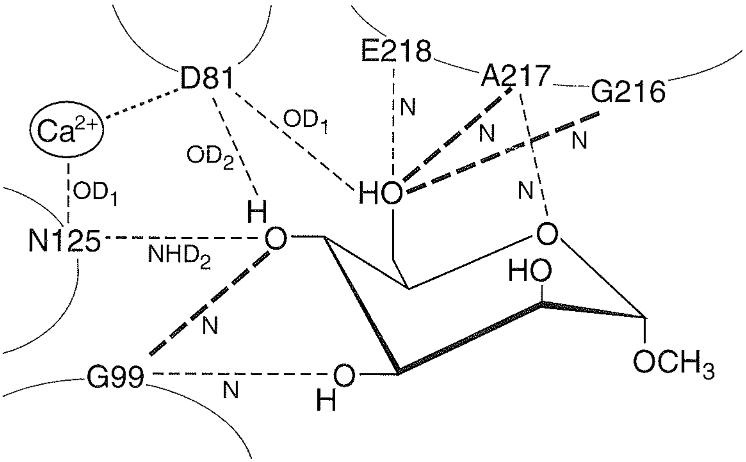

Figure 1. Binding of *α*-methyl-mannopyranoside in the saccharide binding pocket of LOLI (from ref. 5 and 6). Binding is the result of several direct interactions between amino acid residues and the sugar. Direct interactions are represented by the broken lines, amino acid residues by one letter characters and the positions within the protein by numbers. Sugar-peptide linkage interactions are marked with **N** and sugar-amino acid sidechain 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 sidechain (from ref. **5**). This model is consistent with results obtained with favin and PSL [**1,28**]. However, in case of favin the molecular positions are numbered differently, resulting in D82, G100, N126, A212 and E213, respectively.

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.

MATERIALS AND METHODS

Bacterial strains

E.coli strain DH5αF$^+$, supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1, was used for PSL production. Bacteria were grown in Luria Complete medium [**21**] (LC) 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 (Figure 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′ CAA GGT GAA TTC AAC TGA AAC 3′) and primer PSL N125D (5′ GCT TGG ATC CCA TGC AAC ATT ATA GAA AG 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 (Figure 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′ GCT TGG ATC CCA TGC AAC ATT ATA GAA AG 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 (Figure 2B).

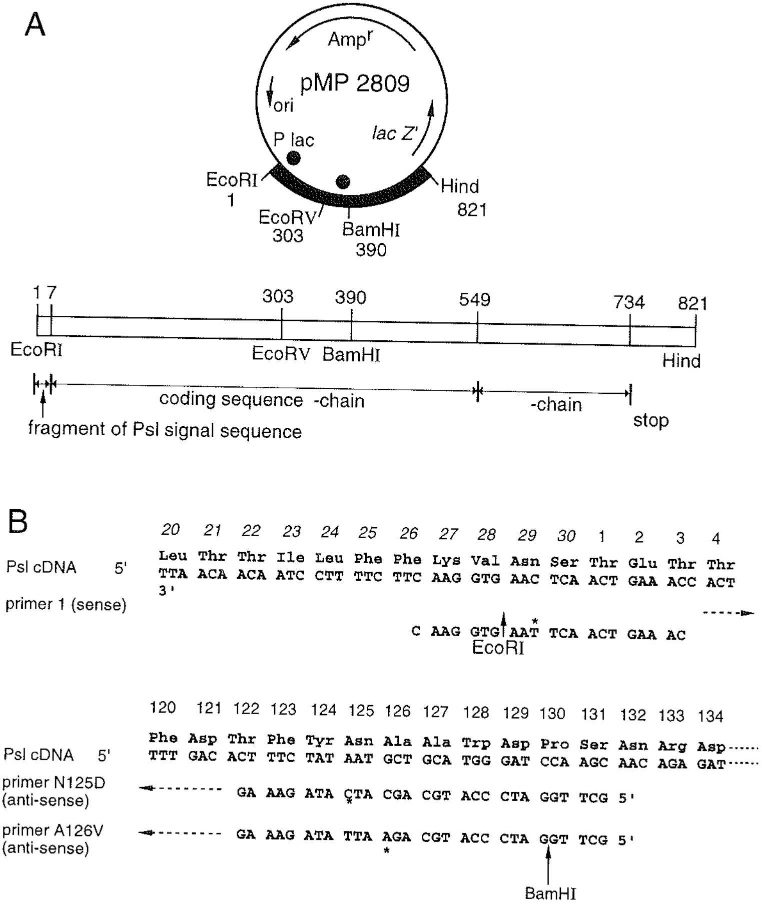

Figure 2. Cloning and mutagenesis of PSL cDNA. **(A)** Map of expression vector pMP 2809 from which high level expression of PSL was obtained. Closed circles show the binding regions of the mutagenic primers used. Transcription directions for the subcloned PSL cDNA, the truncated lac Z' gene and the ß-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 stars. Amino acids are given by three letter characters and numbered from the first Thr residue at the N-terminus of the ß-chain. Italic numbered residues correspond with residues encoded by the PSL signal sequence.

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, Chapter 3]. 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 % (wt/vol) 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 2,000-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-indolyl-phosphate (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^{-1}. 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% (vol/vol) concentrated HNO_3, to a final concentration of 5 mg.ml^{-1}. Aggregates were removed by centrifugation for 10 min at 7,000 rpm. Measurements 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 4, with an average R.S.D. 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^{-1} 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^{-1}. 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 protein, 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 (Figure 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 (Figure 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 kD (Figure 3A, lanes 2-4). This molecular weight corresponds with that of unprocessed PSL from pea plants (Figure 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 kD ß-chains. Since the antiserum used mainly interacts with epitopes of the ß-chain, the 6 kD 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, 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 ß-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 (Figure 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 weight of about 55 kD 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 kD. SDS-PAGE and immunoblotting of the peak I fractions of each PSL form yielded bands with a molecular weight of about 28 kD (Figure 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 Asn 125 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.

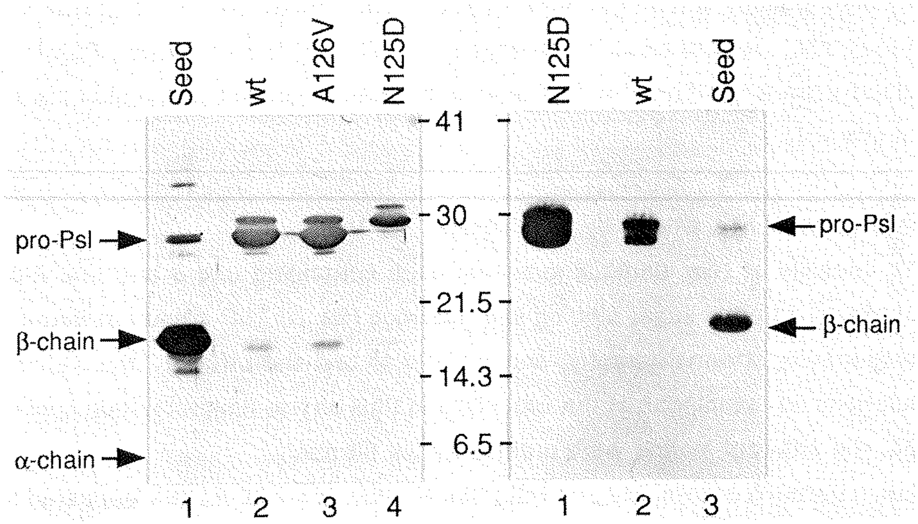

Figure 3. Immunodetection of different PSL forms. **A)** Immunodetection was performed with wt PSL, PSL N125D and PSL A126V, directly after isolation from *E.coli*. Mature pea lectin from seed (seed PSL) was loaded as a reference. Lane 1: seed PSL; Lane 2: wt PSL; Lane 3: PSL A126V; Lane 4: PSL N125D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

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 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

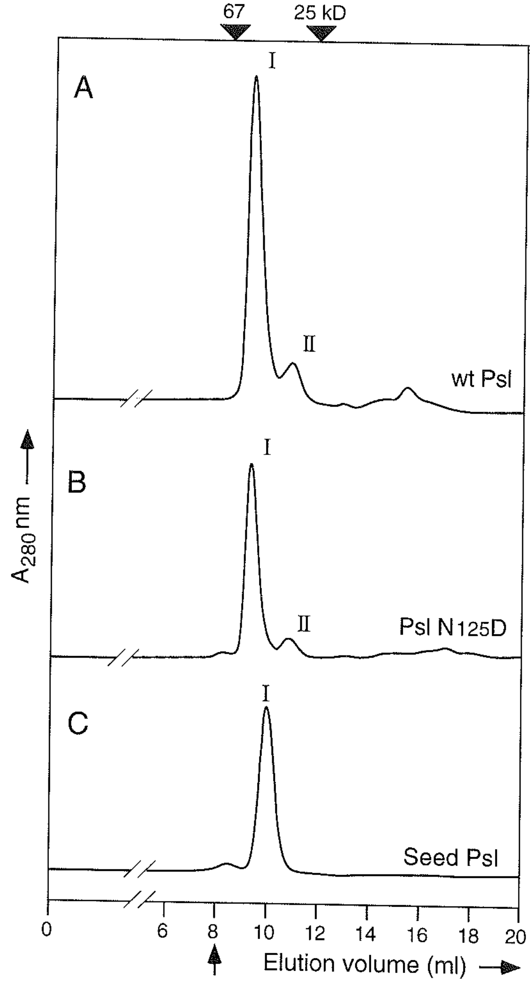

Figure 4. Gelfiltration of PSL after isolation from *E.coli*. Gelfiltration was performed to test dimer-formation by wt PSL, PSL N125D and seed PSL, shown in **A**, **B** and **C**, respectively. Large arrowheads at the top of the figure represent molecular markers, whereas the vertical arrow at the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

concentration of 16 μg/ml or lower. In case of PSL N125D, no agglutination activity could be detected (Figure 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 PSL N125D is coupled with inability to agglutinate erythrocytes.

	Ca^{2+} concentration (mol/mol PSL)	Mn^{2+} concentration (mol/mol PSL)
wt PSL	2.25	2.26
PSL N125D	2.17	2.29

Table 2. Ca^{2+} and Mn^{2+} content in wt PSL and PSL N125D. Ca^{2+} and Mn^{2+} concentrations were determined using ICP-AES (Inductively Coupled argon Plasma Atomic Emission Spectroscopy). Concentrations were calculated using a molecular weight of 55 kD for both wt PSL and PSL N125D.

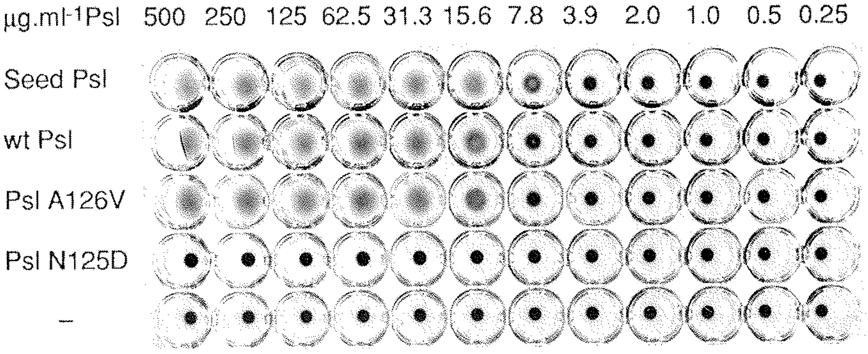

Figure 5. Haemagglutination assay with wt and mutant PSL. The ability to agglutinate a 2 % suspension of human A^+ erythrocytes was tested for seed PSL, wt PSL, PSL N125D and PSL A126V. The assay started from lectin concentrations of 0.5 $mg.ml^{-1}$, which were serially diluted. The photograph was taken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

DISCUSSION

Asparagine 125 is conserved in all legume lectins tested [36]. In two ways, it is thought to be involved in sugar binding, (i) by binding of calcium, the presence of which is necessary for structuring the sugar binding site, and (ii) 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 Asn 125 by aspartate (in other words, replacement of the NHD2 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](Figure 1) apparently is insufficient for sugar binding and that Asn 125 plays an essential role in this process. Since (i) 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 (ii) Asn 125 is conserved in all legume lectins, PSL N125D 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 PSL 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 [23] and [31]), despite the inability of the bacterium to process the protein. The molecular weight has the expected value (Figure 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 (Figure 4), points at either a slight inhibition of dimerization in *E.coli* or, most probably, incomplete dimerization of PSL during renaturation. At present, we can not explain why recombinant PSL appears as a doublet after SDS-PAGE. The slightly lower mobility of crude PSL N125D in comparison with the other PSL preparations (Figure 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 also chapter 1), 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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CHAPTER 7:
GENERAL AND SUMMARIZING DISCUSSION

Introduction

During the past century the sugar-binding properties of lectins, especially legume lectins, have been extensively studied [30,31]. Since legume lectins are found with a wide variety of sugar specificities, and therefore can distinguish surface properties of various cell types, these proteins have become important tools in medical research, with an emphasis on cancer research [30]. Lectins are produced in large amounts in the seeds of legumes, and are easy to isolate and purify [31]. In the last decade, a number of legume lectins have been crystallized and their three-dimensional structures have been determined [2,5,6,10,14,23,27]. The resulting data provided information on the molecular basis of sugar-binding specificity and opened the possibility to study the structure-function relationship of these proteins. For example, attempts have been made to change the sugar-binding specificity of lectins, based on structural and sequential homologies between lectins [1,35].

This thesis describes the study of different properties and functions of legume lectins by using site-directed mutagenesis. Both beneficial properties of lectins, such as the role in host-plant-specific symbiosis of legumes with *Rhizobium* [4,9], and deleterious effects, such as anti-nutritional properties [15,18], have been studied. PSL (for *Pisum sativum* L. lectin) has been used as a model lectin in most of the work described in this thesis. Data obtained with PSL have been applied in a study of PHA-L, the mitogenic lectin from *Phaseolus vulgaris*.

Post-translational processing

Chapter 2 addresses the post-translational processing of PSL. A number of legume lectins, including PSL, are processed in specialized protein storage vacuoles [17]. Conflicting results have been published on processing of PSL [17,24,26]. It was known that both PSL isolectins found in seeds are derived from a single gene [20], and that the difference in charge between these isolectins is located somewhere in the C-terminus of the smaller *α*-chain in each monomer. In Chapter **2**, we show that one residue, lysine 240, is responsible for the charge difference between PSL1 and PSL2. Furthermore, it is shown that PSL from roots is identical to isolectin 2 from seeds. cDNAs obtained from pea roots and seed are identical, proving that root PSL is derived from the same gene as seed PSL. A carboxypeptidase appears to be responsible for the processing step leading to the charge difference, in addition to an Asn-X specific protease which is responsible for the processing of PSL monomers into ß- and *α*- chains. Recently, a similar processing scheme has been proposed for DB58, a lectin from *Dolichos biflorus*, by Etzler *et al.* [**12**]. The reason for this processing is not well understood, since unprocessed lectins generally have the same activity and specificity as their processed counterparts [**32,28**]. Also, processing does not have a significant effect on stability [Chapter **3**]. It is possible that this processing is a first step in a specific degradation pathway, since proteins in plant (storage) vacuoles can be used as a nutrient reserve for the germinating embryo or for young developing organs.

The exact location of PSL inside the roots is still unknown. Presence of PSL on the tips of root hairs has been demonstrated [**9**]. The identical processing of seed and root PSL shows that proteases with identical specificities as those in pea seeds are also present in pea roots.

Lectins as anti-nutritional factors

Animal fodder, based on processed legume seeds, contains lectins. A number of legume lectins have been shown to have anti-nutritional effects [**15,18**]. The lectins can pass the stomach unharmed, and can subsequently bind to the intestinal epithelium. Binding of some lectins to the intestine has been associated with disruption of the brush border, with defects in digestion, and with effects on enzyme activity [**15**]. Therefore, elimination of anti-nutritional proteins, including lectins, is an important element in the processing of animal fodder. Since genetic elimination of lectins from legumes will probably have a negative effect on the *Rhizobium*-legume symbiosis [**9**], we have pursued another goal: destabilization of the lectins. Destabilized lectins should be normally active *in planta* at ambient temperatures, but should be easily inactivated after harvest of the legume seeds. As an alternative approach, our research group studies expression of the pea lectin gene, in order to test the possibility of elimination of expression in seeds with preservation of expression in roots. It has been shown recently that a 22 bp psl promoter element confers seed-specific expression of PSL [8].

In addition to lectins, legume seeds contain a number of other anti-nutritional factors, such as protease and α-amylase inhibitors, tannins, and (poly)phenolic compounds [7,18]. These factors probably do not have a function in regulating the *Rhizobium*-symbiosis, but may have an important role in plant defence [7]. Some of these compounds, like α-amylase inhibitors, are evolutionary related to legume lectins [21,33], and might therefore be eliminated by destabilisation following a similar approach.

Stability of legume lectin

Most research on protein stability has been carried out on α-helical proteins [13]. General rules for stabilizing (or destabilizing) α-helices cannot be applied to legume lectins, because these proteins are ß-barrels lacking α-helices [10,24]. The hydrogen bonds which are formed in ß-sheets are difficult to influence by site-directed mutagenesis, because they are essentially independent of amino acid sidechains. We decided to focus our attention on a large surface loop, containing a conserved box of hydrophobic residues [Chapter 3]. The sidechains of these hydrophobic residues point inward, making contact with the ß-sheet underneath. It has been well established that protein stability depends on differences in entropy and enthalpy between the folded and unfolded state [19]. Removal of bulky apolar sidechains from the hydrophobic core of a protein usually results in destabilization, because of loss of van der Waals contacts and because of the possibility for water molecules to enter hydrophobic cavities [11]. Substitution of valine 103 in PSL for alanine indeed destabilized the protein, resulting in a decrease in denaturation temperature by about 10°C. This result confirms the general idea that a local change in Van der Waals contacts can affect the total structure [13], and demonstrates the possibility of destabilizing legume lectins, and possibly other proteins with similar structures, without the need to introduce mutations in ß-sheets.

The V103A mutation also caused a reversible effect on sugar-binding activity towards erythrocytes at 37°C. Because the mutation is made in a surface loop that contributes to the sugar-binding site, sugar-binding at this temperature may be impaired by an increase in flexibility of the loop, without total denaturation of PSL. This reversible inactivation of PSL could not be found when the less-stable mutants were used to stimulate human lymphocytes at 37°C [Chapter 5], a process also dependent on sugar binding. Stability of PSL may be affected by the growth medium used for these lymphocytes. Alternatively, the affinity of PSL to ligands on erythrocytes may differ from the affinity to lymphocytes.

The destabilizing mutation in PSL was made in a conserved box of amino acids, which opened the possibility of extrapolating the results to other legume lectins. We introduced the corresponding mutation (substitution of leucine 108 for alanine) in PHA-L, the leucocyte-specific *Phaseolus vulgaris* (common bean) lectin, and our results show that this mutation has a similar effect [Chapter 4]. Apparently, this particular mutation can be the basis for a general method for destabilisation of legume lectins, without prior knowledge of the exact three-dimensional structure.

The results described in Chapter 4 demonstrate that PHA-L has a higher denaturation temperature than PSL. This could imply that the effect of a single destabilizing mutation is not sufficient for elimination of the anti-nutritional properties of PHA, but this has yet to be demonstrated experimentally. Introduction of additional destabilizing mutations is possible, also in other loops contributing to the sugar-binding site. On numerous occasions, it has been shown that stabilizing or destabilizing mutations are additive, provided that the residues involved do not directly interact [13]. In this context, one could also think of mutations interfering with metal binding of lectins at higher temperatures.

Effects of destabilizing mutations on the biological activity of PSL

The properties of destabilized mutant lectins *in vivo* should be preserved. For example, a well established beneficial function of PSL is its role in symbiosis of pea plants with rhizobia [9]. Other functions of legume lectins are not yet fully understood, but it is assumed that the proteins are involved in protection against insects and other herbivorous animals [7]. The study of these functions has been hampered until now by the lack of a reliable transformation protocol for large seeded legumes.

To circumvent this problem, we studied the effects of wild type and mutant PSL on symbiosis in a *Trifolium repens* hairy root system, as described by Díaz *et al* [9]. Other *in vivo* properties of PSL were studied in transgenic tobacco (*Nicotiana tabacum*) [Chapter 5]. We were able to show that destabilisation of PSL does not interfere with proper processing and with its ability to improve nodulation on white clover hairy roots by *R.leguminosarum* bv *trifolii* and bv *viciae in planta* at ambient temperatures.

Toxicity of PSL could not be demonstrated for the insect larvae we used (*Manduca sexta*) [Chapter 5]. This result is consistent with a number of reports showing that PSL is hardly toxic, if at all [11,16,22]. Therefore, effects of such destabilizing mutations on toxicity should be studied with more toxic lectins, such as PHA. Nonetheless, the present results indicate that destabilisation of legume lectins in the way described in this thesis is a promising approach for solving the problem of anti-nutritional lectins in legume seeds.

Sugar-binding activity of PSL

In Chapter 6, a PSL mutant is described which is unable to bind sugars. The minor change introduced by substitution of asparagine 125 for aspartic acid completely abolishes sugar-binding activity, without affecting the calcium-binding site. The six amino acids which together form the sugar-binding site of PSL all are essential for sugar-binding activity [34]. Non-sugar-binding PSL mutants are important tools in studying the properties and functions of legume lectins. Sugar-binding activity has proven to be essential for the role of PSL in the *Rhizobium* symbiosis, and for mitogenic stimulation of lymphocytes [Chapter 5; ref. 35 for PHA]. It remains to be tested if such mutants are non-toxic in a feeding assay. If so, the result would finally provide conclusive evidence that sugar binding activity of legume lectins is responsible for toxicity.

Future research

Taken together, the experiments described in this thesis indicate that the anti-nutritional properties of legume lectins might be eliminated without interference with the beneficial properties. By using PSL as a model lectin, we could demonstrate that destabilisation of lectins does not necessarily interfere with their role in the *Rhizobium* symbiosis, which so far seems to be the only established function of legume lectins *in planta*. In order to test the effects of the destabilizing mutations on the toxicity of lectins, the experiments have to be repeated with other, more toxic legume lectins. The results obtained until now with PHA-L seem to corroborate the findings with PSL. Ultimately, feeding tests with affinity-purified wild type and mutant PHA will be necessary to determine the effects of destabilization on interactions with intestinal epithelial cells.

The economical aspects of the described approach in improving the nutritional value of legume seed meal can only be addressed after expression of mutated lectins in legume seeds. Recently, a protocol for the transformation of pea has been developed by Schroeder *et al* [29]. Obviously, pea cultivars that do not produce endogenous lectins will have to be used for transformation with mutant lectin genes. Such PSL-deficient pea cultivars have not been found or made until now.

At present, it can not be tested whether mere sugar-binding activity is sufficient for lectins to function properly. Legume lectins are multivalent, and can therefore agglutinate cells and glycosylated macromolecules. This trait could be essential for the role of lectins in symbiosis and/or plant defence. To address this question, lectin mutants have to be made that are deficient in multimerisation. Monomeric lectins would still be sugar-binding proteins, but will not be able to agglutinate. Attempts in this direction have been unsuccessful until now (Hoedemaeker, unpublished results). In view of the continuous ß-sheet connection between the two PSL monomers, multimerisation might be an essential factor during proper folding of the protein.

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SAMENVATTING

Eiwitten die suikers kunnen binden, en die geen enzymen en geen antilichamen zijn, behoren per definitie tot de **lectinen**. Het woord lectine komt van het latijnse *legere*, dat "uitkiezen" betekent. Lectinen veranderen de suikers die ze binden niet, en kunnen ze ook weer loslaten. Deze suikerbinding is specifiek, dat wil zeggen dat alle lectinen bepaalde suikers heel goed, en andere suikers helemaal niet kunnen binden. Deze definitie van lectinen is heel ruim, en lectinen komen daarom in alle soorten en maten voor in misschien wel alle levende wezens op aarde, van virussen tot de mens. Sommige wetenschappers stellen dat een eiwit tenminste twee bindingsplaatsen voor suikers moet hebben om een lectine te zijn. Dergelijke lectinen kunnen cellen aan elkaar koppelen, door met de ene suikerbindingsplaats één cel en met de andere bindingsplaats een andere cel te binden. Een voorbeeld hiervan is te zien in Figuur 4 van Hoofdstuk 3 van dit proefschrift (pagina 54). Op de foto's is een aantal putjes te zien, met daarin menselijke rode bloedlichaampjes. De bodem van de putjes is u-vormig. Wanneer er geen lectine aanwezig is, of wanneer het lectine niet actief is, zakken de bloedcellen uit onderin het putje. Als er actief lectine aanwezig is, vormen de bloedcellen met het lectine een netwerk, dat over de hele bodem van het putje ligt. De vorming van zo'n netwerk is ongedaan te maken door de specifieke suikers toe te voegen. Dit is een belangrijke methode om de activiteit van lectinen aan te tonen. Lectinen worden dan ook wel "haemagglutininen" genoemd, "bloedcelplakkers". Deze methode is natuurlijk niet geschikt voor het aantonen van de activiteit van lectinen met maar één suikerbindingsplaats.

Het onderzoek aan lectinen is meer dan 100 jaar geleden begonnen. In 1888 mengde Hermann Stillmark, een promovendus aan de Universiteit van Dorpat in Estland, een extract van zaden van de wonderboon (*Ricinus communis*) met bloed, en hij zag haemagglutinatie optreden. De suikerspecificiteit van lectinen werd pas in 1936 ontdekt door James Sumner. In 1948 werden lectinen gevonden die onderscheid kunnen maken tussen de verschillende bloedgroepen. Hiermee was aangetoond dat bloedcellen van verschillende bloedgroepen verschillende suikers aan hun oppervlakte hebben. Het is vooral vanwege deze eigenschap dat de naam "lectine" gekozen is door William Boyd en Elizabeth Shapleigh in 1954. Vervolgens duurde het niet lang voordat ontdekt werd dat met lectinen ook allerlei andere cellen van elkaar kunnen worden onderscheiden, zoals kankercellen van normale cellen. Ook werd ontdekt dat sommige lectinen lymfocyten kunnen activeren en tot deling kunnen aanzetten. Vandaag de dag worden lectinen dan ook over de hele wereld gebruikt in allerlei (bio)medisch onderzoek.

De groep van lectinen die voorkomt in vlinderbloemige planten (behorend tot de familie Fabaceae, beter bekend als Leguminosae) is van alle lectinegroepen het best beschreven. Tot de vlinderbloemige planten behoren alle peulvruchten, zoals erwt, boon, pinda, soja, maar ook klaver, acacia en nog meer algemeen bekende planten. De lectinen van meer dan 600 vlinderbloemigen zijn beschreven, hiervan zijn meer dan 70 gezuiverd en van meer dan 40 soorten is (zijn) ook het gen (de genen)(het DNA dat voor het eiwit codeert) bekend. Bovendien is van acht vlinderbloemige lectinen de precieze drie-dimensionale structuur bepaald. Zoals gezegd bestaat er een grote verscheidenheid aan lectinen in allerlei planten en dieren, maar de vlinderbloemige lectinen lijken onderling sterk op elkaar. In de verschillende figuren in Hoofdstuk 1 (pagina's 10, 11 en 14) is te zien hoe deze lectinen eruit zien. Lectinen bestaan zoals alle eiwitten uit een lange keten aminozuren, die op een speciale manier is opgevouwen. Dit is te zien in Figuur 1. In dit geval is de keten zodanig opgevouwen dat het molecuul een onregelmatige cilindervorm heeft, en dat het begin en eind van de keten (de N-terminus en de C-terminus) vlak naast elkaar liggen. De aminozuurketen is een aantal malen aan elkaar geritst, zodat twee vlakken ontstaan, ook wel ß-sheets genoemd. In Figuur 1 zijn deze in rood en blauw weergegeven. De rest van de keten vormt een aantal lussen, waarvan vier samen de suikerbindingsplaats vormen (Figuur 3). Verder valt nog op dat alle vlinderbloemige lectinen vlak in de buurt van de suikerbindingsplaats twee metaalionen binden, een calcium ion en een mangaan ion (Figuur 2). Het lectine van de erwt (*Pisum sativum* lectine, oftewel PSL) bestaat uit twee van deze moleculen, die zodanig aan elkaar geritst zijn, dat twee ß-sheets in elkaar overlopen

(Figuur 5). PSL is daarom een voorbeeld van een dimeer lectine. Een ander lectine, CON A, is een voorbeeld van een tetrameer lectine: CON A bestaat uit twee dimeren, die "rug-aan-rug" aan elkaar gebonden zijn (Figuur 6). Alle bekende vlinderbloemige lectinen zijn dimeren of tetrameren, zodat ze allemaal ook twee of vier suikerbindingsplaatsen hebben. In de boon is de situatie ingewikkelder, omdat daar twee lectine varianten gevonden worden, PHA-E en PHA-L. Deze verschillende lectinen kunnen samen in een tetrameer voorkomen. In de boon vind je dus lectinen die uit vier E's bestaan of uit 4 L's, of alle denkbare mengvormen.

In vlinderbloemige zaden worden lectinen nadat ze gemaakt zijn naar speciale opslagvacuolen gedirigeerd. Deze opslagvacuolen (in het Engels protein bodies of protein storage vacuoles genoemd) bevatten naast lectinen nog meer eiwitten, die als voedselreserve voor het jonge kiempje worden gebruikt. Vlinderbloemige zaden (bijvoorbeeld erwten en bonen) zijn dan ook zeer eiwitrijk voedsel, en lectinen kunnen tot 10% van deze eiwitvoorraad uitmaken. In deze opslagvacuolen worden de lectinen bewerkt door een aantal eiwitsplitsende enzymen. Dit verandert niets aan de uiteindelijke vorm van lectinen. In PSL wordt elke monomeer een keer in tweeën geknipt, en de C-terminus wordt een stukje ingekort. Het tweede hoofdstuk van dit proefschrift beschrijft precies wat er met PSL gebeurt. Met CON A gebeurt iets bijzonders: De CON A monomeren worden niet alleen een keer doorgeknipt, maar de oorspronkelijke uiteinden van de keten worden weer aan elkaar geplakt. Hierdoor begint en eindigt de aminozuurketen op een heel andere plaats dan bij de meeste andere lectinen, zonder dat dit de vorm van het eiwit verandert. Dit verschijnsel wordt circulaire permutatie genoemd, en het is pas in 1985 voor het eerst beschreven.

Over de functie van lectinen in planten is nog niet erg veel bekend. Er zijn goede aanwijzingen dat lectinen in de wortels van vlinderbloemige planten een rol spelen bij het tot stand komen van een samenwerkingsverband, een *symbiose*, tussen deze planten en stikstof-bindende *Rhizobium* bacteriën. Als ze op stikstof-arme grond groeien, zijn alle vlinderbloemigen in staat om deze bacteriën op te nemen in de cellen van speciale wortelknolletjes. De bacteriën zetten vrije stikstof uit de lucht om in ammonia, een vorm van gebonden stikstof die de plant kan gebruiken. Elke plantesoort heeft een bijbehorende *Rhizobium* soort; de erwterhizobium is bijvoorbeeld anders dan de klaver-rhizobium. Het blijkt dat klaverwortels die PSL maken niet alleen klaver-rhizobia, maar ook erwte-rhizobia binnen kunnen halen, een sterke aanwijzing dat lectinen iets met herkenning van deze bacteriën te maken hebben. Door deze symbiose kunnen vlinderbloemige planten op stikstof-arme grond groeien. De planten zorgen voor hun eigen kunstmest. Dit is op dit moment vooral belangrijk voor landen in de Derde Wereld, waar kunstmest vaak te duur is.

De grootste hoeveelheid lectine zit echter in de zaden van deze planten, zoals al eerder gezegd. Het is bekend dat sommige lectinen giftig zijn voor mens en dier. Bij het ontkiemen van deze zaden komen grote hoeveelheden lectine vrij, die het jonge kiempje mogelijk bescherming geven tegen vraat. Hier komen we meteen op de negatieve kant van vlinderbloemige lectinen: ze worden beschouwd als anti-nutritionele factoren (ANF). Vooral bonelectine is berucht: als je rauwe bonen eet passeren de lectinen onbeschadigd de maag, en binden vervolgens aan darmcellen. Het darmoppervlak kan hierdoor flink beschadigd raken. Voor mensen is dit niet echt van belang, want wij eten geen rauwe bonen. Als de bonen goed gekookt worden gaan de lectinen ook kapot. In veevoer is de situatie anders: rauwe peulvruchten zouden beter veevoer opleveren, als ze geen giftige lectinen zouden bevatten.

Het zou natuurlijk een oplossing zijn om peulvruchten zonder deze giftige lectinen te kweken, maar dan verstoor je ook de symbiose met *Rhizobium* bacterien. Het dillemma is dus dat peulvruchten met lectinen minder wenselijk zijn voor de veevoerindustrie, maar peulvruchten zonder lectine minder wenselijk voor de landbouw.

Dit probleem is het belangrijkste onderwerp van dit proefschrift. We hebben gezocht naar een manier om lectinen te maken die in de plant gewoon hun werk doen, maar die na het oogsten van de zaden gemakkelijk te inactiveren (lees: door verhitting kapot te maken, of te verteren) zijn. De vlinderbloemige lectinen zijn behoorlijk stevige eiwitten, die pas uit elkaar vallen bij een temperatuur boven de 80°C. Om deze eiwitten minder stabiel te maken hebben we gebruikt gemaakt van een techniek die plaatsgerichte mutagenese heet. Zoals de naam al doet vermoeden maakt deze techniek het mogelijk om op een van te voren bepaalde plaats in het eiwit een aminozuur te vervangen door een ander aminozuur. Je brengt deze verandering niet direct in het eiwit aan, maar in het gen. Als het veranderde gen wordt teruggezet in de plant, wordt dit keurig vertaald in een eiwit met de gewenste verandering (mutatie). Daarna kan worden getest of het gewijzigde eiwit andere eigenschappen heeft dan het oorspronkelijke eiwit. In eerste instantie zijn we begonnen met het veranderen van PSL, niet omdat dit zo erg giftig is, maar omdat we al veel wisten van dit lectine. Zo was de structuur van dit eiwit al bekend, en die van bonelectine bijvoorbeeld nog niet. We hebben de mutaties aangebracht in delen van de eiwitketen die in alle vlinderbloemige lectinen (bijna) gelijk zijn, zodat mutaties die in PSL succesvol blijken te zijn ook in andere, meer giftige lectinen kunnen worden toegepast.

Zoals in Hoofdstuk 3 is te lezen, was de eerste mutatie die we in PSL aanbrachten al meteen raak. Als je het aminozuur valine op positie 103 vervangt door een alanine, ontvouwt het resulterende lectine al bij ongeveer 72°C, een verschil van ongeveer 10°C met het oorspronkelijke lectine. Bij kamertemperatuur is het veranderde lectine nog gewoon actief. We zagen ook dat het instabiele lectine bij 37°C geen haemagglutinatie meer vertoont, terwijl het oorspronkelijke PSL bij 45°C nog gewoon actief is. Deze inactivatie is echter niet onomkeerbaar: in tegenstelling tot de inactivatie bij 72°C bleek het gemuteerde lectine na afkoeling weer te werken. Dit zou zelfs kunnen betekenen dat het instabiele lectine helemaal niet meer verhit hoeft te worden. Immers, in darmen van vee is de temperatuur ook ongeveer 37°C.

Een tweede mutatie, het veranderen van fenylalanine 104 in alanine bleek maar een gering effect te hebben. Kennelijk speelt valine 103 een belangrijker rol in de stabiliteit van PSL. We hebben in een derde mutant beide mutaties gecombineerd. Het lectine dat dan gemaakt wordt blijkt zo instabiel te zijn dat het niet in actieve vorm kon worden gezuiverd.

In Hoofdstuk 4 is het aanbrengen van de mutatie, overeenkomstig met de valine mutatie in PSL, in een van de twee bonelectinen (de L variant) beschreven. Het blijkt dat dezelfde mutatie in bonelectine ook hetzelfde effect heeft, een verlaging van de ontvouwingstemperatuur met ongeveer 10°C. Deze verandering is vermoedelijk toepasbaar in alle vlinderbloemige lectinen. Helaas is bonelectine ondanks deze mutatie nog te stabiel om afwijkende activiteit bij 37°C te vertonen. Een tweede mutatie, bijvoorbeeld van het naastgelegen fenylalanine, lijkt noodzakelijk te zijn.

Om te kunnen testen wat het effect van vergrote instabiliteit is op de normale functies van PSL, zou je erwteplanten moeten maken die in plaats van het oorspronkelijke PSL het veranderde PSL bevatten. Helaas kunnen we dit bij de erwt (en bij de boon) nog niet voor elkaar krijgen. De proeven die in de hoofdstukken 3 en 4 beschreven zijn, zijn dan ook gedaan met lectine dat door een bacterie, *E.coli* genaamd, is gemaakt. Het oorspronkelijke gen voor PSL ofwel het veranderde gen is in deze bacterie gezet. Omdat de DNA "taal", de genetische code, in alle levende wezens dezelfde is, maakt de bacterie van het gen precies hetzelfde eiwit als de plant. Dit levert weliswaar informatie op over het veranderde eiwit, maar zegt niets over het functioneren van het eiwit in de plant. Ook is het zuiveren van lectinen uit *E.coli* veel moeilijker dan uit erwten of bonen. We hebben de PSL genen daarom ook in tabaksplanten gezet, om wat te weten te kunnen komen over hoe de veranderde lectinen zich in een plant gedragen. Tabak is een plant waar makkelijk andere genen in aangebracht kunnen worden. Het bleek dat PSL normaal in tabak wordt gemaakt, en het wordt zelfs net als in erwteplanten geknipt. De enzymen die verantwoordelijk zijn voor het knippen van PSL in de erwt komen kennelijk algemeen voor, ook in planten die niet direct verwant zijn aan vlinderbloemigen. Ook de dubbelmutant, die eerder niet in actieve vorm kon worden gezuiverd uit *E.coli*, kan wel uit tabak worden gezuiverd. Dit staat beschreven in Hoofdstuk 5. Ook hebben we PSL, en de varianten met mutaties, laten maken in klaverwortels. Als het lectine in deze wortels goed "werkt", kunnen erwterhizobia hierop wortelknollen vormen, zoals hierboven al is uitgelegd. Het destabiliseren van PSL bleek geen invloed te hebben op de gunstige invloed van het lectine op de symbiose. Dit is belangrijk, omdat dit een van de eisen was die we vooraf aan deze mutatie hebben gesteld. Ook dit is beschreven in Hoofdstuk 5.

In Hoofdstuk 5 staat verder beschreven, welke invloed destabilisering van PSL heeft op activatie van lymfocyten (witte bloedcellen) en op toxiciteit van lectinen voor rupsen. PSL, stabiel of instabiel, bleek lymfocyten tot deling aan te kunnen zetten. Als controle hebben we een PSL mutant getest die geen suikers meer kan binden (zie ook Hoofdstuk 6). Deze mutant bleek geen effect te hebben op de lymfocyten. Suikerbinding is dus noodzakelijk om de lymfocyten tot deling aan te zetten. Het feit dat beide instabiele lectinen lymfocyten kunnen activeren is een interessante waarneming. Deze proeven zijn namelijk bij 37°C gedaan, en de waarneming is in tegenspraak met de eerder beschreven resultaten van de haemagglutinatietest. Kennelijk hangt de stabiliteit van een eiwit af van de omstandigheden waaronder een test wordt uitgevoerd. Mogelijk is er een effect van andere eiwitten in de buurt, de zoutconcentratie, de zuurgraad enz. Het is duidelijk dat we pas kunnen zeggen of we hebben voldaan aan alle eisen, die aan het veranderde lectine worden gesteld, als we erwtemeel met dit lectine in plaats van het oorspronkelijke lectine aan vee te eten geven.

We hebben niet kunnen aantonen dat PSL giftig is voor de rupsen die we gebruikt hebben. Als dat wel gelukt was hadden we een goed modelsysteem gehad om de gifigheid van lectinen te testen. Het is natuurlijk een stuk moeilijker om zo'n test met ratten of varkens op te zetten, omdat je dan vele grammen lectine nodig hebt, hoeveelheden die we (nog) niet kunnen maken. Het effect van destabiliserende mutaties op de toxiciteit van lectinen zal daarom moeten worden onderzocht met meer giftige lectinen die werkzaam zijn in kleinere hoeveelheden. Eerst moeten we een manier vinden om voldoende zuiver, instabiel bonelectine te produceren.

In Hoofdstuk 6 is een mutatie in de suikerbindingsplaats van PSL beschreven. De verandering in de suikerbindingsplaats is minimaal: we hebben een asparagine vervangen door een aspartaat. Asparagine is een aminozuur met een zijketen die er uitziet als een Y. Het ene pootje wordt gevormd door een zuurstofatoom, dat bindt aan het calcium ion vlak in de buurt van de suikerbindingsplaats, en het andere pootje is een stikstofgroep (NH_2), die de suiker vasthoudt. Aspartaat heeft net zo'n Y, maar bij dit aminozuur zijn beide pootjes zuurstofatomen. Eigenlijk vervang je dus maar één atoom, en dat blijkt voldoende te zijn om de suikerbinding volledig te remmen. Deze mutant is niet meer actief, maar bindt nog wel gewoon calcium. Helaas is deze mutant ook niet meer in staat om de *Rhizobium* symbiose te stimuleren (zie proefschrift van collega Ron van Eijsden), en is dus ongeschikt als vervanger van PSL in erwten.

Samenvattend kan worden gezegd dat de verschillende experimenten die in dit proefschrift staan beschreven inzicht hebben verschaft in de structuur van lectinen uit vlinderbloemige planten, en hoe activiteit van lectinen kan worden beinvloed door plaatsgerichte mutaties. Hierbij moet worden aangetekend dat het werk aan PSL model staat voor het veranderen van lectinen die echt giftig zijn. Er is een begin gemaakt met het werk aan bonelectine. Uiteindelijk moeten de veranderde lectine genen natuurlijk ook in erwten en bonen worden teruggezet, zodat de eigenschappen van de mutant- lectinen beter getest kunnen worden.

CURRICULUM VITAE

Philippus Jacobus Hoedemaeker werd geboren op 7 september 1963 te Groningen. In 1981 behaalde hij het diploma Gymnasium-ß aan het Praedinius Gymnasium te Groningen, waarna werd aangevangen met de studie Biologie aan de Rijksuniversteit Groningen. In augustus 1988 werd het doctoraal examen Biologie afgelegd met als hoofdvak Moleculaire Genetica en als bijvakken Immunologie en (gedrags)neuroendocrinologie, onder leiding van respectievelijk prof.dr. G. Venema, prof.dr. T.H. The en prof.dr. B. Bohus. In 1989 bleef hij als gastmedewerker verbonden aan de vakgroep Moleculaire Genetica van de Rijksuniversiteit Groningen. Vanaf december 1989 tot december 1993 was hij in dienst van het Centrum voor Fytotechnologie RUL/TNO, en in die hoedanigheid als AIO verbonden aan het Instituut voor Moleculaire Plantkunde, Rijksuniversiteit Leiden. Van december 1993 tot maart 1995 was hij gastmedewerker bij hetzelfde instituut. Vanaf 1 mei 1995 is hij als post-doc verbonden aan het Ontario Cancer Institute in Toronto, Canada.

NAWOORD

Toen ik een keer met een vriend lunchte in de kantine van het Biologisch Centrum in Haren, nabij Groningen, keek hij de eerste vijf minuten geamuseerd om zich heen om vervolgens te zeggen: "Wat lopen er hier allemaal rare mensen rond!" Inderdaad, zo'n verzameling biologen is een bont gezelschap van allerlei verschillende mensen die op een fantastische manier met elkaar omgaan, niet alleen in Groningen, maar ook in Leiden. Aan de speciale sfeer die Botanie zo bijzonder maakte, en die daarna meeverhuisde naar het Clusius, hebben een groot aantal mensen op hun eigen manier een kleine, of soms een grotere, bijdrage geleverd. Iedereen die op 19 november 1993 het fantastische afscheidsfeest van het Botanisch Lab heeft meegemaakt, en toen de fototentoonstelling heeft gezien, weet ook dat die sfeer er al hing lang voordat ik er m'n eerste schreden had gezet.
Het is aardig dat deze speciale sfeer niet ten koste gaat van de wetenschap op het lab, getuige de vele publicaties die elk jaar worden afgeleverd en het aanzien van de groep in het buitenland. Ik moet nog zien hoe de situatie in Toronto is, als ik over enkele weken in het Ontario Cancer Institute ga werken. Met plezier en met weemoed denk ik terug aan m'n tijd hier. Ik heb hier naast een lieve vriendin een aantal vrienden voor het leven gevonden, die zeker deze kans zullen aangrijpen om een weekje in Canada te komen skien.

Tot ziens!

Flip

druk: Pasmans Offsetdrukkerij B.V., Den Haag

Stellingen, behorend bij het proefschrift "Structure and stability of legume lectin"

- 1 Erwtelectine is geen anti-nutritionele factor.

  Bertrand et al, *Sci Alimen 8: 187-212 (1988).*
  Koninkx et al, *Gastroenterology 102: 1516-1523 (1992).*
  Dit proefschrift.

- 2 De conclusie van Yamauchi *et al*, dat Concanavaline A in *Escherichia coli* op dezelfde manier gemodificeerd wordt als in *Canavalia ensiformis*, is gebaseerd op onvoldoende experimentele gegevens.

  Yamauchi et al, *FEBS Lett 260: 127-130 (1990).*
  Min et al, *FEBS Lett 301: 315-318 (1992).*

- 3 De bewering van Pak *et al*, dat het Blec-1 gen van de erwt codeert voor een vegetatief lectine, is voorbarig.

  Pak et al, *Plant Mol Biol 18: 857-863 (1992).*

- 4 Bourne *et al* gaan voorbij aan de vraag of de bacteriële celwandcomponenten, die door hen geco-kristalliseerd zijn met *Lathyrus ochrus* lectine, ook *in vivo* aan dit lectine binden.

  Bourne et al, *J Biol Chem 269: 9429-9435 (1994).*

- 5 Het model, gepostuleerd door Ardourel *et al*, waarin twee verschillende receptoren voor nodulatiefactoren betrokken zijn bij de regulatie van infectie van alfalfa door *Rhizobium meliloti*, had kunnen en moeten worden onderbouwd met (competitieve) bindingsstudies.

  Ardourel et al, *Plant Cell 6: 1357-1374 (1994).*

- 6 Het trekken van conclusies over functionele homologie tussen eiwitten, uitsluitend op grond van de primaire structuur, is een hachelijke zaak.

  Saier et al, *Mol Microbiol 11: 841-847 (1994).*

- 7 Bij het publiceren van modellen, theorieën en hypotheses in de wetenschappelijke literatuur wordt soms te weinig aandacht besteed aan controleerbare feiten en directe waarnemingen.

  Showalter, Science 267: 490-493 (1995).
  Kurzýnski, FEBS Lett 328: 221-224 en corrigendum 332: 202. (1993)

- 8 Nederlandse universiteiten besteden te weinig aandacht aan het verbeteren van hun imago als objectieve en onafhankelijke bron van kennis.

- 9 Elke bezuinigingsoperatie in het hoger onderwijs zonder invoering van een goede selectie van aankomende studenten "aan de poort" is gedoemd te mislukken.

-10 De fout die de meeste Nederlandse politici maken bij het tegemoet treden van een partij als de CD is dat zij het partijkader te veel en de achterban van de partij te weinig serieus nemen.

-11 Het uitblijven van uitgebreide "Marshall" hulp in de eerste jaren na het vallen van de Muur zou het Westen uiteindelijk het veelvoudige kunnen kosten aan bestrijding van de Russische georganiseerde misdaad.

-12 Het is onzinnig dat aan leidingwater hogere kwaliteitseisen worden gesteld dan aan "bronwater" uit flessen, aangezien het grootste deel van het leidingwater niet voor consumptie wordt gebruikt.

-13 Het luiden van kerkklokken is een verouderde en vooral ook overbodige vorm van massa-communicatie.

-14 De toename van de rekensnelheid van PC's wordt teniet gedaan door slordig geschreven software en door fabrikanten die de aan/uit schakelaar steeds beter weten te verstoppen.

-15 Een afgesloten hek tussen twee verder vrij toegankelijke terreinen, zoals het AZL terrein en het Boerhaave kwartier, heeft geen enkele functie.

-16 Muziek verbroedert meer dan sport.

-17 AIO zijn is geen beroep, maar een roeping.