

The amino acid sequence of peanut agglutinin

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The amino acid sequence of peanut (*Arachis hypogaea*) agglutinin was determined from three major fragments obtained by mild acid cleavage at Asp-Pro peptide bonds. The sequence of 236 amino acids has residues identical to those that form the metal-binding site and the hydrophobic pocket in concanavalin A and other lectins, although the overall similarity is only 42%. In the segments of peanut agglutinin that correspond to the four loops that form the carbohydrate-binding site in concanavalin A and favin, several central residues are homologous, while others show changes to smaller side chains, such as Tyr → Gly. The carbohydrate-binding site of peanut agglutinin may therefore have a similar peptide-backbone architecture, but form a considerably more open cleft.

Peanut agglutinin (PNA) is a legume seed lectin that is specific for D-galactose residues at non-reducing terminal positions of glycoconjugates [1]. This specificity has made it useful for histochemical and cell-surface studies. The lectin recognises both α - and β -galactosides, and the disaccharide of highest known affinity is the T-antigen structure Gal(β 1–3)GalNAc [2] (GalNAc, *N*-acetyl-D-galactosamine). Detailed studies of the interactions of PNA with monosaccharides and disaccharides have been made by UV difference [3, 4], fluorescence [5, 6] and NMR [7, 8] spectroscopy, and a model of the binding process has been proposed [8]. PNA is therefore one of the best characterized lectins in its functional aspects, but far less is known about its structure. It is a tetramer of subunits of molecular mass 27 kDa [9], each with one binding-site [3], and is not a glycoprotein [1]. PNA has been successfully crystallized for X-ray studies in two laboratories [10, 11].

A partial amino acid sequence was reported by Lauwereys et al. [12] who encountered difficulties with multiple genetic forms and poorly soluble peptides. The former are to be expected in view of the multiple bands seen in isoelectric focussing and chromatofocussing experiments [13, 14]. We have reinvestigated the amino acid sequence of PNA and report a complete sequence together with data on the genetic variation at some positions. The sequences of the peptide loops in the putative carbohydrate-binding-site region are discussed in relation to the X-ray results for the D-mannose-specific lectins concanavalin A [15], favin [16] and pea lectin [17]. A preliminary report of a partial sequence from this work has been published [18].

MATERIALS AND METHODS

Purification of the protein

Peanut agglutinin was purified from buffer extracts of delipidated peanut meal by affinity chromatography on a lactose-substituted agarose matrix [3]. The initial PNA sample was material used for spectroscopic studies [3, 4, 7, 8]; subsequent samples were prepared identically from the same batch of peanuts.

Chemical cleavage

The protein was cleaved at Asp-Pro peptide bonds by limited acid hydrolysis [19]. PNA (22 mg) was dissolved in 2.28 ml 88% formic acid, 1.72 ml water was added and the solution was incubated at 37°C for 96 h. The digest was diluted and freeze-dried. The fragments were redissolved in 4 M guanidinium chloride, 0.2 M ammonium bicarbonate and separated by gel filtration on a Sephadex G75 column (1.6 cm $\times$ 80 cm) in the same buffer. The fragments were recovered by dialysis against water using dialysis tubing with a 3.5-kDa cut-off (Spectra/Por 3, Spectrum Medical Industries) and freeze-dried.

For general cleavage at aspartic acid residues, the proteins were digested in 0.25 M acetic acid at 110°C for 8 h [20]. Cleavage at methionine residues with CNBr was carried out in 88% formic acid under argon at room temperature for 24 h. The digest was diluted and freeze-dried, then dissolved and freeze-dried prior to HPLC.

Enzyme cleavage

Tryptic cleavages were carried out in 0.2 M ammonium bicarbonate with trypsin (treated with *N*-tosyl-L-phenylalanine-chloromethane; Worthington) purified by HPLC to remove chymotryptic contaminants [21]. The enzyme substrate ratio used was 1:50, and digestion was carried out at 37°C for 16 h. To restrict cleavage to arginine residues, PNA fragments were treated with equal amounts of succinic anhydride in

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Note. The amino acid sequence data published here have been deposited with the EMBL sequence data bank.

Abbreviations. PNA, peanut agglutinin; GalNAc, *N*-acetyl-D-galactosamine.

0.1 M NaHCO₃, then dialysed into the digestion buffer. Chymotryptic digests were carried out using the same conditions, except the digestion time was 4 h. The tryptic and chymotryptic digests were freeze-dried prior to separation by HPLC.

Peptide separation

The peptide mixtures obtained by the above methods were separated by reverse-phase HPLC, using acetonitrile gradients. The columns used were a C₄ column of 5- μ m material (100 mm $\times$ 4.6 mm, Synchropak RP-4; Synchrom Inc.) in 10 mM ammonium acetate, pH 6.9, and C₁₈ columns (250 mm $\times$ 4.6 mm and 50 mm $\times$ 4.6 mm, Synchropak RP-P; Synchrom Inc.) in 0.1% trifluoroacetic acid. The equipment was comprised of Beckman model 112 pumps, model 421 controller and model 165 ultraviolet analyser.

Amino acid composition

Protein and peptide samples were hydrolysed *in vacuo* at 110°C in 6 M for 22 h. Amino acid analyses were performed with a Durrum D-500 analyser.

Amino acid sequencing

Automated gas-phase sequencing was performed on an Applied Biosystems model 475A sequencing system with on-line identification of the phenylthiohydantoin derivatives or on a model 470A sequencer with identification of the derivatives by HPLC on a Varian Vista-56 chromatograph using a cyanopropyl column (250 mm $\times$ 4.6 mm, IBM Instruments, Inc.) maintained at 37°C and run in 30 mM sodium acetate/5% tetrahydrofuran, pH 4.88, with an acetonitrile gradient. For liquid-phase sequencing, a Beckman model 890C sequencer was used with a 0.5 M Quadrol program.

Mass spectrometry

Molecular mass was determined for the major fragments by Mr. B. N. Green of VG Analytical (Manchester, UK) on a VG ZAB-SE mass spectrometer equipped with a cesium ion gun [22]. The samples were dissolved in *m*-nitrobenzyl alcohol containing 1% trifluoroacetic acid.

RESULTS

Sequencing strategy

In a preliminary experiment with CNBr, it was discovered that the acid conditions were causing cleavage at Asp-Pro peptide bonds. The partial sequence [12] had two such sites at approximately residues 80 and 130. The strategy adopted therefore was to obtain the three acid cleavage fragments and sequence them from their tryptic peptides. The partial sequence [12] gave the overlaps of the acid fragments and these are substantiated by the general similarity of lectins in these areas (see alignment below). Also, the amino acid composition of PNA (Table 1) does not indicate any additional Asp-Pro sites. Initially, the order of the tryptic peptides was established by their similarity to other lectin sequences, as was performed for the *Onobrychis vicifolia* lectin [23]. Confirmatory overlaps of the tryptic peptides were obtained by other chemical or enzymic cleavages, with cleavage at aspartic acid residues by acid hydrolysis being particu-

larly useful for the largest fragment. The overall fragment set that establishes the complete sequence of 236 residues is shown in Fig. 1 and composition data for the three fragments from Asp-Pro cleavage are compared to sequence information in Table 1.

Separation of the major fragments

The products from treatment of PNA with formic acid were separated by gel filtration on Sephadex G75 under denaturing conditions, giving four peaks (Fig. 2). The first peak was mainly an aggregated form of the largest fragment (DP3), which forms the C-terminal half of the protein. The second peak also contained DP3, in monomeric form, together with some unhydrolysed DP1-DP2 adduct. The third and fourth peaks were, respectively, DP1, the N-terminal fragment of 80 residues, and DP2, the central fragment of 50 residues.

Fragment DP1

Half of the sequence of this 80-residue fragment was obtained from gas-phase sequencing of the intact PNA. The remainder was established from tryptic peptides (Fig. 1 and Table 2). Their order was confirmed by extension of the N-terminal sequence by liquid-phase sequencing to residue 48, and analyses of a CNBr peptide and two chymotryptic peptides. Only a small amount of tryptic cleavage was found at the Lys-Asp bond in the C-terminal region. The N-terminal 39-residue tryptic peptide was insoluble, so it could not be sequenced independently. The molecular mass calculated from the sequence was 8769.8 Da, in good agreement with the values of 8789 ± 2 Da obtained by mass spectrometry, assuming a sodium ion is present giving an MNa⁺ species. No genetic polymorphism was detected in this region of the protein.

Fragment DP2

This 52-residue fragment had only one lysine residue and no arginine. The overlap of the two tryptic peptides was established from N-terminal sequencing of DP2 (Fig. 1 and Table 2). Major (DP2a) and minor (DP2b) forms of this fragment could be separated by reverse-phase HPLC (Fig. 3). The amino acid compositions of the two forms differed by only a few residues, including Glu and Val (Table 1). The sequence differences were not investigated completely, but a chymotryptic peptide was obtained as an impurity in DP3 that indicated one difference was Glu92 $\rightarrow$ Val. Further sequence changes would be required to produce the composition seen for the minor form.

Fragment DP3

This 104-residue fragment contains the hydrophobic regions that gave difficulties in previous sequencing work [12]. Though peptides could be obtained by direct tryptic digestion (Fig. 1 and Table 3), the yields of peptides from cleavage at the arginine residues in the C-terminal half of the fragment were better from succinylated protein. The order of the tryptic peptides was confirmed from overlapping peptides obtained by acid cleavage at Asp residues (Fig. 1). The C-terminal sequence was extended by a chymotryptic peptide. Peptides obtained from pepsin and thermolysin digestions were also in agreement with the sequence shown (data not reported).

Table 1. Amino acid composition of PNA and major fragments

Values in parentheses are the number of residues deduced from the sequence. For PNA, the Thr, Ser and Val values are extrapolated from 24-h, 48-h and 66-h hydrolyses. Trp values are from hydrolysis in 4 M methanesulfonic acid. AA, amino acids

Amino acid	Amount in polypeptide				
	DP1 (AA 1–80)	DP2a (AA 81–132)	DP2b (AA 81–132)	DP3 (AA 133–236)	PNA (AA 1–236)
	mol/mol				
Asp	13.31 (14)	7.07 (7)	6.89	9.81 (11)	31.43 (32)
Thr	4.90 (5)	3.78 (4)	4.39	9.40 (13)	23.20 (22)
Ser	9.57 (10)	3.86 (4)	4.51	11.66 (14)	26.80 (28)
Glu	4.38 (5)	3.86 (4)	4.71	3.20 (3)	11.93 (12)
Pro	1.76 (2)	3.27 (3)	2.99	2.99 (4)	8.03 (9)
Gly	5.63 (5)	8.74 (9)	10.72	5.84 (7)	20.28 (21)
Ala	5.17 (5)	3.96 (4)	3.99	5.00 (5)	13.34 (14)
Val	7.92 (8)	3.32 (3)	4.77	11.75 (15)	24.95 (26)
Met	1.73 (2)				2.36 (2)
Ile	3.97 (4)	3.82 (5)	4.62	5.81 (7)	15.13 (16)
Leu	5.11 (5)	0.94 (1)	1.18	5.28 (6)	11.69 (12)
Tyr	1.80 (2)	2.09 (2)	2.26	0.89 (1)	5.20 (5)
Phe	6.63 (7)	4.19 (4)	5.33	3.29 (3)	13.81 (14)
His		0.86 (1)	1.06	1.29 (2)	2.88 (3)
Lys	3.20 (3)	1.11 (1)	1.58	4.58 (6)	9.77 (10)
Arg	1.93 (2)			3.84 (5)	6.96 (7)
Trp	0.9 (1)			2.4 (2)	3.3 (3)

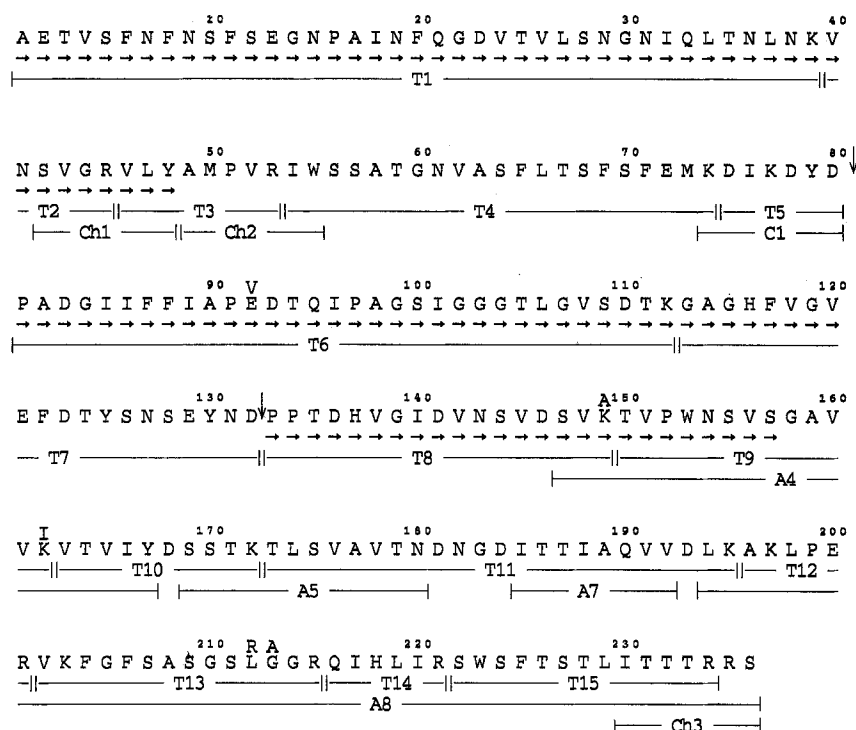


Fig. 1. Amino acid sequence of PNA and its peptide fragments. The two cleavage points that gave the three large fragments, DP1–DP3, are indicated with vertical arrows. Horizontal arrows indicate N-terminal sequencing of the intact protein, DP2 and DP3. The peptides labelled T are from cleavage with trypsin of the DP fragments, A from acid cleavage at Asp residues, C from CNBr and Ch from chymotrypsin. All were sequenced completely, with the exception of T1, and insoluble peptide, and the acid peptide A8 which was sequenced to residue 232. Where two residues assignments are shown, the upper one is from minor peptides

Molecular mass data could not be measured accurately for this fragment, and a value of $11\,123 \pm 30$ Da is the best estimate. This is close to the value calculated for the major sequence of 11 083.6 Da, particularly if a sodium ion form is

again present. Other evidence to substantiate residue 236 being at the C-terminus could not be obtained by carboxypeptidase digestion or other C-terminal methods. Termination at this position is consistent with other lectin sequences, however.

Table 2. Amino acid composition of tryptic peptides from fragments DP1 and DP2

Values in parentheses are the number of residues deduced from the sequence. T1 was an insoluble peptide, and was purified for analysis by size-exclusion HPLC in 6 M guanidine/HCl. AA, amino acids

Amino acid	Amount in peptide						
	T1 (AA 1–39)	T2 (AA 40–45)	T3 (AA 46–53)	T4 (AA 54–74)	T5 (AA 75–80)	T6 (AA 81–112)	T7 (AA 113–132)
	mol/mol						
Asp	8.93 (9)	1.06 (1)		0.89 (1)	2.78 (3)	3.34 (3)	3.92 (4)
Thr	2.85 (3)			1.12 (2)		2.80 (3)	1.12 (1)
Ser	3.64 (4)	0.99 (1)		2.68 (5)		1.98 (2)	2.00 (2)
Glu	3.30 (4)			0.63 (1)		2.11 (2)	2.06 (2)
Pro	0.91 (1)		1.11 (1)			2.38 (3)	
Gly	3.76 (3)	1.07 (1)		1.02 (1)		5.59 (6)	3.24 (3)
Ala	2.12 (2)		1.02 (1)	1.28 (2)		2.96 (3)	1.10 (1)
Met			0.99 (1)	0.05 (1)			
Ile	1.92 (2)			0.54 (1)	0.99 (1)	4.12 (5)	
Leu	2.87 (3)		0.95 (1)	0.85 (1)		1.13 (1)	
Tyr			1.04 (1)		1.05 (1)		1.96 (2)
Phe	4.32 (4)			2.18 (3)		2.28 (2)	2.02 (2)
His							0.75 (1)
Lys	1.59 (1)			0.92 (1)	1.03 (1)	1.10 (1)	
Arg		0.94 (1)	0.90 (1)				
Trp				(1)			

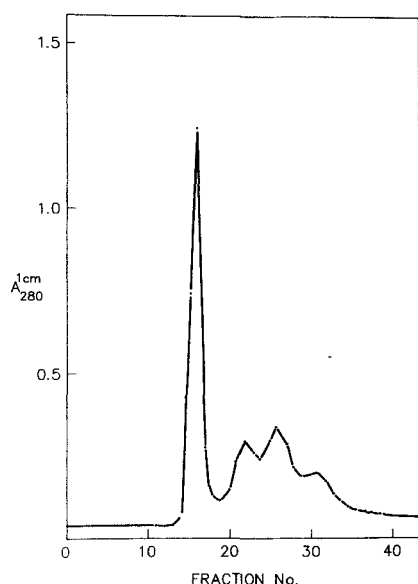


Fig. 2. Separation of mild-acid-cleavage products on Sephadex G75

Sequence variation was indicated at four positions in this fragment from minor peptides (Fig. 1), but separation of isomorphous forms could not be achieved by HPLC.

DISCUSSION

The PNA sequence was determined using two less common methods, limited acid cleavage at Asp-Pro peptide bonds [19] and stronger acid cleavage at Asp residues [20]. Both gave excellent results and deserve to be used more widely. One of the Asp-Pro sites (positions 132 and 133, respectively) is conserved in legume lectins and forms part of the calcium and carbohydrate-binding sites [15–17]. Hence, this cleavage should be of general use in lectin sequencing. The sequence

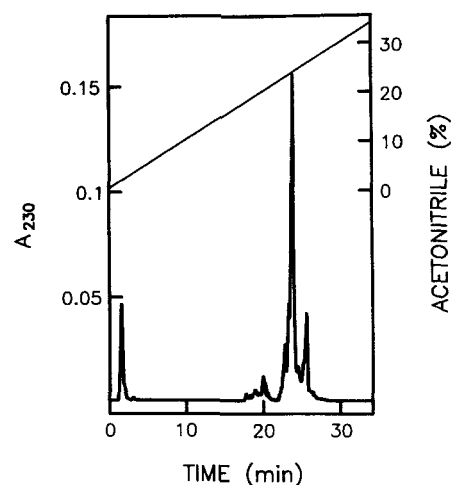


Fig. 3. Separation of major and minor forms of DP2. The C₄ HPLC column was run in 10 mM ammonium acetate, pH 6.9, with the acetonitrile gradient shown at the top

data were confirmed by measurement of the molecular masses of the large fragments by mass spectrometry, which has recently become capable of providing data for polypeptides of this size. From the separation of fragment DP2 into major and minor forms and the minor peptides encountered in DP3, PNA appears to have one major form and one or more minor forms. These are responsible for the isoelectin species seen in isoelectric and chromatofocussing [13, 14]. Heterogeneity was definitely established at five positions, 92, 149, 162, 212 and 213, four of which were changes between neutral and charged residues. Some of the variation occurs near one of the binding-site loops (see below) and may have functional consequences.

The PNA sequence is compared to those of concanavalin A and the pea lectin in Fig. 4. These two lectins had similar levels of identity to PNA as most of the legume lectins that have been sequenced, and were chosen because their three-

Table 3. Amino acid composition of peptides from fragment DP3

Values in parentheses are the number of residues deduced from the sequence. Peptides shown are the tryptic peptides plus the C-terminal acid-cleavage peptide A8. AA, amino acids

Amino acid	Amount in peptide								
	T8 (AA 133–149)	T9 (AA 150–162)	T10 (AA 163–172)	T11 (AA 173–195)	T12 (AA 196–201)	T13 (AA 202–215)	T14 (AA 216–221)	T15 (AA 222–234)	A8 (AA 194–236)
	mol/mol								
Asp	4.04 (4)	1.17 (1)	0.74 (1)	3.80 (5)					
Thr	0.98 (1)	0.75 (1)	1.66 (2)	4.10 (4)				5.02 (5)	4.93 (5)
Ser	1.89 (2)	1.73 (2)	1.72 (2)	1.88 (1)		2.89 (3)		3.93 (3)	6.03 (7)
Glu				1.33 (1)	1.00 (1)		1.11 (1)		2.06 (2)
Pro	1.91 (2)	0.89 (1)			0.97 (1)				1.01 (1)
Gly	1.15 (1)	1.17 (1)		1.11 (1)		4.07 (4)			4.01 (4)
Ala		0.79 (1)		1.68 (2)	0.89 (1)	1.04 (1)			2.17 (2)
Val	3.90 (4)	2.53 (4)	1.72 (2)	3.75 (4)		0.87 (1)			0.78 (1)
Met									
Ile	0.86 (1)		0.68 (1)	2.43 (2)			1.81 (2)	0.83 (1)	3.34 (3)
Leu				2.19 (2)	0.97 (1)	1.16 (1)	1.04 (1)	0.91 (1)	5.19 (5)
Tyr			1.05 (1)						
Phe						1.98 (2)		1.41 (1)	3.34 (3)
His	1.03 (1)						0.91 (1)		0.93 (1)
Lys	1.09 (1)	1.12 (1)	1.14 (1)	0.96 (1)	0.99 (1)	0.94 (1)			2.88 (3)
Arg					1.18 (1)	1.02 (1)	1.03 (1)	0.99 (1)	4.72 (5)
Trp		(1)						(1)	(1)

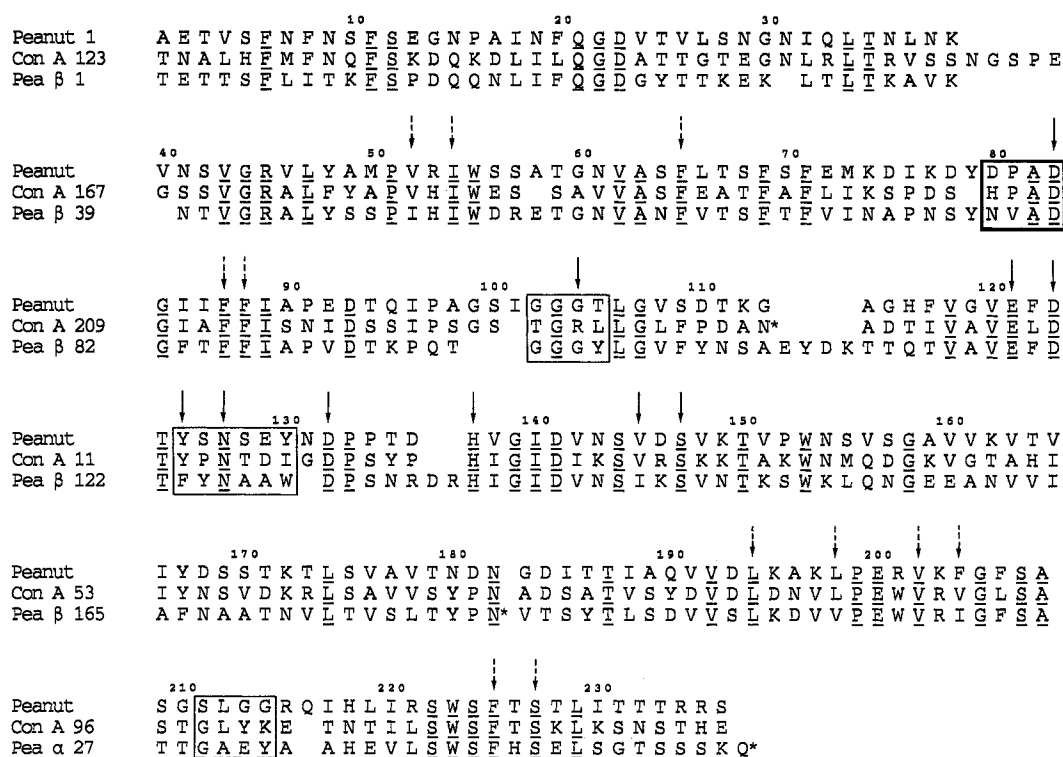


Fig. 4. Sequence comparison of PNA, concanavalin A and pea lectin. Residues homologous in all three sequences are underlined, and spaces represent gaps introduced to maximise similarity. The residues that form the double-metal site in concanavalin A (con A) are indicated with arrows, and those that form the hydrophobic pocket with dashed arrows [32]. The segments that form the carbohydrate-binding site are boxed. The sequences shown for concanavalin A [24] and pea lectin [25] were obtained by cDNA sequencing, but are shown in their post-translationally processed forms, i.e. concanavalin A in its N-terminal-ligated form, and pea lectin as α and β chains, with their C-termini indicated with asterisks

dimensional structures have been solved by X-ray crystallography [15–17]. The sequence of concanavalin A is shown in its circularly permuted alignment [24] which arises from

post-translational changes that include peptide ligation. The pea lectin sequence [25] is also shown in its processed form, i.e. separate α and β peptide chains. Three major features of

their structures are the metal sites that bind Ca^{2+} , and Mn^{2+} or Mg^{2+} , a hydrophobic pocket, and the carbohydrate-binding site. The pea lectin and favin [16] are virtually identical in these regions. The residues that interact with the metals in concanavalin A and the pea lectin are fully conserved in PNA. Analyses of PNA have shown that it has only Mg^{2+} [3], rather than the mixture of Mn^{2+} and Mg^{2+} found in most legume lectins [26]. In view of the sequence similarity, the lack of Mn^{2+} in PNA may reflect its availability in the plant rather than structural differences. Since the residues in this region are so conserved, the *cis* peptide bond arrangement that occurs in concanavalin A may be expected in PNA. The similarity of the residues that form the hydrophobic site is also high (Fig. 4). This site in concanavalin A was discovered through its ability to bind iodophenylglycosides [27], but has as yet no known function. Its concentration in PNA is a little higher than for interior residues in general.

A general model of the PNA-binding-site for galactosides can be derived from the sequence data. The carbohydrate-binding sites in concanavalin A and the pea lectin are formed from four peptide loops, which are shown boxed in Fig. 4. Despite the differences in sequence, the loops in these two lectins are of very similar geometry. The $\text{C}\alpha$ positions are within 0.1 nm (root mean square), and the residues that contact the ligand methyl- α -D-mannoside are homologous in the two structures [15, 28]. It is noteworthy that several of the H bonds between the ligand and the lectin involve atoms from the peptide backbone. Assuming that the PNA carbohydrate-binding site is in the same location, some sequence and structural differences are to be expected in PNA, since it has a quite different sugar specificity. However, the four PNA segments are as similar to those of either concanavalin A or the pea lectin as the latter are to each other. The D-galactose-specific PNA site appears to be more similar to these D-mannose-specific sites than it is to lectins specific for N-acetyl-D-galactosamine, such as soybean agglutinin and *Dolichos biflorus* agglutinin. The four residues that form the centre of the binding site, Asn14, Ala207, Asp208 and Gly227 in the concanavalin A sequence, are all conserved. Two of the PNA segments resemble concanavalin A, and the other two resemble the pea lectin. The major effect of the sequence differences would be to create a more open site in PNA than in concanavalin A. Several of the residues with large side chains in concanavalin A are replaced by glycine residues in PNA. These include concanavalin A residues Tyr100, Thr226 and Arg228.

On ligand binding, PNA develops a strong ultraviolet difference spectrum [3], but does not have as great a change in its CD spectrum in the near-ultraviolet region as concanavalin A [29]. These effects arise from tyrosine residues in the binding sites. One of the two concanavalin A tyrosines, Tyr12, is conserved in PNA as Tyr125, while the other, Tyr100, is changed to a glycine, Gly213. A second tyrosine residue occurs in the PNA site, Tyr130, which is in the position of Trp128 in the pea lectin, a residue that may contribute to the recognition of glycopeptides by this lectin [28]. Tyr125 is therefore likely to be responsible for the ultraviolet difference effects seen on monosaccharide binding, while the larger changes seen with disaccharides could be due to involvement of the second tyrosine. Based upon its structural similarities, it may be possible to model the binding site of PNA in more detail, as has successfully been done for immunoglobulins [30]. This is presently under investigation.

Preliminary X-ray studies of PNA [11] have shown that the protomers are arranged in the tetramer in the same way

as in concanavalin A. The PNA tetramer is more stable than that of concanavalin A, and does not dissociate into dimers until much lower pH [9, 31]. In concanavalin A, the dissociation is mainly due to the ionization of a cluster of histidine residues, the four His127 residues. The corresponding residue in PNA is Ser5, which would be expected to increase the acid stability.

In summary, the overall similarity of PNA to concanavalin A is 99 identical residues out of 236 or 42%, and with pea lectin 94 residues or 40%, with 64 residues (27%) being conserved in all three. Nevertheless, the major features of these lectins are strongly conserved in PNA, including many aspects of their carbohydrate-binding site.

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