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Crystal Structure of Concanavalin B at 1.65 Å Resolution. An “Inactivated” Chitinase from Seeds of Canavalia ensiformis

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Seeds of Canavalia ensiformis (jack bean) contain besides large amounts of canavalin and concanavalin A, a protein with a molecular mass of 33,800 which has been named concanavalin B. Although concanavalin B shares about 40% sequence identity with plant chitinases belonging to glycosyl hydrolase family 18, no chitinase activity could be detected for this protein. To resolve this incongruity concanavalin B was crystallised and its three-dimensional structure determined at 1.65 Å (1 Å = 0.1 nm) resolution. The structure consists of a single domain with a \((\beta/\alpha)\)₈ topology. A 30 amino acid residue long loop occurs between the second \(\beta\)-strand of the barrel and the second \(\alpha\)-helix. This extended loop is unusual for the \((\beta/\alpha)\)₈ topology, but appears in a similar conformation in the structures of the seed protein narbonin and several chitinases as well. Two non-proline \(\text{cis}\)-peptide bonds are present in the structure of concanavalin B: Ser34-Phe, and Trp265-Asn. This structural feature is rarely observed in proteins, but could also be identified in the three-dimensional structures of family 18 chitinases and narbonin in coincident positions. In the chitinases the aromatic residues of the non-proline \(\text{cis}\)-peptides have been proposed to have a function in the binding of the substrate. The region in concanavalin B, where in chitinases the active site is located, shows two significant differences. First, the catalytic glutamic acid is a glutamine in concanavalin B. Second, although part of the substrate binding cleft of the chitinases is present in concanavalin B, it is much shorter. From this we conclude that concanavalin B and family 18 chitinases are closely related, but that concanavalin B has lost its enzymatic function. It still may act as a carbohydrate binding protein, however.

Keywords: \((\beta/\alpha)\)-barrel structure; chitinase; seed protein; X-ray crystallography; \(\text{cis}\)-peptide bonds

Introduction

A major fraction of the proteins in seeds of legumes has been classified as storage proteins. These proteins are synthesised during maturation of the seed, deposited in protein bodies and degraded during germination. Apparently, they are unable to catalyse any enzymatic reaction. One of these proteins is narbonin, a seed protein from Vicia narbonensis. Although the crystal structure of narbonin (Hennig et al., 1992, 1995) revealed that it has the “TIM-barrel” \((\beta/\alpha)\)₈-fold (Banner et al., 1975), which was found exclusively amongst enzymes, the three-dimensional structure did not give any clues to an enzymatic activity. In an effort to obtain additional information on the biological function of this seed protein, further narbonin-like proteins from various plant sources were purified to compare their biochemical and structural properties. The first such protein was narbonin from Vicia pannonica, which shows 85% sequence identity to narbonin from Vicia narbonensis and which also lacks enzymatic activity. X-ray crystallography to

Abbreviations used: Endo F₁, endo-β-N-acetylglucosaminidase F₁; r.m.s., root-mean-square.
1.9 Å (1 Å = 0.1 nm) resolution revealed close similarity between the three-dimensional structures of the two narbonins (M.H. and B.S., unpublished results). Both amino acid sequence and three-dimensional structure, are particularly well conserved between the two narbonins in that region of the molecule where the active site in (β/α)₈-barrel enzymes are generally found, i.e. the C-terminal ends of the β-strands in the barrel and the subsequent loops.

Concanavalin B from jack beans (Canavalia ensiformis) is biochemically similar to narbonin. It was originally purified by crystallisation from crude seed extracts by dialysis against water and, as narbonin, classified as a storage protein (Sumner, 1919). The crystallisation of this protein, with ammonium sulphate as precipitant, has been published (Morrison et al., 1984). However, its three-dimensional structure has not been reported. The recently determined amino acid sequence of concanavalin B (Schlesier et al., 1995) showed a striking homology of concanavalin B to several plant chitinases that belong to the glycosyl hydrolase family 18 (see Henrissat & Bairoch (1993) and Beintema (1994) for a classification of glycosyl hydrolases).

Crystal structures are known for several family 18 chitinases: the bacterial endo-β-N-acetylglucosaminidase F₁ (Endo F₁) secreted by Flavobacterium meningosepticum (Van Roey et al., 1994) and chitinase A from Serratia marcescens (Perrakis et al., 1994) were determined at 2.0 and 2.3 Å resolution, respectively. Hevamine, a plant chitinase from the latex of Hevea brasiliensis was analysed to 2.2 Å resolution (Terwisscha van Scheltinga et al., 1994).

All these enzymes show a (β/α)₈-folding topology in the functionally important domain. The putative catalytic residue is a glutamic acid located in the cleft formed by the loops connecting the parallel β-strands of the barrel with the subsequent α-helices (Beintema, 1994).

Here we report on the crystallisation and crystal structure analysis at 1.65 Å resolution of concanavalin B. Several novel structural arguments for

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**Figure 1.** (a) Ribbon representation of the folding of concanavalin B with the numbering of secondary structural elements as used in the text. The secondary structure elements were identified using the program DSSP (Kabsch & Sander, 1983) as follows: strand β₁, 6-11; helix α₁, 19-24; strand β₂, 30-36; strand β₂', 38-39; helix α₂, 66-74; strand β₃, 78-84; strand β₃', 89-90; helix α₃, 95-109; strand β₄, 125-129; helix α₄, 139-153; strand β₅, 158-161; helix α₅, 173-176; strand β₆, 183-187; helix α₆, 202-214; strand β₇, 222-227; helix α₇, 241-247; strand β₈, 259-265; helix α₈, 267-273; helix α₈', 275-282. The four disulphide bridges are shown in ball and stick representation with carbon atoms as open and sulphur atoms as filled circles. (b) Stereo view of the concanavalin B molecule rotated by 90°. The cleft formed by the β₂ loops is at the top (program MOLSCRIPT: Kraulis, 1991).
the classification of “family 18” chitinases, like the identification of two conserved non-proline cis-peptide bonds and a characteristic extended loop following β-strand 2, are revealed. These features demonstrate that concanavalin B belongs to this family. However, the catalytic glutamic acid residue in chitinases is replaced by a glutamine residue in concanavalin B. This presumably is the main reason for its lack of catalytic activity. Possible functions for concanavalin B are discussed.

Results

Overall structure of the molecule

The overall structure of concanavalin B is shown in Figure 1. The molecule is very compact and shows no protrusions beyond an ellipsoid with dimensions of 30 Å × 40 Å × 50 Å. A total of 288 water molecules were identified in the asymmetric unit, but no electron density for metal ions or other ligands was detected. Concanavalin B consists of an eight-stranded parallel β-barrel surrounded by α-helices. Figure 1(a) illustrates the nomenclature of the α-helices and β-strands. The connecting loops will, in the following, be referred to as βxxα for loops from β-strand x to α-helix x and αxxβx + 1 for loops from α-helix x to β-strand x + 1, respectively. Four disulphide bridges (Cys41–Cys93, Cys54–Cys62, Cys23–Cys72, Cys165–Cys194) connect several βx loops (Figure 1).

Compared to the canonical (β/α)8-barrel (Brändén & Tooze, 1991) several βx loops contain insertions. The loop β2α2 resembles an antiparallel β-hairpin, although no direct hydrogen bonding is observed. The strands are connected by two hydrogen bonds mediated by water molecules. Figure 2 shows the backbones and hydrogen bonds

Figure 2. Stereo view of parts of the loops β2α2 and β3α3 forming the parallel β-sheet {2'-β3}. Only backbone atoms are shown, except for residues Cys41, Ile43, Pro46 and Cys93. The disulphide bridge Cys41–Cys93 stabilises the conformation (program MOLSCRIPT: Kraulis, 1991).

Figure 3. Ramachandran φ,ψ diagram for the refined structure of concanavalin B. Glycine residues are represented by triangles. Dark areas correspond to the energetically most favourable φ,ψ regions (see the text). This diagram was produced using the PROCHECK suite of programs (Laskowski, 1993).
Figure 4. Stereo view on the additional disulphide bridge of concanavalin B and its environment. Cys93 is the only residue with $\phi, \psi$ angles in a “forbidden” region of the Ramachandran plot (Figure 3). The electron density, contoured at 1.5$\sigma$ (blue) and 5.0$\sigma$ (red), was calculated with coefficients ($2F_o - F_c$) and $\alpha$, phases from the refined 1.65 Å structure.

of the $\beta_2\alpha_2$ and $\beta_3\alpha_3$ loops. These two loops are partially in $\beta$-strand conformation ($\beta_2$ and $\beta_3$, respectively) and together they form a two-stranded parallel $\beta$-sheet. Cys41 lies in a type I $\beta$-turn which is followed by a bulge containing the residues Ile43 to Pro46. A disulphide bridge, Cys41–Cys93, stabilises the loops $\beta_2\alpha_2$ and $\beta_3\alpha_3$. Cys93 is the only residue with unfavourable dihedral angles ($\phi = 65^\circ$, $\psi = -65^\circ$), but has well defined electron density (Figures 3 and 4).

Figure 5. Stereo view of the non-proline cis-peptide bonds Ser34-Phe (Top) and Trp265-Asn in concanavalin B. The electron density in blue (contoured at 3$\sigma$) was calculated with coefficients ($F_o - F_c$) and $\alpha$, phases from the refined 1.65 Å structure with the residues involved in the cis-peptide bond as well as water molecules (Top) and residues Pro227-Ala228 omitted. Atoms are shown in stick representation coloured yellow, red and blue for carbons, oxygens and nitrogens, respectively. Water molecules are depicted as red spheres (Program “O”, Jones et al., 1991).
Figure 6. Active site of concanavalin B. Carbon atoms are drawn in white, oxygen in red, nitrogen in blue and oxygen of the water molecules in green. Only side-chains of residues are drawn, with the exception of Ser34-Phe35 and Trp265-Asn266 to illustrate the two non-proline cis-peptide bonds (program MOLSCRIPT: Kraulis, 1991).

After helix \( \alpha2 \) the typical \( \beta2\beta \) folding topology is only interrupted by an additional short \( \alpha \)-helix \( \alpha8' \) in the loop \( \beta8\beta8 \). The additional \( \alpha \)-helix seems to be a common feature of several \( (\beta/\alpha)_8 \) barrel proteins (Farber, 1993). In two proteins involved in tryptophan biosynthesis, indoleglycerolphosphate synthase and phosphoribosylanthranilate isomerase, a phosphate binding site is located at the N terminus of this helix \( \alpha8' \) (Wilmanns et al., 1992). It is part of the substrate binding site. In concanavalin B this charge compensation is achieved by the side-chain of residue Asp16 from the neighbouring loop \( \beta1\alpha1 \) and a main-chain hydrogen bond of the peptide NH of Arg267 (\( \alpha8' \)) and the main-chain carbonyl group of Tyr10 (\( \alpha1\alpha1 \)). The close proximity to loop \( \beta1\alpha1 \) leaves no room for the binding of a ligand at the N terminus of helix \( \alpha8' \). In the structures of narbonin and hevamine this helix is present as well, but as in concanavalin B a ligand binding can be excluded (Hennig et al., 1995; Terwisscha van Scheltinga et al., 1994). In order to estimate the structural similarity of concanavalin B and hevamine the r.m.s. differences in 254 C\(^\alpha\) positions after optimal superposition of the \( (\beta/\alpha)_8 \)-molecule were calculated to be 1.4 Å (Program "O", Jones et al., 1991).

At four locations the electron density distribution could only be interpreted in terms of cis-peptide bonds. In the refinement the \( \omega \)-angles converged to values close to 0°. Only two of these conformations involve proline residues (Ser167-Pro and Tyr216-Pro). The other two cis-peptide conformations are found in the positions Ser34-Phe35 and Trp265-Asn266 (Figure 5).

Structure of the cleft formed by the \( \beta/\alpha \) loops

In \( (\beta/\alpha)_8 \)-barrel enzymes the active site is always located in a cavity at the C-terminal end of the parallel \( \beta \)-barrel, with the \( \beta\alpha\beta \)-loops giving the enzyme its substrate and reaction specificity. In concanavalin B a cleft is also present (Figure 1(b)). It is rather flat and about 7 Å deep, 8 Å wide and 30 Å long (Figure 6). The loops \( \beta3\alpha3 \) and \( \beta7\alpha7 \) are diametrically opposite and form the side walls of the cleft. They both have a proline residue at the top (Pro86 and Pro234). Loop \( \beta5\alpha5 \) shapes the major part of the bottom of the cleft with the backbone and side-chains of the residues Gly164 to Tyr171. This loop is stabilised by a disulphide bridge Cys165–Cys195. In addition, several side-chains from other loops delineate the cleft such as Gln131 and Arg187, which lie in close proximity in the central region of its bottom (Figure 6). An aromatic cluster formed by the side-chains of Trp265, Phe35 and Tyr189 borders the cleft at one end. The indole of Trp265 is accessible to the solvent and divides the cleft in two sections. Phe35 and Trp265 seem to require distinct structural constraints, because they are involved in cis-peptide bonds connecting Ser34 with Phe35 and Trp265 with Asn266. The other end of the cleft is a flat depression only. It is bordered by the backbone atoms of loop \( \beta2\alpha2 \) and by the side-chains of Gln13, Thr32, Asn266 and Gln268 (Figure 6).
Crystal Structure of Concanavalin B

Table 1. Alignment of the two conserved regions in family 18 chitinases for proteins with known three-dimensional structure

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>β3</th>
<th>β4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin B</td>
<td>77</td>
<td>VKVFLALGGPK 122 ALDGIFDIQKP</td>
</tr>
<tr>
<td>Hevamine</td>
<td>72</td>
<td>KVMLSAGGGI 118 VLDGFIDIEHG</td>
</tr>
<tr>
<td>Narbonin</td>
<td>78</td>
<td>VKVVISIGRRG 123 LDGDIYHEHI</td>
</tr>
<tr>
<td>Chitinase A</td>
<td>266</td>
<td>LKILPSGDMWT 306 FDGVQDDDEEP</td>
</tr>
<tr>
<td>Endo-F1</td>
<td>86</td>
<td>IKVILSILGHN 123 NLIDGFVFDDDEYS</td>
</tr>
</tbody>
</table>

Sequences shown are from *Canavalia ensiformis* concanavalin B (sequence Schlesier et al., 1995, structure this paper), *Hevea brasiliensis* hevamine (Jekel et al., 1991; Terwisscha van Scheltinga et al., 1994), *Vicia narbonensis* narbonin (Norg et al., 1995; Hennig et al., 1995), *Serratia marcescens* chitinase A (Jones et al., 1994) and *Flavobacterium meningosepticum* endo-β-acetylglucosaminidase F1 (Iarentino et al., 1992; Van Roey et al., 1994). Conserved residues are drawn in bold. The residue numbers and the secondary structure assignment correspond to the concanavalin B sequence and structure, respectively. The proposed catalytic residue in hevamine, chitinase A and endo-β-acetylglucosaminidase F1 is highlighted with an arrow.

Discussion

Concanavalin B is yet another member of the fast-growing family of structures having (β/α)₈-barrel topology (Farber, 1993). In addition to narbonin from the seeds of *Vicia narbonensis* and *Vicia pannonica* it is the second representative of the family with no demonstrated catalytic activity. The structures of the plant proteins concanavalin B and narbonin are highly similar to that of hevamine, a plant enzyme with both lysozyme and chitinase activity (Beintema, 1994; Terwisscha van Scheltinga et al., 1994). All these plant proteins and the bacterial members of family 18 chitinases such as endo-β-N-acetylglucosaminidase F₁ (Van Roey et al., 1994) and chitinase A (Perrakis et al., 1994) have (β/α)₈ topology and an extended β₂x₂-loop. These structural features can therefore be considered common characteristics. On the other hand there are also clear differences between these proteins. The bacterial enzyme chitinase A has two additional domains and endo-β-N-acetylglucosaminidase F₁ lacks helices α₅, α₆ and α₈β.

In view of the 39% sequence identity and the close similarity in three-dimensional structure between concanavalin B and hevamine, the question arises whether concanavalin B could have any chitinase activity. All (β/α)₈-barrel enzymes for which catalytic activity has been observed, have their active sites positioned in a cleft formed by the βx loops of the molecule. Surprisingly, this is also the region where the highest sequence identity between concanavalin B, hevamine and narbonin is observed (Coulson, 1994). However, if we look at essential residues for chitinase activity then it appears that these have been replaced in concanavalin B (Table 1). For instance, mutagenesis analysis identified Glu204 as an essential residue for activity in chitinase A₁ of *Bacillus circulans*. The mutation Glu204-Gln completely inactivated chitinase A₁ (Watanabe et al., 1993). The three-dimensional structure of hevamine assigned Glu127, which is equivalent to Glu204 of the *B. circulans* chitinase A₁, as a catalytic residue (Beintema, 1994; Terwisscha van Scheltinga et al., 1994). The corresponding residue in concanavalin B is Gln131 (Figure 7). This replacement might explain why concanavalin B has no chitinase activity. In narbonin a glutamate (Glu132) is present in an equivalent position to the catalytic glutamate in hevamine and chitinase A (Table 1). However, it is involved in a salt bridge with Arg87, an interaction which seems to be incompatible with catalysis. In agreement with
solution studies it is thus unlikely that concanavalin B and narbonin have chitinase activity. Nevertheless, these proteins may still have a function in binding chitin or other carbohydrates. Another conserved residue in chitinases (Gln181 in hevamine) was originally proposed to bind a putative catalytic water molecule for an inverting mechanism (Terwisscha van Scheltinga et al., 1994). However, recent results show unambiguously that hevamine acts via a retaining mechanism (Terwisscha van Scheltinga et al., unpublished results), refuting a catalytic role for the water molecule bound to Gln181.

How similar are the potential substrate binding regions in hevamine, concanavalin B and narbonin? In hevamine, the side walls of the substrate binding cleft are formed by the glycine-rich loops β3β3' and β7α7. In concanavalin B, either loop contains one proline (Pro86 and Pro234, Figure 6). Such Gly-Pro replacements will certainly have an effect on the flexibility of these loops, and they affect substrate binding, but they do not necessarily prevent binding of carbohydrates. In narbonin, the loops that form the side walls of the cleft are connected by a salt bridge formed by Arg87 (β3α3), Asp231 (β7α7) and Glu132 (β4α4) (Hennig et al., 1992, 1995). This salt bridge has to be disrupted to make the cleft accessible for oligosaccharides. Presumably, this salt bridge prevents narbonin from acting as a chitinase, in spite of the presence of a catalytic glutamic acid.

However, there are also several residues that are fully conserved. For example, the aromatic cluster Trp265, Phe35, Tyr10 and Tyr189 has almost identical conformations in concanavalin B, hevamine and narbonin. Two of these residues, Phe35 and Trp265, are involved in cis-peptide bonds in concanavalin B (Figures 6 and 7). This unusual geometry normally involves proline residues. Non-proline cis-peptide bonds have rarely been reported for proteins. Examination of the Brookhaven Protein Data Bank revealed only 0.05% of the non-proline peptide bonds to be cis (Stewart et al., 1990). Interestingly, in narbonin the Gly30–Phe31 and Trp261–Asn262 peptide bonds have this geometry as well, while recent further refinement of hevamine identified exactly the same feature there (A.C.T.S. & B.W.D., unpublished data). Moreover, also for the chitinase A from Serratia marcescens, the occurrence of three cis-peptide bonds has been reported (Perrakis et al., 1994). Two of these correspond to those described above. In another

bacterial chitinase, endo-β-acetylglucosaminidase F1, at least one cis-peptide bond has been observed (Van Roey et al., 1994). The positions of the cis-peptide bonds in the sequences are summarised in Table 2. The finding of this unusual geometry in five crystal structures in structurally identical positions is a striking common characteristic of chitin binding proteins of “family 18”. The residues involved are implicated in substrate binding in hevamine, as shown by the complex structure with N,N',N''-triacytldchitotriose (Terwisscha van Scheltinga et al., 1994). The phenylalanine and tryptophan residues can be assumed to be key residues in substrate recognition.

Which functions do concanavalin B and narbonin have in the seeds of legumes? First, the earlier classification of concanavalin B and narbonin as storage proteins can no longer be maintained. Recent biochemical investigations demonstrated that both proteins are very stable against proteolytic attack during germination of the seeds. In addition, the proteins are found in rather small amounts and thus cannot serve as a significant nitrogen source for the developing plant (Schlesier et al., 1995). Second, concanavalin B and narbonin exhibit close structural similarity to enzymes of family 18 glycosyl hydrolases. However, no chitinase activity could be demonstrated for them. This is in agreement with the finding that the catalytic glutamic acid residue in chitinases is a glutamine in concanavalin B.

Although in narbonin a glutamic acid residue is present at the equivalent position, this protein lacks a functional binding cleft for chitin-like substrates. A possibility could be that concanavalin B and narbonin interact with small saccharides. Especially a cluster of highly conserved aromatic residues, two of which are involved in cis-peptide bonds, suggests a function in carbohydrate binding. Seeds of legumes may contain substantial amounts of carbohydrate binding proteins, such as lectins. Little is known of the physiological function of legume lectins. It has been hypothesized that lectins can protect seeds against herbivorous animals (Chrispeels & Raikhel, 1991). Moreover, lectins are released upon imbibition of dry seeds, and therefore they are present in the vicinity of germinating seeds, and can contribute to the protection of the young seedlings (Chrispeels & Raikhel, 1991). In addition, it has been shown that lectins can play a role in the nitrogen-fixing symbiosis of legumes with Rhizobium bacteria (Diaz et al., 1989).

### Table 2. Local sequences around residues involved in non-proline cis-peptide bonds

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Concavavalin B</th>
<th>Hevamine</th>
<th>Narbonin</th>
<th>Chitinase A</th>
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</thead>
<tbody>
<tr>
<td>cis-peptide bond</td>
<td>34 SFL</td>
<td>31 AFL</td>
<td>30 GPA</td>
<td>190 GFF</td>
</tr>
<tr>
<td>Position</td>
<td>←β2→</td>
<td>←β8→</td>
<td>←β8→</td>
<td>←β8→</td>
</tr>
</tbody>
</table>

The proteins listed are identical to those of Table 1. The secondary structure corresponds to concanavalin B. Arrows show the positions of the cis bonds. A cis-peptide bond between Phe45 and Ser46 (β2 region), but none for the β8 region, have been reported for endo-β-acetylglucosaminidase F1.
Future work will be focused on systematic investigations of the carbohydrate binding properties of concanavalin B and narbonin. In addition, heterologous expression and purification of the proteins pave the way for site-directed mutagenesis to exchange tentative active site residues to those observed in glycohydrolases in order to determine the catalytic and carbohydrate binding properties of such mutants.

Materials and Methods

Enzymatic characterisation

Chitinase activity was assayed spectrophotometrically with CM-Chitin-RBV (Loewe Biochemica, München) as a substrate (Wirth & Wolf, 1990) using McIlvaine buffer (McIlvaine, 1921), 0.1 M Tris-HCl and 0.1 M acetate at pH values between 3.0 and 9.5 at 40°C for two hours. Lysozyme activity was determined by monitoring the turbidity between 3.0 and 9.5 at 40°C. Original and tenfold-diluted McIlvaine buffers at pH 5.0 and 8.0 were used. The crude extracts of seed flour from *Canavalia ensiformis* and *Vicia narbonensis* exhibit chitinase activity with pH-optima of 5.5 and 6.6, respectively. This activity is no longer present in purified concanavalin B preparations.

Purification and crystallisation

Concanavalin B was purified as described (Schlesier et al., 1995). Briefly, flour from mature seeds was stirred for 30 minutes at room temperature with 2.5 parts (v/w) of 0.1 M Tris-HCl buffer (pH 9.5) containing 0.5 M NaCl. After centrifugation for 30 minutes at 6000 × g for 30 minutes at room temperature with 2.5 parts (v/w) of McIlvaine buffer, the supernatant was dialysed against an excess of distilled water at 4°C in five days. The content of the dialysis bag was diluted by five parts (v/v) of 0.1 M Tris-HCl buffer (pH 8.0), 0.27 M NaCl and the microcrystals of concanavalin B were collected by sedimentation. The crystals were dissolved in 0.1 M citric acid and subsequently the pH was increased from pH 3.0 to pH 5.0 at 4°C in steps of 0.5 pH unit by dialysis against McIlvaine buffer. Crystallisation by pH-shift is a suitable method for the growth of well-diffracting crystals for high resolution X-ray analysis (Hennig & Schlesier, 1994). Crystals with a maximum size of 4 mm × 0.3 mm × 0.3 mm grew within a week. The crystals diffract to better than 1.4 Å resolution. The structural analysis revealed that they belong to space group *P6*1, with unit cell parameters *a* = 81.4 Å, *c* = 102.2 Å, *α* = *β* = 90°, *γ* = 120°. One molecule in the asymmetric unit corresponds to a Vm value of 2.88 Å³/Da and a solvent content of about 57% using the sequence derived molecular mass of 33,828 (Matthews, 1968).

Data collection and processing

A needle-shaped crystal was mounted in a glass capillary and X-ray diffraction data to 1.65 Å resolution were measured on a MAR-Research image plate area detector system mounted on a modified Elliott GX-20 rotating anode generator (CuKα, 40 kV and 50 mA). Data evaluation was performed with the XDS program package (Kabsch, 1988). The integrated intensities were converted into CCP4 format by ROTAPREP, scaled with the program ROTAVATA and merged using AGROVATA. The reflection intensities were finally converted to structure factor amplitudes using TRUNCATE (SERC, 1994). The excellent quality of the diffraction data is indicated by an *R*sym of 4.2% for the entire data set. Details on data collection parameters and statistics of the measured data are summarised in Table 3.

<table>
<thead>
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<th>Table 3. Data collection statistics</th>
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<td>Unique reflections</td>
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<td>Reflections with I &gt; 3σ (%)</td>
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<tr>
<td>Completeness (%)</td>
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<td>Rsym</td>
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\[
R_{sym} = \frac{\sum_{hkl} |I(hkl)| - \langle I(hkl)\rangle}{\sum_{hkl} \langle I(hkl)\rangle}.
\]

Structure determination

Co-ordinates of the structures of narbonin (R-factor 15.9% for data between 10.0 and 1.8 Å, r.m.s. deviations from ideal bond length 0.015 Å, Brookhaven PDB code 1NAR) and hevamine (R-factor 16.9% for data between 10.0 and 2.2 Å, r.m.s. deviations from ideal bond length 0.014 Å, Brookhaven PDB code 1HVM) were used as models in molecular replacement. Several search models were tried for cross-rotation function calculations using AMoRe (Navaza, 1994). Cross-rotation calculation gave no

<table>
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<th>Table 4. Statistics of the final model</th>
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</thead>
<tbody>
<tr>
<td>No. of protein atoms</td>
</tr>
<tr>
<td>No. of water molecules</td>
</tr>
<tr>
<td>r.m.s. deviation from ideal distances (Å)</td>
</tr>
<tr>
<td>Mean B-factor for protein atoms (Å²)</td>
</tr>
<tr>
<td>Mean B-factor for water molecules (Å²)</td>
</tr>
<tr>
<td>R-factor* (10.0–1.65 Å, all data) (%)</td>
</tr>
</tbody>
</table>

\[
R-factor = \sum_{hkl} |F(hkl)| - F(hkl)|/\sum_{hkl} F(hkl).
\]

* R-factor*
significant solution, neither for narbonin nor hevamine models. The orientations of the molecule corresponding to 50 cross-rotation peaks with the highest correlation were tried in a translation search. The highest signal-to-noise ratio for the correlation coefficient and lowest R-factor were observed with a modified hevamine structure in which the residues 40 to 58 and 107 to 112 (hevamine numbering) had been removed and residues non-identical to concanavalin B had been substituted for serine residues (alanine or glycine residues in hevamine were not changed). The overall sequence identity between hevamine and concanavalin B is about 39% (Schlesier et al., 1995). A Patterson integration radius of 15 Å and a resolution range 15 to 4.5 Å gave the best signal-to-noise ratio in the rotation function calculation. A clear helix or loop region lowered the R-factor to 4.0 Å. This rigid body refinement reduced the R-factor from 49.0 to 45.2%. Extension of the resolution to 1.65 Å further and allowed extension of the resolution to 1.65 Å resolution. R-factor for all data in this resolution range of 51.3%.

Refinement
The position and orientation of the molecule, as a single rigid entity, were refined for ten cycles with X-PLOR (Brünger, 1992a) using reflections in the resolution range of 10.0 to 6.0 Å. This rigid body refinement reduced the R-factor from 49.0 to 45.2%. Extension of the resolution to 3.0 Å and further refinement of 21 rigid segments of the molecule (each segment containing at least one strand or helix or loop region) lowered the R-factor to 43.6%. The free R-factor (Brünger, 1992b), for 10% of the observed X-ray data not used in the refinement, was 47.1% after this step confirming the correctness of the solution.

After energy minimisation of the model (preparation step) and determination of a suitable weighting scheme a simulated annealing run was carried out. An initial temperature of 3000 K was lowered to 300 K by 25 K per cycle. Each cycle contained 40 time steps of 0.5 fs. This slow cooling refinement reduced the R-factor to 33.1% for all data between 8.0 and 2.2 Å. 2Fo − Fc and Fo − Fc, electron density maps were calculated and the first manual corrections of the model were performed using the molecular graphics program FRODO (Jones, 1985). After positional and B-factor refinement σA-weighted Fo − Fc and 2Fo − Fc, electron density maps (Read, 1986) were calculated and the model was extended and corrected further. Eight cycles of conventional positional and B-factor refinement, together with manual rebuilding, lowered the R-factor further and allowed extension of the resolution to 1.65 Å. The refined model includes residues 1 to 284 of the reported cDNA sequence (EMBL data library accession number X83426). The last 15 residues at the C terminus are not visible in the electron density map. The R-factor is 17.2% for all 39,989 unique reflections between 10.0 and 1.65 Å (no σ cut-off) with excellent stereochemistry (Table 4). Dihedral angles of the polypeptide backbone for all residues except Cys93 lie in allowed regions of the Ramachandran plot (Figure 3). Figures 4 and 5 show representative regions of the molecule with electron density superimposed. The atomic co-ordinates of the final model have been deposited with the Protein Data Bank, Brookhaven National Laboratory (reference: 1CNV).

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References


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