

Crystal Structure of a Hypoallergenic Isoform of the Major Birch Pollen Allergen Bet v 1 and its Likely Biological Function as a Plant Steroid Carrier

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Bet v 1l is a naturally occurring hypoallergenic isoform of the major birch pollen allergen Bet v 1. The Bet v 1 protein belongs to the ubiquitous family of pathogenesis-related plant proteins (PR-10), which are produced in defense-response to various pathogens. Although the allergenic properties of PR-10 proteins have been extensively studied, their biological function in plants is not known. The crystal structure of Bet v 1l in complex with deoxycholate has been determined to a resolution of 1.9 Å using the method of molecular replacement. The structure reveals a large hydrophobic Y-shaped cavity that spans the protein and is partly occupied by two deoxycholate molecules which are bound in tandem and only partially exposed to solvent. This finding indicates that the hydrophobic cavity may have a role in facilitating the transfer of apolar ligands. The structural similarity of deoxycholate and brassinosteroids (BRs) ubiquitous plant steroid hormones, prompted the mass spectrometry (MS) study in order to examine whether BRs can bind to Bet v 1l. The MS analysis of a mixture of Bet v 1l and BRs revealed a specific non-covalent interaction of Bet v 1l with brassinolide and 24-epicastasterone. Together, our findings are consistent with a general plant-steroid carrier function for Bet v 1 and related PR-10 proteins. The role of BRs transport in PR-10 proteins may be of crucial importance in the plant defense response to pathological situations as well as in growth and development.

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Introduction

Plants respond to pathogen attacks, mechanic or environmental stress by activating or upregulating

a large array of genes.¹ The most remarkable event in all phases of the defensive response is the increased expression of pathogenesis-related proteins (PRs), a diverse class of proteins with various functions and activities which represent a generalized plant defense response against a range of pathogens.² The classification of PRs now includes 14 families.³ The PR-10 family is a ubiquitous group of defense-related proteins whose biological function has not yet been elucidated.⁴ The PR-10 genes encode a family of intracellular polypeptides,

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Abbreviations used: PR, pathogenesis-related protein; StAR, steroidogenic acute regulatory protein; BR, brassinosteroids; LRR, leucine-rich repeat.

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over-expressed after fungal or bacterial infection, which are different from all previously described plant defense-related proteins.^{5,6} Bet v 1, the major birch pollen allergen, is a representative of the PR-10 protein family whose members have been identified in more than 70 species of flowering plants, both mono- and dicotyledons.⁴

The hypoallergenic isoform Bet v 1l is a member of a small gene family.⁷ The relative abundance of Bet v 1l in pollen is shown by mass spectroscopy (MS) to be about 7% of the total mass of pollen Bet v 1^{8,9} (and unpublished results). Bet v 1 and its homologues from other tree pollen, such as alder, hazel and hornbeam, and from various vegetables and fruits, such as celery and apple, are best known and studied for their allergenic properties.¹⁰ Yet, no conclusive study has been reported concerning the biological activity of PR-10 proteins. Biochemical studies suggest diverse functions, such as a ribonuclease activity of PR-10 proteins from ginseng^{11,12} and white lupin,¹³ and Bet v 1 from birch pollen^{14,15} as well as the enzymatic activity in phenylpropanoid biosynthesis or in a secondary metabolism such as shikonin production.¹⁶ However, the three dimensional structure of Bet v 1a variant¹⁷ shows no similarity to known enzymes with ribonuclease activity nor displays arrangements of amino acid residues resembling known catalytic sites. The first indication that Bet v 1l may be involved in the transport of steroid compounds came from the discovery of a high structural similarity between Bet v 1 and the START domain of human MLN64 protein, which is steroid binding domain related to steroidogenic acute regulatory protein (StAR) involved in cholesterol translocation.¹⁸ This finding led Neudecker *et al.* to test by NMR spectroscopy whether Pru av 1, a major cherry allergen similar to Bet v 1l, can bind plant brassinosteroid homocastasterone. On the basis of overlapping Heteronuclear single quantum coherence spectra it was concluded that Pru av 1 and Bet v 1l are probably steroid-carriers.¹⁹ Very recently, an other study aimed at elucidating physiological role of birch pollen Bet v 1 protein was conducted and showed that a wide range of physiologically active ligands, such as fatty acids, flavonoids and cytokinins, were able to displace fluorescence probe ANS bound in the Bet v 1l cavity.²⁰

Only a few three dimensional structures of the members of the PR-10 class of proteins are known to date: crystallographic and NMR structure of a major birch pollen Bet v 1 protein,¹⁷ NMR structure of a major cherry allergen Pru av 1¹⁹ and recently, the crystallographic structures of two proteins from yellow lupine.²¹ These structures show similar fold consisting of seven-stranded β sheet bending around a long α helix, as expected in view of the high sequence identity (beyond 40%). All of these structures represent free unliganded proteins. Here we report a crystal structure of a complex formed between Bet v 1l and two molecules of the bile salt ligand, deoxycholate, bound in a

large hydrophobic cavity of the protein. Notably, deoxycholate is structurally very similar to brassinosteroids (BRs), an important family of plant steroids. Together, our complex structure and the results from BRs binding studies revealed by MS experiments, strengthen the view that Bet v 1l is involved in the transport of plant steroids.

Results and Discussion

The crystal structure of Bet v 1l in complex with deoxycholate

The crystal structure of the natural, low-allergenic isoform Bet v 1l was determined to a resolution of 1.9 Å (Table 1) using the structure of the Bet v 1a variant 2801 (PDB accession code: 1BV1) as the molecular replacement probe.¹⁷ There are nine amino acid differences between Bet v 1l and Bet v 1a (in brackets): Ala9 (Thr9), Met18 (Leu18), Lys28 (Asn28), Val30 (Phe30), Asn57 (Ser57), Val91 (Ile91), Cys112 (Ser112), Val113 (Ile113), Asn125 (Asp125), as confirmed by the structure of Bet v 1l (Figure 1(a)). The final model of Bet v 1l corresponds to a full-length protein, from Gly1 to Asn159. The overall fold consists of a seven-stranded antiparallel β -sheet (β 1– β 7) which curves around a long C-terminal α helix α 3, while two additional short α helices (α 1 and α 2) interconnect β 1 with β 2 strand (Figure 1(a)). A large Y-shaped hydrophobic pocket is present inside the protein,

Table 1. Data collection and refinement statistics

Data collection statistics		
Space group	P2 ₁	P2 ₁
X-ray source	Rot. anode	Synchrotron
Resolution range (Å)		
Total	30.0–1.97	40.0–1.37
Outer shell	2.07–1.97	1.39–1.37
Total No. of reflections	32,201	121,704
Unique reflections	9670	27,483
Intensity ($I/\sigma(I)$) ^a	10.2 (1.8)	34.5 (3.7)
Completeness (%) ^a	95.4 (59.6)	97.1 (64.2)
R _{sym} (%) ^a	11.7 (37.1)	3.7 (25.2)
Refinement statistics		
Resolution range (Å)	30.0–1.97	40.0–1.9
Number of reflections	9659	9565
R _{crys} /R _{free} (%) ^b	19.7/24.0	18.6/20.8
No. of protein atoms	1228	1228
No. of water molecules	96	146
No. of ligand atoms	56	56
rmsd from ideal values		
Bond lengths (Å)	0.009	0.009
Bond angles (deg.)	1.33	1.29
Improper (deg.)	0.92	0.91
% Average B factor (Å ²)	16.3	16.3
Average B-factor (Å ²)		
Protein atoms	15.1	14.7
Solvent atoms	30.5	29.2
Ligand atoms	16.7	19.1

^a Values in parentheses refer to the highest resolution shell.

^b R_{free} calculated with 10% of reflections that were not used for refinement.

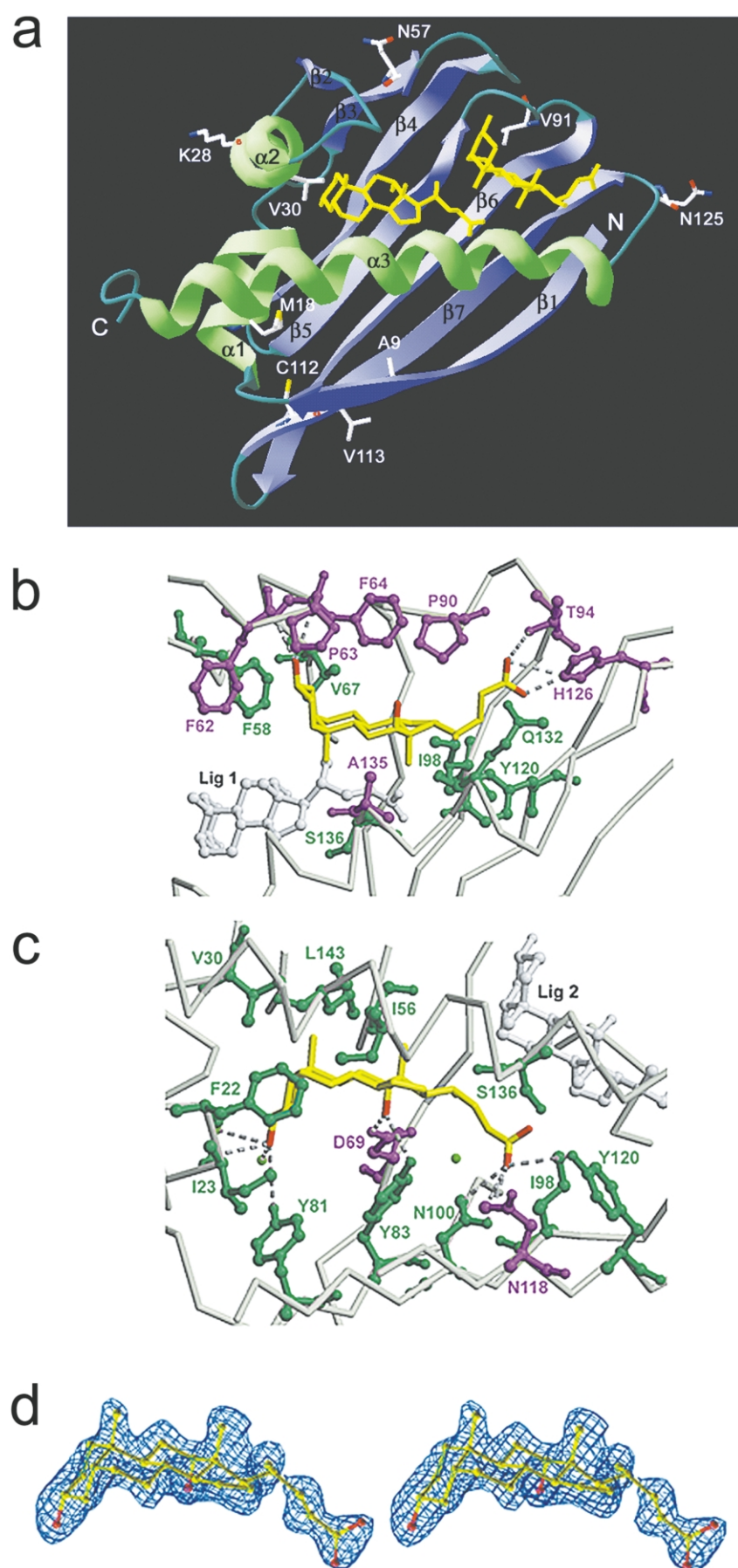


Figure 1. Crystal structure of Bet v 1l in complex with deoxycholate. (a) Overall structure represented by a ribbon diagram consists of a seven-stranded antiparallel β sheet and three α helices. The hypoallergenic isoform Bet v 1l differs from high-IgE binding Bet v 1a in nine amino acid positions, which are shown in stick representation. The two deoxycholate molecules bound in the hydrophobic pocket of the protein are colored yellow. (b) The atomic model of deoxycholate (yellow) bound in the outer binding niche. Protein residues which are conserved in >70% of known PR-10 proteins are colored green, non-conserved ones in purple. Most of the residues in contact with the outer ligand are not strictly conserved. The inner ligand (gray) is labeled Lig 1. (c) The atomic model of deoxycholate bound in the inner binding niche. The color scheme of amino acid residues is the same as in (b). Most of the residues in contact with the inner ligand are strictly conserved. The outer ligand is colored gray and labeled Lig 2. (d) Close-up stereo view of the electron density map associated with the inner deoxycholate ligand. A final SigmaA-weighted $2F_o - F_c$ map⁴³ (contoured at 1σ) has been calculated with the ligand omitted. Figures were generated using the programs Swiss-PdbViewer,⁴⁴ Molscript⁴⁵ and Raster3D.⁴⁶

formed between the residues lining the interior face of the β sheet and the three α helices. The amino acid substitutions do not alter significantly the structure of Bet v 1l compared to the Bet v 1a variant, the rmsd being 0.97 Å for 159 C_{α} atoms.

The most striking new structural information revealed by the structure of Bet v 1l, as compared to that of Bet v 1a variant, is the presence of a residual electron density located in the 30 Å deep, Y-shaped hydrophobic pocket. After careful refinement, two molecules of deoxycholate could be unambiguously modeled into the residual electron density (Figure 1(a)–(d)). Remarkably, sodium deoxycholate (Figure 2(a)), which was used as a reagent in the solubilization of the recombinant protein, remained bound to the protein throughout the purification process, indicating a very high affinity towards Bet v 1l. The two deoxycholate molecules are bound in tandem, and their relative orientation differs by an almost 180° rotation in the polycyclic ring plane (Figure 1(a)–(c)). One ligand molecule is almost completely inaccessible to solvent (12.3 Å² accessible surface, referred to as “inner” ligand), while the other deoxycholate exposes part of the steroid skeleton, side-chain, and the whole carboxylate moiety to the bulk solvent (59.0 Å², “outer” ligand). The inner deoxycholate forms hydrogen bonds (H-bonds) with several amino acids residues: one H-bond between the hydroxyl group at C3 and the carbonyl oxygen of Ile23 (α 1), two between hydroxyl group at C12 and Asp69 (β 4) and Tyr83 (β 5), and three between the terminal carboxylate and residues Asn100 (β 6), Asn118 (β 7) and Tyr120 (β 7). The outer deoxycholate molecule forms two H-bonds between the hydroxyl group at C3 position and Phe64 (loop between β 3 and β 4), and two between the terminal carboxylate and residues Thr94 (β 6) and His126 (loop between β 7 and α 3). Both ligands interact with Bet v 1l *via* extensive van der Waals contacts between the hydrophobic side-chains lining the binding cavity (Phe22, Val30, Ile38, Val41, Leu43, Ile56, Phe58, Phe62, Pro63, Phe64, Val67, Val85, Pro90, Leu95, Ile98, Tyr120, Met139 and Leu143) and the polycyclic ring system of deoxycholate.

The amino acid differences between Bet v 1l and Bet v 1a variant do not involve ligand-contacting residues and hence, the binding mode for both protein–ligand complexes is expected to be the same.

Deoxycholate versus brassinosteroids

Deoxycholate promotes lipid metabolism in mammals and is, to our knowledge, not found as a metabolite in plants. Thus, it cannot be the natural ligand for Bet v 1 and its homologous PR-10 proteins. However, deoxycholate shares a striking structural similarity with BRs, a family of ubiquitous plant steroid hormones (Figure 2(a)) that are implicated in plant development and growth processes.^{22–26} The distinctive structural features of BRs are confined to the substituents at positions

2, 3, 6, and 7 of the steroid skeleton and in the hydrophobic side-chain (Figure 2(a)). Mutant plants deficient in BRs biosynthesis show phenotypes of dwarfism, reduced fertility and light independent development.²³ The polycyclic ring structure and stereochemistry are analogous for deoxycholate and most BRs, while higher variability is found in the chemical nature of the A and B rings and side-chain substituents.²⁷ Evidently the chemical differences seen in BRs (Figure 2(a)) do not significantly alter the structural features of these molecules whose functional groups can interact with Bet v 1l *via* hydrogen bonding pattern similar to that observed with deoxycholate. The same interactions are not possible for sterol molecule, such as cholesterol, due to differences in both functional groups and stereochemistry of the A–B ring junction. Therefore, it is suggestive that deoxycholate acts as a molecular mimic of BRs, which are probably the natural ligands of Bet v 1l and homologous PR-10 proteins.

Docking brassinolide into the hydrophobic cavity of Bet v 1l

How would the binding of brassinolide to Bet v 1l differ from that which we observed with deoxycholate? We chose to model brassinolide into the Bet v 1l binding cavity, since it is the BR displaying the highest biological activity. The brassinolide model was built by using the coordinates of the crystal structure of the (22S,23S)-homobrassinolide²⁸ (CCD reference code BRASSA01) as a template. The docking of brassinolide as the inner and outer ligand was performed by the superimposition of rings C and D of the brassinolide moiety on the respective rings of inner and outer deoxycholate molecules. Brassinolide could be easily accommodated in the binding cavity of Bet v 1l without any steric hindrance (Figure 2(b)). All possible substituents at ring positions 2, 3, 6 and 7 of the steroid skeleton, which are distinct for BRs, could be fitted for the inner and the outer ligand without alteration of the binding mode observed with deoxycholate.

The alkyl side-chain of the inner brassinolide was modeled optimizing the interactions with the hydrophobic residues Val67 (β 4), Val85 (β 5), Ile98 (β 6) and Tyr120 (β 7) lining the binding cavity. Its conformational freedom is further restricted by the presence of the outer ligand. The hydroxyl groups at positions 22 and 23 of brassinolide form hydrogen bonds with the amino acid Tyr83 (β 5) and Asn100 (β 6), respectively. The hydroxyl groups at positions 2 and 3 are nearly within hydrogen bonding distance with the strongly conserved residues Lys54 (β 3) and Tyr81 (β 5). No similar hydrophilic residues were observed at equivalent positions of the proposed cholesterol binding site, as shown by the structure of the START domain, involved in cholesterol binding and translocation in humans.¹⁸ The alkyl side-chain of

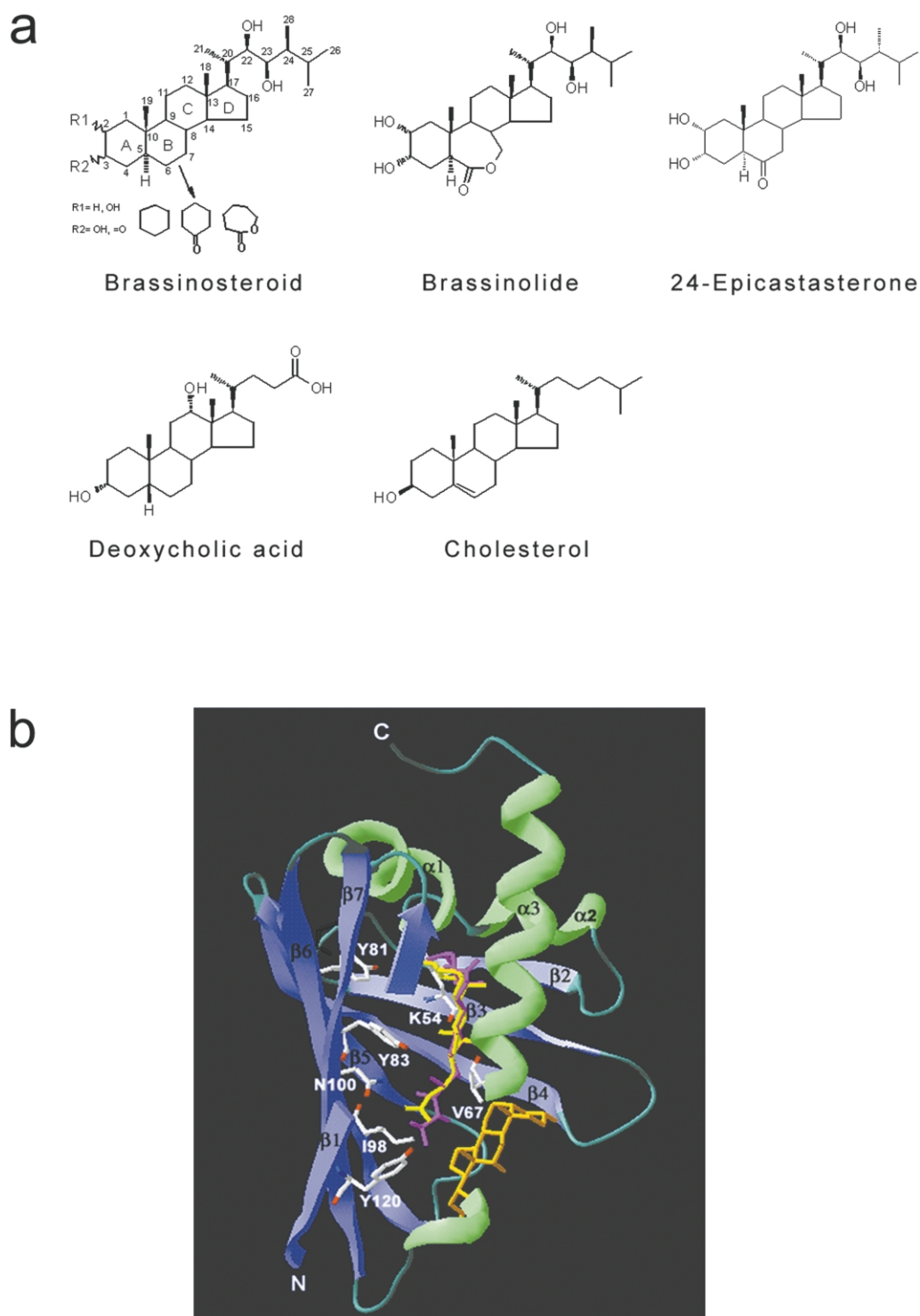


Figure 2. (a) Chemical structures of some plant steroids (brassinosteroids BRs, brassinolide and 24-epicastasterone), deoxycholate and cholesterol. For simplicity, only a simple C27 brassinosteroid structure is displayed together with the nomenclature of its skeleton (one or two additional methyl groups can be found at positions 27 or 28). Brassinolide, the BR with the highest activity, shows strong structural similarities with deoxycholic acid. Cholesterol, steroid molecule found in mammals is shown for comparison. (b) Ribbon representation of Bet v 1l with brassinolide shown as stick (magenta) modeled in the inner binding niche. Protein residues which interact with brassinolide are colored white. The bound inner (light yellow) and outer (dark yellow) deoxycholate molecules are shown for comparison. Part of the strand $\beta 1$ and helix $\alpha 3$ are omitted for clarity. The arbitrary orientation of Bet v 1l is chosen to optimize viewing of the protein–ligand interactions.

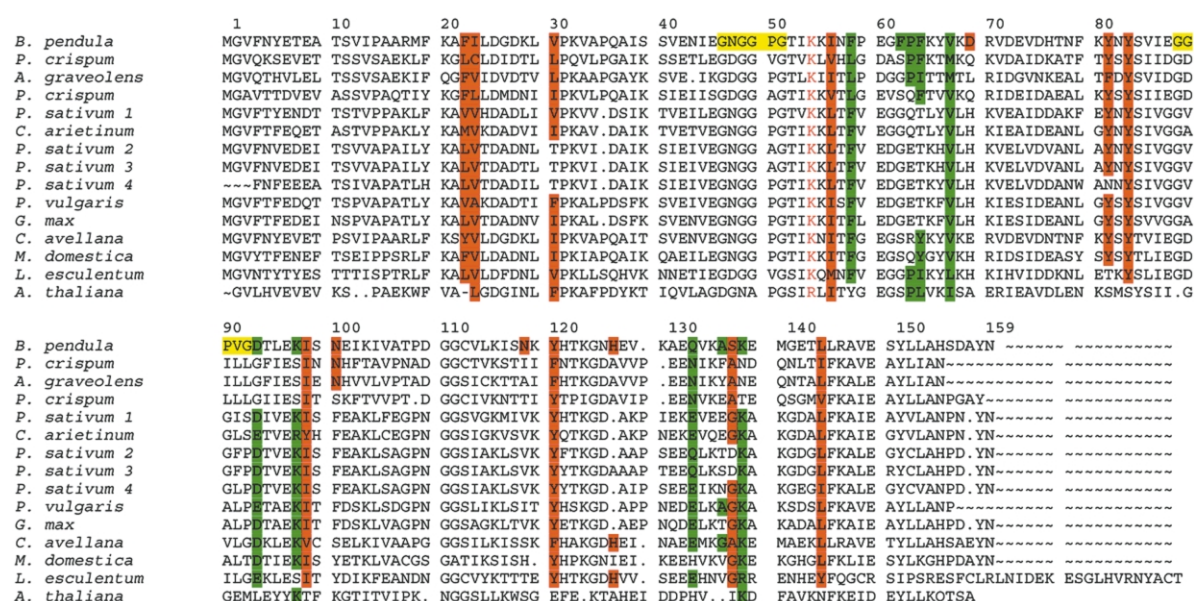


Figure 3. Sequence alignment of selected PR-10 proteins homologous to Bet v 1l (identified using a BLAST search of all protein and nucleic acid sequence databases). Fourteen representative proteins are listed from top to bottom: *B. pendula*, birch; *P. crispum*, parsley; *A. graveolens*, celery; *P. sativum*, garden pea; *C. arietinum*, chickpea; *P. vulgaris*, bean; *G. max*, soybean; *C. avellana*, hazelnut; *M. domestica*, apple; *L. esculentum*, tomato; and *A. thaliana*, thale cress. Amino acid sequence numbering is indicated on top of the alignments and refers to Bet v 1l. A residue conserved (either identical, or similar in size and hydrophilic/hydrophobic character) in >70% of the sequences is highlighted in green if contacting the outer ligand, and in red if contacting the inner ligand. The Gly rich loops are highlighted in yellow. Residue Lys54, proposed to interact with the 2-hydroxyl substitute of BRs, is colored pink.

the outer brassinolide molecule favorably interacts with hydrophobic residues Leu95 ($\beta 6$) and Val128 (loop between $\beta 7$ and $\alpha 3$; data not shown). The hydroxyl substituents at positions 22 and 23 are in a favorable orientation for hydrogen bonding interactions with Gln132 (not shown).

The observed mode of deoxycholate binding to Bet v 1l differs, however, from that derived from the modeling of the docking of two homocastasterone molecules into the NMR structure of the major cherry allergen Pru av 1, a homologue of Bet v 1.¹⁹ These authors have shown qualitatively by NMR titration experiments that Pru av 1 can interact with the plant steroid homocastasterone in solution. However, no quantitative data are available on the homocastasterone–Pru av 1 interaction and thus, in view of the large size of hydrophobic cavity there are several possible orientations in which two, or more, steroid ligands can be accommodated. Therefore, our structure of the deoxycholate–Bet v 1l complex represents the first experimental example of the possible docking of steroid molecules into the cavity of Bet v 1 and related PR-10 proteins.

An alignment of the primary structure of the 14 representative plant homologous of Bet v 1l present in the database (Figure 3) shows that all residues interacting with the inner ligand molecule are conserved in both chemical character and size, except for Asn100 which forms the hydrogen bond with the carboxylic group of inner deoxycholate (Figure 1(c)) and with the hydroxyl group at position 23 of the modeled inner brassinolide

(Figure 2(b)). Evidently, the absence of the carboxylic group in BRs and their precursors is not likely to alter significantly the binding of the natural ligands to Bet v 1l as compared to that observed with deoxycholate. In contrast, the outer ligand is bound more weakly and the residues of Bet v 1l contacting the outer ligand are not conserved in character or shape throughout the PR-10 protein family (Figure 3). In particular, the substitutions of small amino acid side-chains with bulky, aromatic residues is likely to result in an obstruction of the outer binding niche, preventing binding of the second ligand molecule. Thus, some members of the PR-10 family are more likely to display a 1:1 binding stoichiometry with steroid ligands.

On the basis of these results it is tempting to suggest that the interactions between functional groups of the inner ligand, which is bound more strongly than the outer ligand, with conserved Bet v 1l residues can be taken to represent the binding of BRs to different PR-10 proteins.

Mass spectroscopy of Bet v 1l interaction with brassinolide and 24-epicastasterone

The method of nanospray ionization MS was employed to examine whether brassinolide, biologically the most active BR, and 24-epicastasterone would bind to Bet v 1l. For these experiments, the unliganded Bet v 1l protein was prepared in the absence of deoxycholate. Our attempts to study non-covalent ligand–protein interaction by MS were hampered by a very low

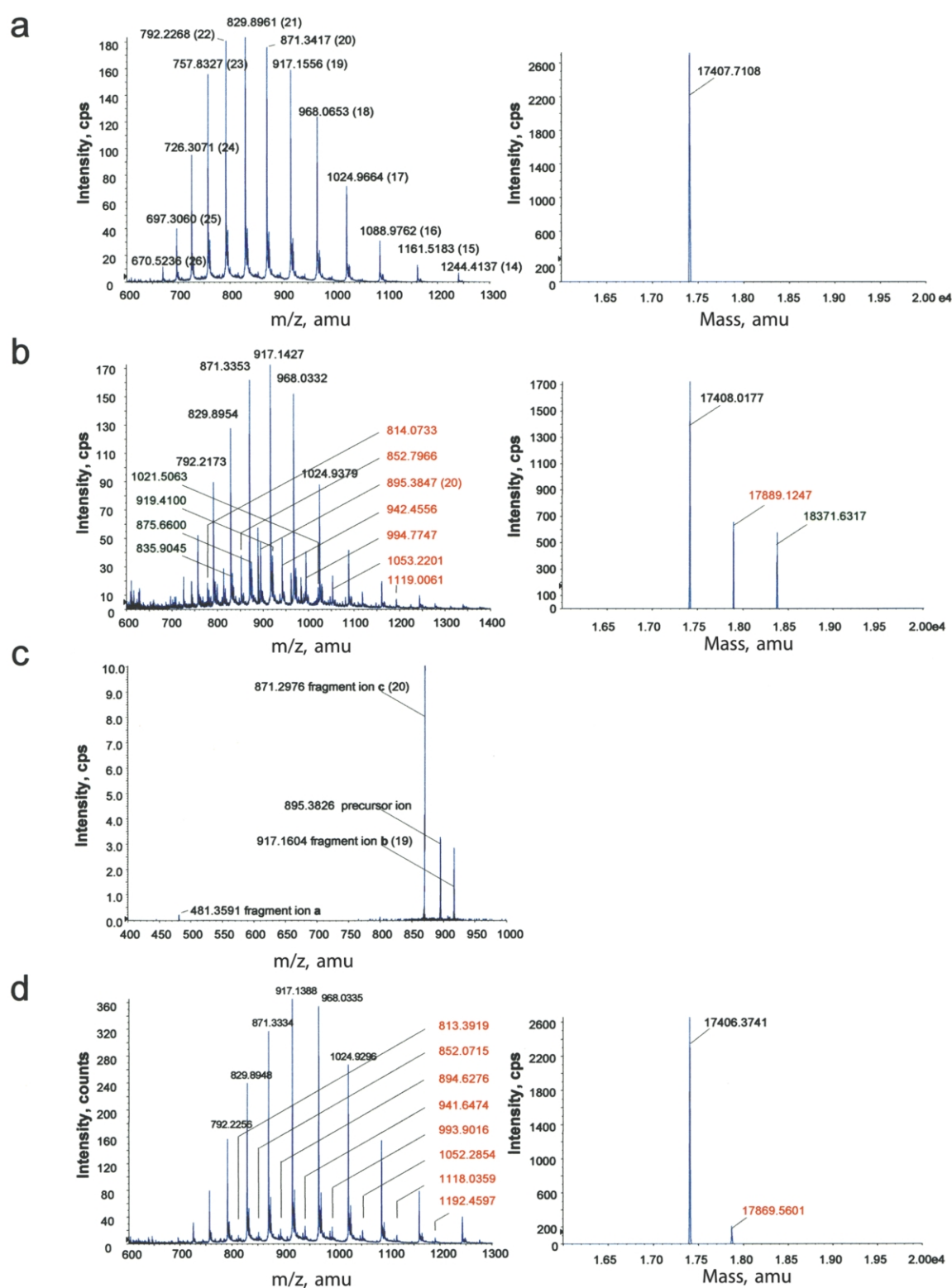


Figure 4. (a) Nanoelectrospray MS of the protein Bet v 1l, (b) the brassinolide-protein mixture, (c) nanoelectrospray tandem MS of the precursor ion m/z 895,38 originating from the protein–brassinolide–mixture, and (d) the 24-epicastasterone–protein mixture. (a) NanoMS spectra of free Bet v 1l (60 μ M; charge states indicated in brackets) deconvoluted to a molecular weight of 17407,7108 Da (right hand side of graph). (b) NanoMS spectra representing the charge distribution of Bet v 1l (black numbers), Bet v 1l plus one molecule brassinolide (red numbers) and Bet v 1l plus two molecules brassinolide (green numbers). Deconvoluted spectra are shown on the right hand side of the graph. (c) Low energy MS/MS (15 eV) of precursor ion m/z 895,38 resulting in the separation of brassinolide (fragment ion a) m/z 481,3591 and the protein Bet v 1l (fragment ion b) m/z 917,1604 and (fragment ion c) m/z 871,2976. (d) NanoMS spectra representing the charge distribution of Bet v 1l (black numbers) and Bet v 1l plus one molecule 24-epicastasterone (red numbers). Deconvoluted spectra are shown on the right hand side of the graph.

solubility of BRs in aqueous solvents. For brassinolide MS measurements were only possible in 70% (v/v) methanol at pH 2.5 (Figure 4). Far-UV circular dichroism (CD) spectroscopy was used to verify whether the native protein fold was retained under the solvent and pH conditions used for the MS analysis. The CD spectra of Bet v 1l recorded at three different solvent conditions (5 mM phosphate buffer (pH 7.0), 70% (v/v) methanol (pH 7.0), 70% (v/v) methanol (pH 2.5)) showed similar shapes and amplitudes (data not shown), indicating the absence of gross secondary structure changes of Bet v 1l in the solvent used for the MS experiments.

The MS spectra were recorded with a solution of free Bet v 1l (Figure 4(a)) and a mixture containing 60 μ M of Bet v 1l and 700 μ M of brassinolide (Figure 4(b)). In Figure 4(a) the different charge states of Bet v 1l were deconvoluted to an average mass of 17407,7108 Da (error deviation of 1 ppm, 5 ppm to the calculated mass MW 17407,787). The mass analysis of the protein–brassinolide mixture (Figure 4(b)) showed a presence of a free Bet v 1l (17408,0177 Da) and two complexes with a deconvoluted average mass of 17889,1247 Da (mass increase of 481.1 Da) and MW 18371,6317 Da (mass increase of 963.6 Da), revealing the attachment of one and two molecules of brassinolide ($[M + H]^+$ 481,3 Da).

Additional confirmation of a 1:1 binding stoichiometry came from the fragmentation of the precursor ion m/z 895,3826 (Figure 4(c)) which resulted in the two ion fragments of different sizes: m/z 48,13,591 corresponding to $[M + H]^+$ of brassinolide (error deviation 14 ppm) and m/z 91,71,604 as well as m/z 871,2976 corresponding to Bet v 1l charge state 19 and 20 (see Figure 4(a)).

To further investigate the specificity of brassinolide interaction with Bet v 1l a similar MS analysis was performed with highly related steroid 24-epicastasterone (Figure 2(a)). In this experiment a four fold higher concentration of 24-epicastasterone (3 μ M) in comparison to the former analysis of brassinolide was chosen for the protein–steroid mixture. Figure 4(d) shows the mass spectra of the interaction analysis, indicating the addition of one molecule 24-epicastasterone to Bet v 1l. The deconvoluted mass peak of Bet v 1l binding one molecule of 24-epicastasterone (17869,56 Da) shows a three times lower intensity than the deconvoluted mass peak of Bet v 1l binding one molecule of brassinolide (Figure 4(b), 17889,1247 Da) pointing to a lower binding affinity. Cholesterol was also used as a control ligand to check for the specificity of brassinolide and 24-epicastasterone interactions with Bet v 1l. Because of low solubility, the cholesterol was dissolved in 100% methanol (pH 2.5). No binding of cholesterol could be detected in the mixture of 60 μ M Bet v 1l and 300 μ M cholesterol whereas a clear interaction of brassinolide and Bet v 1l could be detected under similar experimental conditions (in 100% methanol, data not shown).

These results reveal a specific non-covalent interaction of Bet v 1l with brassinolide (1:1 and 1:2 stoichiometry) and 24-epicastasterone (1:1 stoichiometry). The fact that Bet v 1l binds not only the biologically most active brassinolide but also less active 24-epicastasterone, although with lower binding affinity, points to the role of Bet v 1l as a general steroid carrier. This is in agreement with our structural studies which showed that two molecules of deoxycholate are bound to Bet v 1l, the inner deoxycholate interacting more extensively with protein than the outer one.

Biological implications

Brassinolide was the first BR identified from rape pollen.²² The biological function of BRs was defined as regulating the processes of growth, development and gene expression.^{24,25,29} BRs elicit a large number of diverse biological activities in plants, including stem elongation, pollen tube growth, leaf bending and unrolling, and xylogenesis. BRs are important signaling molecules present at moderate levels (0.5 pg–30 ng/g fresh weight) in seeds, fruits, shoots and leaves, in contrast to significantly higher levels (5 ng/g–190 ng/g) found in pollen.³⁰ It is remarkable that the high expression of Bet v 1 proteins in birch pollen⁸ correlates well with the high levels of BRs detected in pollen. This observation is in agreement with a view that Bet v 1l and PR-10 proteins are involved in the binding and transport of hydrophobic BR molecules. This hypothesis is supported by the results presented here and the studies by Tsujishita *et al.*¹⁸ and Neudecker *et al.*¹⁹ It is conceivable that such a steroid carrier system could allow the availability of high levels of signaling molecules which could be readily delivered to their receptor(s) for quick response to external stimuli. The recent identification and characterization of the *Arabidopsis* receptor kinase BRI1, revealed that its extracellular leucine-rich repeat (LRR) and the transmembrane domain (70-amino acid “island”) are implicated in BRs binding which, upon binding, activates intracellular signaling events.³¹ This is a first example of a membrane-bound steroid receptor which possibly interacts with the BRs in complex with a steroid-carrier protein.²⁶

Mammalian steroid receptors are modular, soluble proteins with distinct domains interacting with DNA and the ligand, and analogous receptors have been suggested in plants.³² The role of PR-10 proteins in plants may well be to shuttle the BRs to a soluble receptor, both increasing the apparent concentration of the ligands and providing the means for a specific interaction. Further studies aimed at characterizing the binding affinity and specificity of PR-10 proteins for different BRs, as well as genetic studies of the phenotype induced by PR-10 mutants with abolished ligand binding properties are required to confirm these hypotheses.

Allergenicity

Nine naturally occurring Bet v 1 isoforms (a to g, j and l) were shown to differ in their ability to bind IgE from birch pollen allergic patients and to induce type I allergic symptoms.⁹ The low IgE binding isoform Bet v 1l show nine sequence differences (Figure 1(a)) with respect to the high IgE-binding isoform Bet v 1a. Sequence comparison of Bet v 1 isoforms with low and high IgE-binding capability combined with the site-directed mutagenesis studies revealed that IgE binding activity *in vivo* and *in vitro* depends on at least six amino acid residues at positions 9, 30, 57, 112, 113 and 125 of the Bet v 1 molecule.³³ This finding led to the design of a hypoallergenic six-point mutant which displayed significantly reduced IgE binding capability but still interacted with allergen specific T cells, and thus could be used in immunotherapy for the induction of immune tolerance without risks of allergic side effects.^{9,33,34}

The naturally occurring hypoallergenic isoform Bet v 1l contains all of these six amino acid exchanges and three additional ones at the positions 18, 28 and 91 as compared to Bet v 1a. The six exchanges revealed by mutagenesis studies (positions 9, 57, 91, 112, 113, 125) are located at opposite edges of the antiparallel β sheet floor whereas residues 18, 28 and 30 are located in the α helices 1 and 2. Although all nine residues are part of the molecular surface and thus, conceivably a part of the Bet v 1l epitope(s), only four are fully exposed to solvent (Lys28, Asn57, Val113 and Asn125). The Asp125Asn exchange, at the tip of the loop connecting β 7 with α 3 (Figure 1(a)), is found in all low allergenic isoforms of Bet v 1, indicating that it may be important for lower IgE binding despite the conservative character of the amino acid substitution. A similar dramatic effect of conservative exchange on antibody binding has been observed in anti-lysozyme IgG antibodies, where a single Arg to Lys substitution reduced antibody binding affinity by a factor of 10^3 .³⁵ None of the nine residues are part of the dominant Bet v 1 epitope revealed from the crystal structure of Bet v 1 in complex with a Fab fragment from a murine monoclonal IgG antibody.³⁶ There is only partial agreement with the three potential B cell epitopes defined by the contiguous surface patches which consist of residues fully conserved among all *Fagales* major pollen allergens.¹⁷ The exact identification of Bet v 1l epitope(s) must await further studies.

Concluding remarks

The hypoallergenic isoform of Bet v 1 is a paradigm of PR-10 proteins whose function has long been elusive. Here we present the first 3D structure of the stable, high affinity complex between Bet v 1l and two molecules of deoxycholate, a steroid molecule very similar to plant BRs. Guided by the locations of the inner and outer deoxycholates

bound in the hydrophobic cavity of Bet v 1l it is shown that two brassinolide ligands can be easily modeled in very similar orientations and locations. The binding of deoxycholate together with the results from MS study which revealed specific non-covalent interaction between Bet v 1l and two BRs brassinolide and 24-epicastasterone, suggest the role of Bet v 1l as the general, rather than specific, steroid carrier. The remarkable correlation of the high expression of both Bet v 1 and BRs in pollen further supports the view that Bet v 1 proteins fulfill a BR-carrier function.

In view of the observed stronger binding of inner ligand and the highly conserved nature of the involved amino acid residues it is tempting to propose that the interactions of Bet v 1l with the inner deoxycholate provide plausible model of the mode of interaction of BRs with Bet v 1 and homologous PR-10 proteins. Conceivably, the role of the PR-10 proteins in BR biology is similar to other transport proteins, that is, to sequester water insoluble hydrophobic ligands from the cytosol and deliver them to their receptors(s). It is probable that the Bet v 1-like fold has evolved for a steroid-binding function, and is conserved in both the plant and animal kingdoms.

Material and Methods

Crystallization

Recombinant Bet v 1l was purified from *Escherichia coli* harbouring the plasmid pMW175/Bet v 1l as previously described,³⁷ except for the addition of sodium deoxycholate to the resuspending buffer to a final concentration of 0.5% (w/v). Monoclinic crystals of Bet v 1l were obtained with the hanging drop vapor diffusion method using 100 mM sodium acetate buffer (pH 4.6), 20 mM ammonium sulfate and 30% (v/v) PEG-MME 2000 as precipitant.

Data collection, structure solution and refinement

X-ray diffraction data to a resolution of 1.9 Å were collected at room temperature on a rotating anode generator coupled to a Mar345 detector using the Cu K α radiation ($\lambda = 1.5418$ Å). High-resolution data at 1.37 Å were collected at the X31 beamline of the EMBL/DESY synchrotron ($\lambda = 1.05$ Å), at 100 K after brief soaking of the crystals in precipitant solution containing 20% (v/v) glycerol. Data were indexed, integrated and reduced using Denzo & Scalepack.³⁸ The crystal structure of Bet v 1a variant with PDB code 1BV1 (EMBL accession number Z80104, new isoform nomenclature Bet v 1.2801) was used as search model. This variant differs from Bet v 1a at position 62 where Leu is found instead of Phe. All solvent molecules and the loop with two conformations were removed from search model, the mutated side-chains truncated to alanine and the temperature factors reset to 12 Å². Rotation and translation functions were calculated using AMoRe.³⁹ After an initial simulated annealing procedure, the model was refined by a cyclic process of manual adjustment of the model into σ A-weighted $2F_o - F_c$, $F_o - F_c$, and annealed omit maps using the program O⁴⁰ followed by a conventional

energy minimization using CNS.⁴¹ Progress of the refinement was monitored by the decrease of the free R -factor while minimizing the divergence between R_{cryst} and R_{free} . In the third round of refinement two deoxycholate molecules could be unambiguously modeled in difference Fourier maps. Solvent molecules were added in the latest stages of refinement at positive peaks in $F_o - F_c$ density maps $> 3\sigma$, within hydrogen bonding distance to a donor or acceptor, and with a temperature factor $< 50 \text{ \AA}^2$ after refinement. The model quality was checked with PROCHECK⁴² which showed that 91% of all residues are within the most favored regions of the Ramachandran plot with no residue found in a disallowed conformation. Details of the refinement to high resolution (at 1.37 Å) will be published elsewhere.

Nanoelectrospray MS/MS

Dried protein was redissolved in water, desalted with ZipTipC4 pipette tips (Millipore, Eschborn, Germany) and eluted with 70% (v/v) methanol in 0.1% (v/v) formic acid. Brassinolide and 24-epicastasterone (Beak Technologies Inc., Brampton, Canada) were dissolved in methanol, cleaned with ZipTipC18 pipette tips (Millipore, Eschborn, Germany) and eluted with the same solvents as the protein. Nanoelectrospray tandem mass spectra were recorded on an API QSTAR Pulsar hybrid Quadrupole TOF instrument (Perkin Elmer Sciex, Foster City, USA). Precoated borosilicate nanospray needles from Protana were used in this set of experiments (needles: medium; flow rate 16 nl/min–20 nl/min, Odense, Denmark). Typical instrument settings were: electrospray voltage 1 kV; collision gas N₂; collision energy 15 eV. Data acquisition time was two minutes or until ion intensities were satisfactory. Analysis of the mass spectra was aided by the Bioanalyst software (Perkin Elmer Sciex, Foster City, USA).

Far UV circular dichroism of Bet v 11

The circular dichroic spectra of Bet v 11 under the solvent and pH conditions used for the mass spectrometric analysis, were recorded with a J-715 spectropolarimeter (JASCO, Inc., Easton, MD) using a 1.0 mm path length flat quartz cuvette. Far-UV (195–250 nm) spectra were recorded at 25 °C: resolution 0.2 nm, bandwidth 2.0 nm, sensitivity 20 mdeg., scan rate 50 nm/min and time constant two seconds. Each circular dichroic profile represents an average of ten scans. The baselines obtained with appropriate buffers(s) in the absence of protein were subtracted from the sample spectra.

Coordinates

Coordinates of Bet v 11 have been deposited for the structure at 1.97 Å resolution with the PDB, accession code 1FM4.

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