

Expression in *Escherichia coli*, Purification, and Spectroscopic Characterization of Two Mutant Bet v 1 Proteins

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Bet v 1 is the major birch pollen allergen. A highly efficient expression and purification scheme for mutant forms of this protein was developed on the basis of the pET expression system in order to provide the high quantities of protein needed for spectroscopic and structural work. Bet v 1 (M139L) protein could be purified at high yield (approx. 30 mg from 1 liter of LB medium) in a two-step procedure by the use of metal-affinity chromatography. Matrix assisted laser desorption ionisation mass spectroscopy, and size exclusion chromatography demonstrate the homogeneity and purity of the prepared protein. Spectroscopic methods were used to show that Bet v 1 (M139L) is structurally similar to wild type Bet v 1.

Furthermore, we investigated the influence of the nature of amino acid 139 on the thermodynamic behaviour of the protein by replacing the leucine residue by alanine. While there appears to be no global structural effect of this mutation, the thermostability of Bet v 1 is greatly decreased.

Key words: Allergen / His-tag / NMR-spectroscopy / Thermostability.

Introduction

About 20% of the population of developed countries suffer from type I allergies caused by allergens from pollen of grasses, trees, weeds, dander from domestic animals and insects, and spores of molds (Kaplan, 1985). Type I allergies are characterized by the immense production of IgE by B cells upon stimulation with antigen. IgE-synthesis is induced in a T cell dependent manner (Vercelli and Geha, 1989) and leads to the well described allergic reactions such as hay fever, dermatitis, asthma, and even anaphylactic shock. Especially the trees within the order Fagales contain potent allergens, the most common of which is Bet v 1, the main allergen of white birch (*Betula verrucosa*). 90% to 96% of the sera of tree pollen allergy patients react with Bet v 1, with 60% reacting solely to Bet v 1 (Larsen *et al.*, 1992; Jarolim *et al.*, 1989).

Recently, more than 24 different isoforms of Bet v 1 have been separated in extracts from birch pollen and it has been shown that each tree produces a different subset of isoforms (Löwenstein *et al.*, 1995). As B cell epitopes are mainly surface structures (Benjamin *et al.*, 1984), a model was proposed (Löwenstein *et al.*, 1995) in which amino acid substitutions are responsible for local perturbations of the allergen surface, causing differences in epitope structure.

Various isoforms of Bet v 1 have been investigated towards their ability to activate T cells and their IgE-binding characteristics. While isoforms a, e, and j show high IgE-binding activity, other isoforms such as d or l show very low binding activity to IgE. The antigenicity to T-cells, however, appears to be arranged in reverse order (Ferreira *et al.*, 1996).

Homology to pathogenesis-related proteins in pea and parsley indicates that Bet v 1 is involved in plant resistance (Breiteneder *et al.*, 1989), and that its expression can be induced upon stress (Scheiner, 1992). While there is no conclusive evidence on the function of Bet v 1 yet, recent reports point to the possibility that Bet v 1 might act as an RNase (Moiseyev *et al.*, 1994; Bufe *et al.*, 1996), a feature that might be common among pathogenesis-related proteins and other major allergens (Bufe *et al.*, 1995).

The isoform a of Bet v 1 is composed of 159 amino acid residues. It has a molecular mass of approximately 17 kDa, and has first been cloned and sequenced by Breiteneder *et al.* (1989). It shows sequence similarity to the pollen allergens of hazel (Breiteneder *et al.*, 1993), hornbeam (Larsen *et al.*, 1992), and alder (Breiteneder *et al.*, 1992), and there exists strong cross reactivity between these allergens (Rohac *et al.*, 1991). It was shown recently by ¹H-¹H and ¹H-¹⁵N-NMR-spectroscopy that Bet v 1a is a globular monomer in solution that consists of three α -helices and two β -sheets, two of the helices being located at the C-terminus (Faber *et al.*, 1996). This finding was confirmed by the elucidation of the tertiary structure of the protein by NMR-spectroscopy (F. Poulsen, pers. comm.).

In this report, we describe the overexpression of a mutant form of Bet v 1 (M139L) with a yield of approximately 30 mg from 1 liter of culture. Spectroscopic investigation of Bet v 1 (M139L) shows that the structure is similar to the wild type protein (Faber *et al.*, 1996). Therefore, we consider the expression system suitable to obtain Bet v 1 protein for structural studies. As residue 139 is located at a crucial position in a kink between two α -helices, we investigated the influence of the removal of an isopropyl group by replacement of leucine by alanine. We find that the alanine mutant displays a greatly reduced thermosta-

Fig. 2 Expression of Mutant His-tag Fusion Bet v 1 Protein in LB Medium Containing 100 $\mu\text{g}/\text{ml}$ Ampicillin. Lane 1: molecular weight standard; Lane 2: total cell lysate without IPTG, Lane 3: total cell lysate 4 h after IPTG addition.

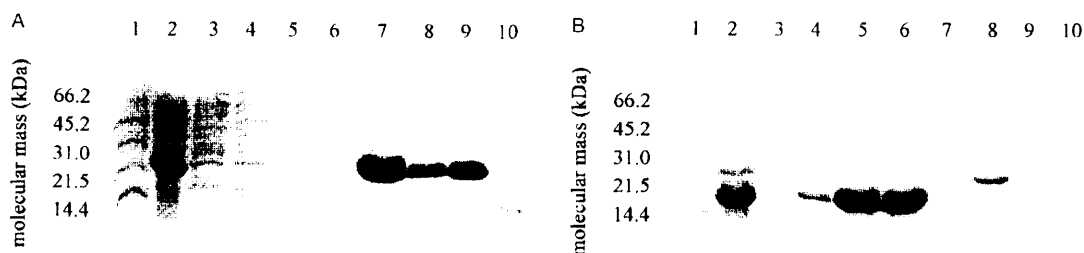


Fig. 3 Purification of Mutant Bet v 1.

(A) Purification of His-tag fusion Bet v 1 protein on a Ni^{2+} -sepharose column. Lanes 1, 10: molecular weight standard; Lane 2: cell extract; Lane 3: flow through; Lanes 4, 5: wash step with 5 mM imidazole; Lane 6: wash step with 100 mM imidazole; Lanes 7, 8: elution of fusion protein with 1 M imidazole; Lane 9: dialysate.

(B) Purification of mutant Bet v 1 protein on a Ni^{2+} -sepharose column. Lyophilized fusion protein was cleaved with CNBr, and the cleavage products subjected to metal chelate affinity chromatography. Lanes 1, 10: molecular weight standard; Lane 2: Lyophilized cleavage reaction; Lane 3: flow through; Lanes 4, 5, 6: elution of Bet v 1 with 5 mM imidazole; Lane 7: wash step with 100 mM imidazole; Lanes 8, 9: elution of uncleaved fusion protein with 1 M imidazole.

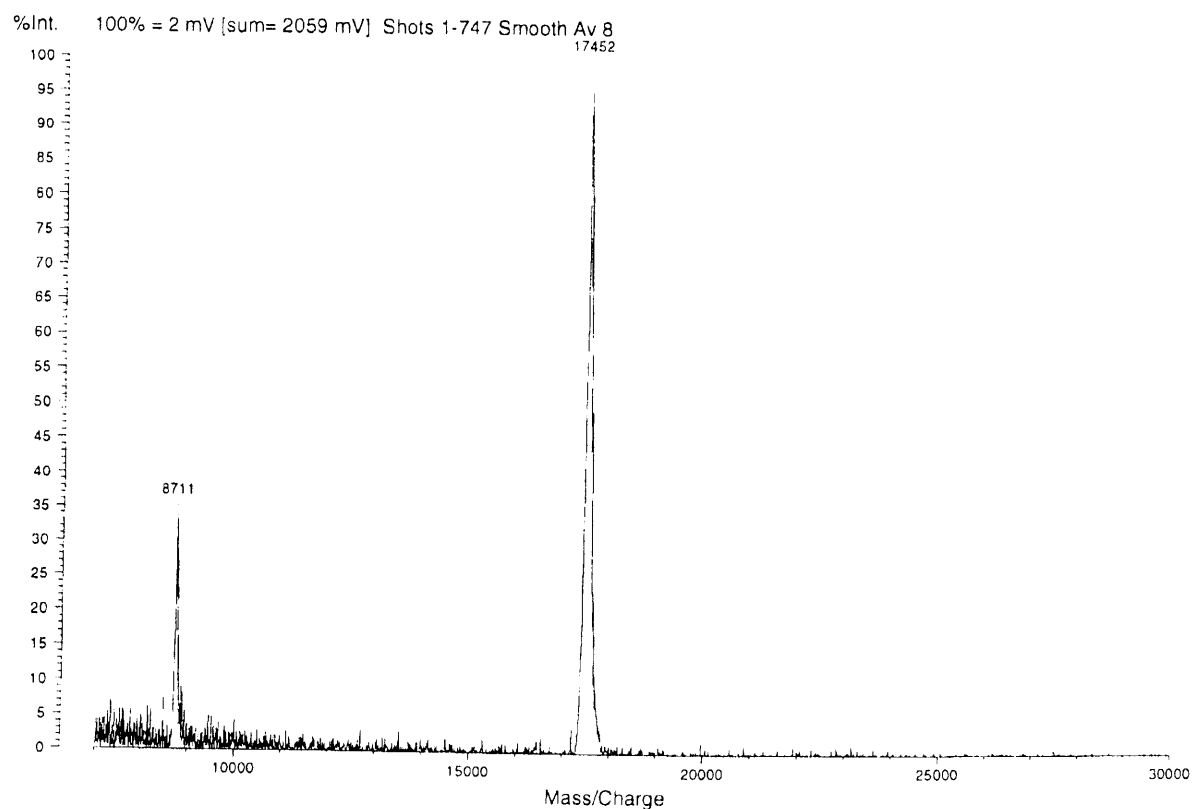


Fig. 4 MALDI-MS Spectrum of Bet v 1.

The matrix was sinapinic acid and spectra were recorded on a Kompact MALDI II mass spectrometer. The peak with half mass/charge ratio represents the double charged protein.

thereby be documented, the clone was stored as glycerol stock at -80°C until further use.

Fermentation

E. coli BL21/DE3-*pbet* and *E. coli* BL21/DE3-*pbetL* were both fermented on a 7 l scale in LB/Amp medium at 37°C . The bacteria grew to an OD_{600} of approximately 0.8 to 1.0

within 3.5 hours. After induction of the expression of Bet v 1 with 1 mM IPTG, the culture reached an OD_{600} of 4.9 within 4 additional hours. As no further growth was detectable, and the amount of Bet v 1 produced in the cells showed no further increase, cells were harvested after 4 hours. We obtained between 34 and 43 g of whole cells per 7-liter fermentation.

Purification of Bet v 1

The fusion of a tag of ten histidine residues to the Bet v 1 sequence allowed the rapid purification of the fusion proteins with immobilized metal ion affinity chromatography. As cellular proteins were not or only weakly bound by the Ni^{2+} equilibrated chelating sepharose column, they were removed by washing with buffer containing 5 mM or 100 mM imidazole (Figure 3A). The fusion protein eluted in buffer with 1 M imidazole. About 10 to 15 mg of fusion protein were obtained from 1 g of cells. The his-tag was removed by CNBr cleavage, and in a second Ni^{2+} -affinity chromatography step the tag as well as uncleaved protein was separated from recombinant Bet v 1 (Figure 3B). The purification resulted in the isolation of about 6 mg Bet v 1 protein from 1 g of cells. Those results were also obtained in a test-fermentation in minimal medium, where a purification according to the above protocol led to the isolation of 20 mg pure Bet v 1 from 500 ml medium (data not shown). After the stepwise removal of urea in Tris-buffer at pH 7.5 at protein concentrations less than 1 mg/ml monomeric, native Bet v 1 was formed, as judged by MALDI-MS (Figure 4) and size exclusion chromatography (Figure 5).

The molar extinction coefficient at the wavelength of 278 nm was determined by the method of Gill and van Hippel (1989) to be $10279 \text{ M}^{-1} \text{ cm}^{-1}$.

Spectroscopic Characterization of Recombinant Bet v 1 (M139L)

In order to test whether the newly generated expression system was suitable for the preparation of native Bet v 1 protein, we recorded one- and twodimensional ^1H -NMR-spectra of Bet v 1 (M139L) protein, and compared those to the spectra generated from recombinant wild type Bet v 1a (Ferreira *et al.*, 1993). The 1D-spectrum (Figure 6) is largely identical with 1D-spectra of wild type Bet v 1 as recorded in our lab (unpublished results), and as published by Kungl *et al.* (1996). Slight differences in the amide proton region of the 1D-spectrum could be due to the difference in pH values. Structural similarity is further demonstrated in the 2D-TOCSY spectrum (Figure 7A and B). Both proteins display the same peak pattern in the fingerprint region, characteristic of the same overall structure. While no complete assignment has yet been made, we therefore consider the two proteins to have the same structure in solution. Hence, by the use of NMR-spectroscopy we were able to show that the expression of Bet v 1 as a His-tag fusion protein in an insoluble form, and refolding at alkaline pH after its purification under denaturing conditions yields native protein with the same NMR-characteristics as wild type Bet v 1, overexpressed in *E. coli*, and purified in a native form.

Investigation of the Thermodynamic Behaviour of Bet v 1 (M139A)

After we had demonstrated that the expression and purification scheme was suitable to isolate native protein, we

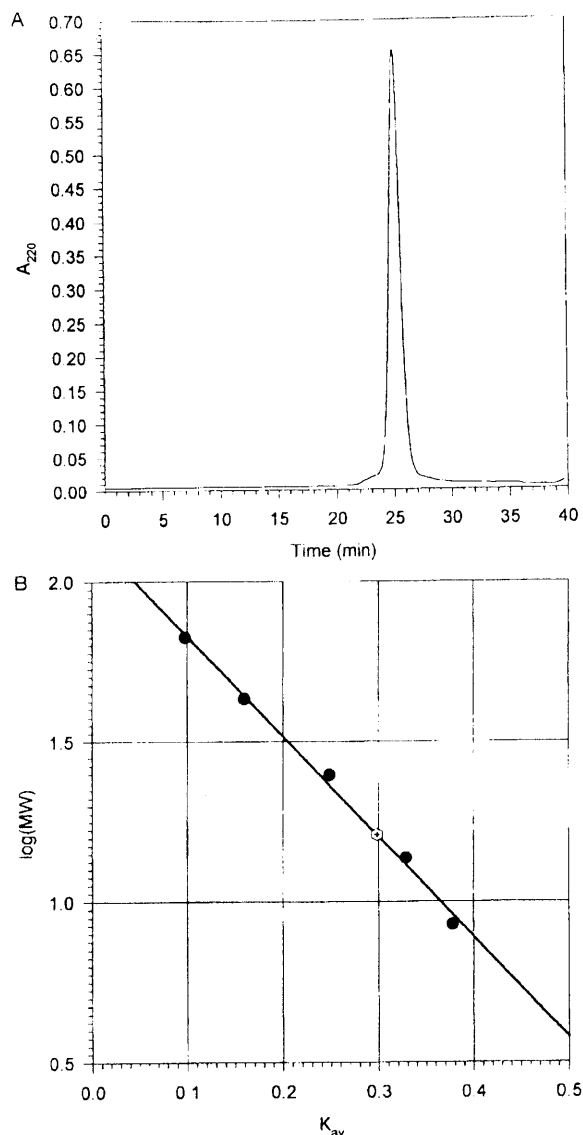


Fig. 5 Size-Exclusion Chromatography of Mutant Bet v 1. Bet v 1 in 20 mM Tris/HCl, pH 7.5, 200 mM NaCl was subjected to a Superdex 75 HR 10/30 (Pharmacia, Freiburg) Column. (A) The chromatogram of mutant Bet v 1 shows a single peak with no protein or nucleic acid contaminations present as judged by measurement of the A_{220} . (B) Evaluation of the molecular weight of Bet v 1. The calibration curve was recorded by applying BSA, Ovalbumin, Chymotrypsinogen, RNase A and Ubiquitin to the column and measuring their retention volumes (filled circles). The exclusion volume of the column was determined with Blue Dextran. The calculated value for mutant Bet v 1 (open hexagon) was 16.4 kDa.

investigated if the nature of the amino acid at position 139 influences the structural or thermodynamic characteristics of the protein. According to our previous structural studies (Faber *et al.*, 1996), this residue is positioned in a kink between two α -helices at the C-terminus of the protein, with its side chain packing against the preceding helix. If this packing interaction indeed plays a role in stabilizing the local structure, shortening the side chain could lead to a destabilized form of Bet v 1. To address this ques-

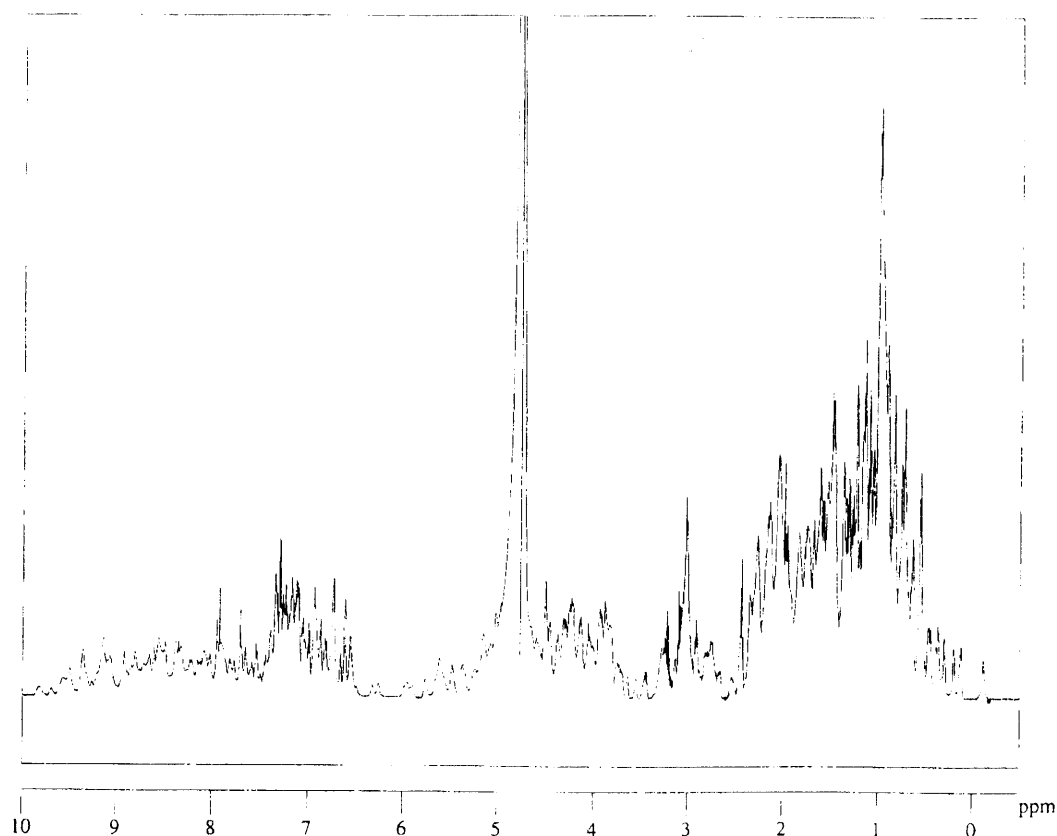


Fig. 6 ^1H -NMR-Spectrum of 1.2 mM Bet v 1 (M139L) Protein in 10 mM Potassium-Phosphate, pH 7.0, 10% (v/v) D_2O . The spectrum was recorded on a Bruker AMX600 NMR-spectrometer at 308 K with 11 ppm sweepwidth.

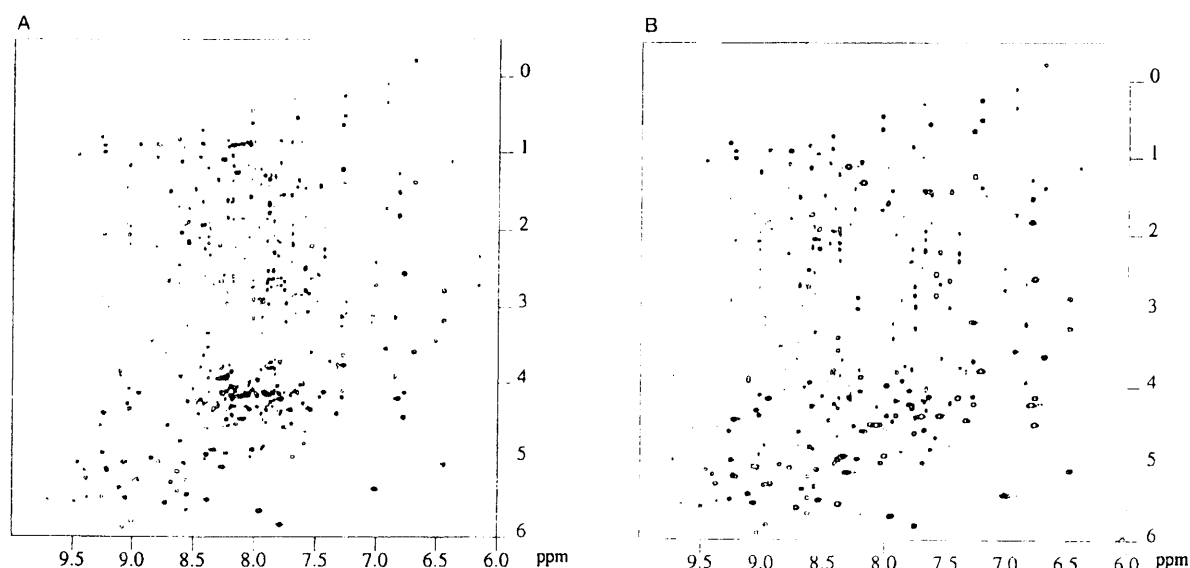


Fig. 7 2D-TOCSY-Spectrum of Bet v 1 (M139L) (A) and Wild Type Bet v 1 (Ferreira *et al.*, 1993) (B). Both Recorded on a Bruker AMX600 NMR-Spectrometer at 308 K.

(A) 1.2 mM Bet v 1 (M139L) in 10 mM potassium-phosphate, pH 7.0, 10% (v/v) D_2O . (B) 4 mM wild type Bet v 1 in 50 mM sodium-phosphate, pH 6.3, 5 mM NaCl, 10% (v/v) D_2O .

tion, we replaced the leucine by an alanine residue. This is structurally equivalent to the removal of an isopropyl group from the protein.

Bet v 1 (M139A) was expressed, purified and refolded in the same way as Bet v 1 (M139L). To investigate the spectroscopic behaviour of Bet v 1 (M139A), we recorded CD-

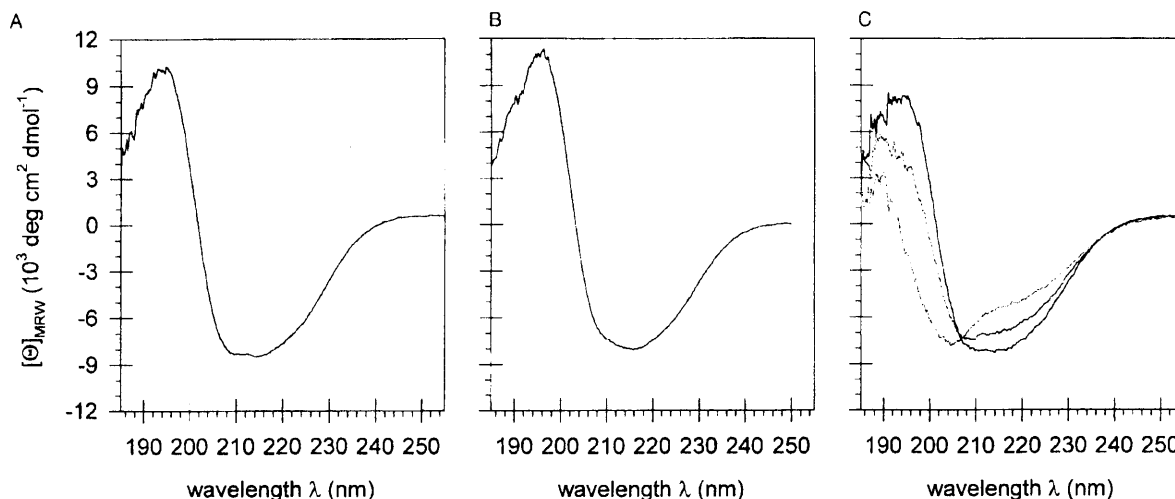


Fig. 8 Far-UV-CD-Spectra of 17 μM Bet v 1 (M139A) in 5 mM NH_4OAc , pH 7.0.

All spectra were recorded on a Jasco-600 spectropolarimeter. (A) CD-spectrum at 22 °C, 10 scans. (B) Reference spectrum of 10.5 μM Bet v 1 (Ferreira *et al.*, 1993) in H_2O , 8 scans, 22 °C. (C) Thermally induced loss of secondary structure. Spectra at 42 °C (solid line), 52 °C (dotted line), and 62 °C (gray line), 10 scans.

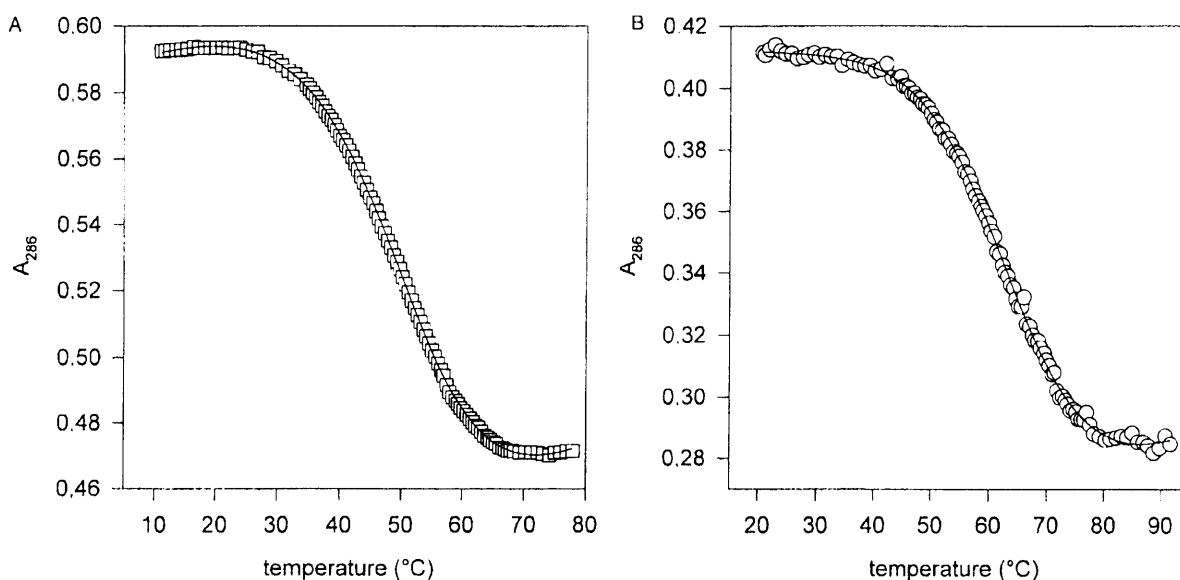


Fig. 9 Thermally Induced Unfolding of Bet v 1 (M139A) and Bet v 1 (M139L).

A Hewlett-Packard 8425 spectrophotometer supplied with a Peltier element temperature control at a wavelength of 286 nm was used. The midpoint, T_m , of the thermally induced unfolding reaction was calculated as described (Mayr *et al.*, 1993). (A) 81 μM Bet v 1 (M139A) solution in 10 mM potassium-phosphate, pH 6.5 (B) 56 μM Bet v 1 (M139L) solution in 10 mM potassium-phosphate, pH 6.5.

spectra in the far-UV-region. As the CD-spectra for Bet v 1 (M139A) (Figure 8A), and for wild type Bet v 1 protein (Ferreira *et al.*, 1993; Figure 8B) at 22 °C are largely superimposable, there appears to be no global effect of the mutation on the structure. Thermal unfolding of the mutant Bet v 1 (M139A) protein was first probed by CD-spectroscopy: Up to a temperature of 42 °C the CD-spectra for Bet v 1 (M139A) show no major structural rearrangements, except for a decrease in the maximum value at 194 nm. Between 42 °C and 62 °C, however, denaturation is indica-

ted by the progressive loss of regular secondary structure (Figure 8C).

In order to quantify the thermodynamic destabilization effect of the lack of the isopropyl group, the two mutant Bet v 1 proteins M139L and M139A were used to record UV-melting curves. UV-spectra of protein solutions taken at 22 °C and at 72 °C, respectively, show a maximum change in the absorption at 286 nm upon unfolding, a wavelength where usually a change of the environment of aromatic residues can be observed. Thermal denaturation

shows that the midpoint of the temperature transition, T_m , for Bet v 1 (M139A) is at approximately 48 °C (Figure 9A), while the mutant Bet v 1 (M139L) has a melting point of 65.5 °C (Figure 9B). This value is close to the value measured for the wild type protein (Klostermeier, 1995). As the Leu-Ala exchange is the only difference between Bet v 1 (M139L) and Bet v 1 (M139A), the difference in the T_m value has to be attributed to the lack of an isopropyl group in the alanine mutant. This finding is supported by the fact that Bet v 1 (M139L) is thermodynamically as stable as the wild type protein. Leucine is structurally comparable to methionine, and consequently the thermodynamic properties of wild type Bet v 1 and Bet v 1 (M139L) are similar. From the experimental evidence presented, we conclude that position 139 is crucial for the proteins' stability. The bulky side chain is required to keep up the local structural architecture, and the positioning of the two helices and the kink in between. Shortening of the side chain, on the other hand, is accompanied by a reduction in those hydrophobic interactions necessary to retain the native structure at elevated temperatures, and, therefore, leads to a lower thermal stability of the whole protein.

Additional evidence for this view comes from a comparison with other isoforms of Bet v 1 that either show conservation at that position (isoforms a, j, f/i, d/h, g, e. and l) or a replacement of methionine by lysine (isoforms b, k, c, and m/n; Swoboda *et al.*, 1995). To further test the influence of amino acid substitutions at this sensitive position, engineering of other mutant proteins and characterization of their thermodynamic behaviour is under way. Furthermore, Bet v 1 (M139A) will be characterized by 2D-NMR spectroscopy at various temperatures to elucidate the nature of the structural effects of the substitution of leucine by alanine in greater detail.

Materials and Methods

Chemicals

Unless otherwise noted, chemicals were obtained from Merck (Darmstadt).

Cloning and Expression of Mutant Bet v 1

Construction of the Plasmids *pbet* and *pbetL* A synthetic *XhoI/Bpu1102I*-DNA fragment of the *bet v 1* gene was purchased from Oligos Etc., Inc. (Wilsonville, OR, USA). As the codon ATG at position 424 to 426 in the DNA was replaced by GCT, methionine 139 was replaced by alanine in the protein Bet v 1 (M139A). The DNA fragment was ligated with *XhoI/Bpu1102I* cut *pET16b* (Novagen, Madison, WI, USA) to obtain the plasmid *pbet*. The plasmid *pbet* was then used as a template to create the plasmid *pbetL*, encoding for the mutant protein Bet v 1 (M139L).

A mutagenesis primer (5'-AAAGCTTCGAAGAAGCTGGGTG-AAACCC-3', mutated nucleotides in bold letters) was purchased from Eurogentec Bel sa (Seraing), and part of the *pbet* DNA-sequence was amplified with the mutagenesis primer and the T7 terminator primer by a standard PCR procedure using Vent-DNA polymerase. The amplified sequence was purified by agarose gel electrophoresis, and digested with *Bsp119I* and *Bpu1102I*. The

double-digested insert was then ligated with *Bsp119I/Bpu1102I* digested *pbet* to generate the plasmid *pbetL*. Both plasmids were transformed into *E. coli* DH5 α cells, and Ampicillin-resistant clones were picked and grown overnight in LB medium with ampicillin. Plasmid DNA was prepared and restricted with *Bpu1102I* and *XhoI* to screen for recombinant plasmids. The plasmid DNA was isolated, and the insert was sequenced by the cycle sequencing method using T7 promoter and terminator primers with digoxigenin labels, Taq DNA polymerase, and the GATC system (GATC, Konstanz). *pbet* and *pbetL* were transformed into *E. coli* BL21/DE3 for expression.

Expression Fermentation of *E. coli* BL21/DE3-*pbet* and *E. coli* BL21/DE3-*pbetL* was performed at 37 °C in a 7-liter working volume of LB medium containing ampicillin at 50 μ g/l. Expression of Bet v 1 was induced at an OD₆₀₀ of 0.8 to 1.0 by the addition of IPTG to a final concentration of 1 mM. Following induction, cells were grown for 4 h, harvested by centrifugation at 6000g for 10 min at 4 °C and stored at -20 °C until needed.

Purification and Refolding of Bet v 1

Protein purification All purification steps were carried out at room temperature, unless otherwise specified. Frozen cells were suspended in 4 ml buffer A (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 6 M urea) per gram wet cell weight and stirred for 24 h. Insoluble cell debris was removed by two centrifugation steps: First at 10000g, 4 °C for 30 min, and second at 120000g, 4 °C for 30 min. The supernatant was applied to a 45 ml chelating sepharose column (Pharmacia, Freiburg), loaded with 200 ml 50 mM NiSO₄ and pre-equilibrated with buffer A. The column was washed with 4 column volumes of buffer A and 8 volumes of buffer A with 100 mM imidazole. Fusion protein was eluted with 2 column volumes of buffer A with 1 M imidazole. Bet v 1 containing fractions were pooled and subsequently dialysed against 5 l 100 mM HCl and 5 l 10 mM HCl for 7 h each, and lyophilized.

Cleavage of the fusion protein was performed by dissolving lyophilized protein to a final concentration of 2 mg/ml in 70% (v/v) formic acid and adding solid CNBr to get a molar ratio of fusion protein to CNBr of 1:2. The cleavage reaction was allowed to proceed for 14 h in the dark under vigorous shaking. Then, the mixture was diluted tenfold with water and lyophilized to remove remaining CNBr, and volatile cleavage products. The lyophilisate was redissolved in buffer A and subjected to a 45 ml chelating sepharose column (Pharmacia, Freiburg), prepared as above. The column was washed with 2 column volumes of buffer A and 2 volumes of buffer A with 100 mM imidazole. Uncleaved fusion protein was eluted with 2 column volumes of buffer A with 1 M imidazole. Bet v 1 containing fractions were pooled. Urea was sequentially removed and the protein refolded by dialyzation steps against 50 mM Tris/HCl, pH 7.5, 220 mM NaCl, with concentrations of urea of 4 M, 2 M, and 0 M, respectively. The solution was dialyzed once against 25 mM imidazole, pH 7.4, twice against 5 mM NH₄OAc, pH 7.5, and lyophilized. The protein concentration was 0.4 mg/ml during all refolding steps.

MALDI-MS 2 μ l of the dialysate were subjected to matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometric analysis on a Kompact MALDI II mass spectrometer (Kratos Analytical, Manchester, UK) with sinapinic acid as matrix.

Size-Exclusion Chromatography A Superdex 75 HR 10/30 (Pharmacia, Freiburg) column fitted to an HPLC-system (Kontron, Eching) was preequilibrated with 50 mM potassium-phosphate, pH 7.0, 100 mM Na₂SO₄ buffer. Blue Dextran was used to obtain the exclusion volume, and sample proteins (BSA, Ovalbumin, Chymotrypsinogen, RNase A, and Ubiquitin) were applied to determine a calibration curve. The molecular weight and purity of

mutant Bet v 1 in 20 mM Tris/HCl, pH 7.5, 220 mM NaCl were specified.

¹H-NMR-Spectroscopy of Bet v 1 (M139L)

A 1D-NMR-spectrum, and a Clean-TOCSY 2D-spectrum with a mixing time of 80 ms were recorded on an AMX600 NMR-spectrometer (Bruker, Karlsruhe) at 35 °C. The sample was 1.2 mM of Bet v 1 (M139L) in 10 mM potassium-phosphate, pH 7.0, 10% (v/v) D₂O.

Thermal unfolding of Bet v 1 (M139A)

CD spectroscopy Spectra were recorded on a Jasco J-600 spectropolarimeter with a stepwidth of 0.2 nm and a bandwidth of 1 nm. The spectral range was 185 to 255 nm. 5 scans were accumulated for each spectrum at various temperatures ranging from 22 °C to 62 °C. The concentration of Bet v 1 (M139A) was 17 µM in 5 mM NH₄OAc, pH 7.0.

UV melting curve The thermal denaturation of a 81 µM solution of Bet v 1 (M139A), and of a 56 µM solution of Bet v 1 (M139L), both in 10 mM potassium-phosphate, pH 6.5, respectively, was investigated in a Hewlett-Packard 8425 spectrophotometer supplied with a Peltier element temperature control unit. The actual temperature was detected by a temperature sensor inserted into the 1 cm cell. Before recording the melting curve, UV-spectra of Bet v 1 (M139A) were taken at 22 °C and 72 °C, respectively. From those spectra, a wavelength of 286 nm was deduced to measure protein denaturation. The Bet v 1 (M139A) sample was heated at a rate of 1 °C per minute between 11 °C and 78 °C, except for the interval from 35 °C to 68 °C where the rate was reduced to 0.5 °C per minute. The respective ranges for Bet v 1 (M139L) were 1 °C intervals between 20 °C and 92 °C, except for 44 °C to 78 °C (0.5 °C steps). The data were fitted to a temperature fit equation, and the midpoint T_m, of the thermally induced unfolding reaction was calculated as described (Mayr *et al.*, 1993).

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