The Secondary Structure of the Calcium binding Birch Pollen Allergen Bet v 4

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Hay fever is widely spread across Northern Europe. The reason are allergies against pollen from trees, grass and other plants. One prevalent origin of allergens is birch pollen. Several allergens from birch pollen have been isolated and characterized so far.

One of them is Bet v 4 with a molecular weight of 9.4 kDa. It binds Ca^{2+} ions and is supposed to possess two EF−hands. We determined the secondary structure of Bet v 4. It has a high content of α−helical structure elements. We could show this using bioinformatic tools like secondary structure prediction and homology modelling. Experimental conformation was obtained by CD (circular dichroism) and NMR (nuclear magnetic resonance) spectroscopy.

Introduction

Up to 20 % of the adult population in industrialized countries suffer from allergic diseases like hay fever and bronchial asthma. These IgE mediated allergic disorders are mainly caused by airborne pollen from trees, weeds, grass and other plants. In Northern Europe allergy against birch pollen is a widely spread phenomenon. Several birch pollen (*Betula verrucosa*) allergens have been isolated and characterized so far: the major birch pollen allergen Bet v 1, birch pollen profilin Bet v 2, a 3 EF−hand calcium binding protein termed Bet v 3, Bet v 6, an isoflavone reductase related protein and cyclophilin Bet v 7.

One member of the birch pollen allergen family is Bet v 4. Bet v 4 is believed to possess two calcium binding sites of EF−hand type. Proteins with homology to Bet v 4 have been identified in olive, *Brassica rapa*, *Brassica napus*, alder and Bermuda grass. Therefore these two EF−hand calcium binding proteins form a group of cross−reacting plant allergens.

We investigated the birch pollen allergen Bet v 4, a protein consisting of 85 amino acids with a molecular weigth of 9.4 kDa, using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. Further information was deduced using sequence alignment, secondary structure prediction and homology modelling.

Results

Sequence similarities and multiple alignment

Several calcium binding pollen allergens with EF−hand motifs have been described so far (fig. 1). They show high sequence identities compared to Bet v 4: Aln g 4 from alder (90 %), Ole e 3 from olive trees (82 %), Bra r 1 and Bra n 1 from turnip and oilseed rape, respectively (78 %), Cyn d 7 from Bermuda grass (69 %) and timothy grass (Phl p 7, 67 %). A remarkable sequence similarity to domains of Calmodulin from *Arabidopsis thaliana* and bovine, human and other calmodulins suggests that Bet v 4 and other pollen allergens may play a role in regulation of cellular Ca^{2+} levels.

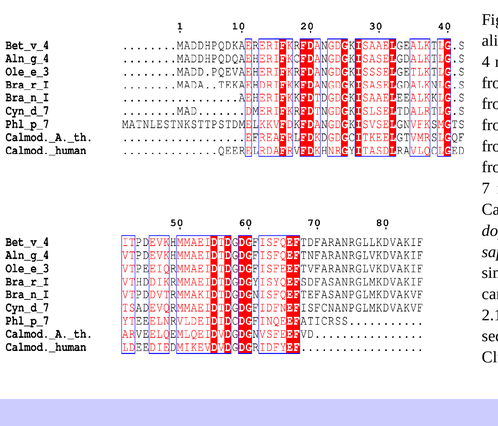

Fig. 1: Multiple sequence alignment of several Bet v 4 related proteins: Aln g 4 from alder pollen, Ole e 3 from olive trees, Bra r 1 from turnip rape, Bra n 1 from oilseed rape, Cyn d 7 from Bermuda grass, Phl p 7 from timothy grass and Calmodulin from *Arabidopsis thaliana* and *Homo sapiens*, respectively. The similarity search was carried out with Psi−Blast 2.1. For the multiple sequence alignment Clustal W was used.

Secondary Structure Prediction

Fig. 2: The secondary structure prediction for Bet v 4 was performed with Jpred[2] (http://jura.ebi.ac.uk : 8888/). Violet barrels indicate predicted α−helices, while yellow arrows indicate β−strands.

DSC prediction (dsc), PHD prediction (phd), PREDATOR prediction (predator), Zpred prediction (zpred), Jnet prediction (jnetpred), Jnet alignment prediction (JNETALIGN), Jnet PSIBLAST pssm profile prediction (JNETPSSM) and Jnet PSIBLAST frequency profile prediction (JNETFREQ). "Consensus" shows the consensus prediction over all methods.

Secondary structure prediction is a suitable tool for estimation of content and distribution of α−helical elements, β−strands, loop and coiled regions. Modern algorithms work with an accuracy of over 70 %. Fig. 2 shows the results of several algorithms and a consensus prediction for the secondary structure of Bet v 4.

The consensus secondary structure prediction includes 5 α−helices: α1 (9−19), α2 (29−38), α3 (44−54), α4 (64−72) and α5 (77−82). Several algorithms predict short β−strands near the NH_2−terminal caps of helices α2 and α4.

An EF−hand usually consists of two α–helices linked by a loop with a length of about 12 amino acid residues. The loop regions include acidic amino residues, especially aspartate, which help to chelate Ca^{2+} ions. The secondary structure prediction suggests loops 20−28 and 55−63 to form the calcium binding sites. A short linker which connects the two EF−hands is predicted to consist of amino acids 39−43.

Circular Dichroism Spectroscopy and Thermal Stability

One suitable experimental access to the secondary structure of proteins is far UV circular dichroism (CD) spectroscopy. α−helical proteins have a typical CD spectrum with minima at 208 and 222 nm and a maximum at 190 nm.

The CD spectra of Bet v 4 (fig. 3a−b) indicate a high content of α−helical secondary structure elements.

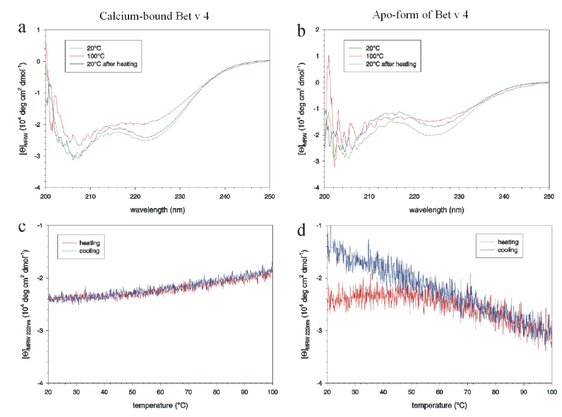

Fig 3: The far UV CD spectra of both the Ca^{2+} bound and the apo−form indicate Bet v 4 to be an α−helical protein (a, b). Heating of Bet v 4 leads to a slight loss of α−helicity (a, b), but no unfolding is observed, neither in the presence nor in the absence of Ca^{2+} ions (c, d).
CD spectra of 3 µm Bet v 4 solutions in 2 mM $CaCl_2$ or 1 mM EGTA in a cuvette with a light path of 1 cm were recorded on a Jasco J810 CD spectropolarimeter equipped with a Peltier temperature control unit.

The overall secondary structure of Bet v 4 remains intact when calcium ions are removed by addition of a chelating agent like EGTA, but a slightly different CD spectrum is observed (cf. Engel et al. 1997). Boiling of aqueous Bet v 4 solutions does not lead to an unfolding of the protein (fig 3c d). Only a slight loss of α−helicity is observed (fig. 3 a−b). Parvalbumins and other EF−hand proteins also show a high thermal stability.

Thus Bet v 4 can be considered an extremely stable protein. Ca^{2+} ions are not responsible for the stability of Bet v 4. Ferreira et al. (1999) have shown that Ca^{2+} is not required for IgE binding to Bet v 4, although most IgE antibodies bind at the COOH terminal Ca^{2+} binding site.

Homo− and heteronuclear NMR spectroscopy

One application of nuclear magnetic resonance (NMR) spectroscopy is the determination of the three−dimensional structure of biomolecules. One can easily derive secondary structure information from the chemical shifts of α− and amide proton resonance signals. The α−proton resonances in the one−dimensional NMR spectrum of Bet v 4 are shifted to high field (fig. 4). This is typical for α−helical proteins as well as the low dispersion of amide proton resonances. Only few NH resonances > 9 ppm are obeserved.

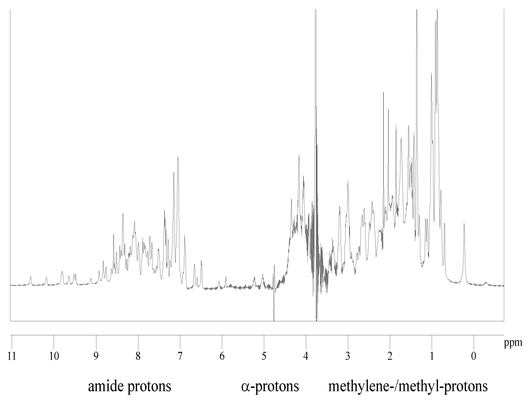

Fig. 4: One−dimensional NMR spectrum of 1 mM Bet v 4 in 8 mM $CaCl_2$ recorded on a Bruker Avance DRX600 spectrometer. The Hα resonances are shifted to high field. This is typical for α−helical proteins. Additional information about the secondary structure is given by the position of the amide proton resonances. Only few signals are observed at lower field than 9 ppm, which is indicative of an α−helical protein, too.

The crowded resonances in the 1D NMR spectrum can be split up in two dimensions by use of ^{15}N labelled protein samples. Fig. 5 shows an HSQC spectrum (heteronuclear single quantum coherence spectroscopy) of uniformly ^{15}N labelled Bet v 4. The amide proton signals in this spectrum are split up along the ^{15}N chemical shift axis. The amide proton resonances of two glycines which are part of the EF−hand loops are extremly shifted to low field.

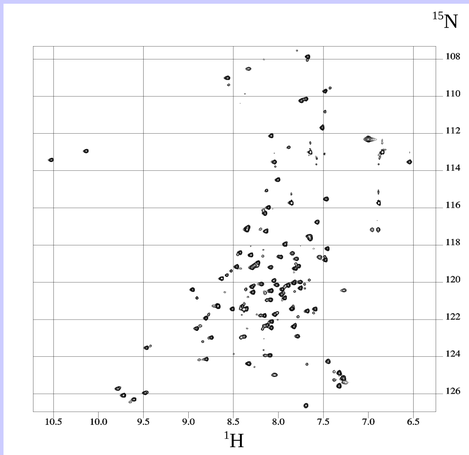

Fig. 5: HSQC spectrum of 2 mM ^{15}N labelled Bet v 4 in 25 mM $CaCl_2$. Most amide proton signals are found at chemical shifts < 9 ppm. This is typical for proteins without β−sheets. The two signals at low field originate from glycine residues in the EF−hand loops. The extreme chemical shifts of these glycine amide protons are only observed in the calcium−bound form of Bet v 4.

Homology modelling

High sequence similarity to calmodulin and experimental confirmation of a high helical content justify the creation of a structure model of Bet v 4 on the basis of known calmodulin structures.

The structure model of Bet v 4 (fig. 6) shows four α−helices: α1 (5−18), α2 (28−35), α3 (43−53) and a long COOH−terminal α−helix, α4 (63−84). α1 and α2 form the NH_2−terminal EF−hand, while the more immune−reactive carboxy−terminal EF−hand consists of α3 and α4. The two helices of each EF−hand are orthogonally packed on each other and connected through a short linker (36−42). The EF−hands are brought together by a short β−sheet (β1: 25−26; β2: 61−62).

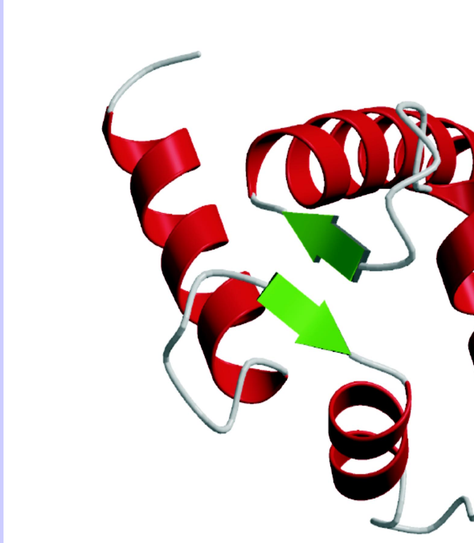

Fig. 6: Structure model of Bet v 4. The Homology model was created with Swiss−Model on the Expasy−server using different calmodulins as templates.

Summary and Future Directions

The birch pollen allergen Bet v 4 is a calcium binding protein. Its biological function remains unknown, but the sequence homology to calmodulin and other calcium binding proteins like Troponin suggests that Bet v 4 plays a role in the regulation of the Ca^{2+} level.

Bet v 4 has two calcium binding sites of EF−hand type. The EF−hands, each consisting of two orthogonally stacked α−helices, are connected by a short linker and brought together via a short β−sheet. This conformation is the basis for an extreme thermostability.

The homology model of Bet v 4 is consistent with the predicted secondary structure and experimental data. Still, the homology model has to be confirmed by the determination of a high−resolution structure, the first one of a plant allergen of EF−hand type.

The structure of an allergen antibody complex will give us a better understanding of the molecular basis of allergy.

Acknowledegement

We thank Fatima Ferreira for kindly providing the Bet v 4 clone.