

Cloning and expression of the cyclophilin Bet v 7, and analysis of immunological cross-reactivity among the cyclophilin A family

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Abstract

Allergic symptoms in sensitized individuals are caused by proteins named allergens. We report here the cloning and the production of the cyclophilin Bet v 7, one of the birch pollen allergens. Recombinant Bet v 7 was produced in bacteria and used to raise a rabbit anti-Bet v 7 antiserum. With this antiserum we detected cyclophilin A in several pollen species and we demonstrated immunological cross-reactivity among those plant cyclophilins A by immunoblot and ELISA inhibition experiments. However, we could not detect cyclophilins in extracts of animal or mould origin with our anti-Bet v 7 antiserum. By inhibition experiments with purified mould cyclophilins, we confirmed the absence of cross-reactivity between plant cyclophilins and non-plant cyclophilins. In addition, our results indicate that the level of immunological cross-reactivity correlates with the level of sequence identity among the cyclophilin A family. This allowed us to define the plant cyclophilin A sub-family as being immunologically distinct, which might have implications at the clinical level in the allergy practice.

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1. Introduction

Birch (*Betula verrucosa*) pollen is a major cause of allergic symptoms in early spring in Northern countries. Allergy can be defined as an IgE-driven pathogenic response of the immune system towards otherwise innocuous proteins named allergens. In birch pollen several allergens have been identified. The major allergen is Bet v 1, related to the PR-10 group of pathogenesis-related proteins, recognized by more than 95% of birch pollen allergic patients (Breiteneder et al., 1989). This allergen is highly involved in allergic cross-reactions between pollen and foods (Vieths et al., 2002). Other birch pollen allergens are known, like the birch profilin Bet v 2 (Diez-Gomez et al., 1999; Valenta et al., 1991),

two calcium-binding proteins, Bet v 3 and Bet v 4 (Seiberler et al., 1994; Twardosz et al., 1997), a protein of the reductase family, Bet v 6 (Karamloo et al., 2001), and a pectin esterase, Bet v 8 (Mahler et al., 2001). The description and characterization of new allergens is more than the mere construction of a catalog. This indeed brings new tools for diagnosis and therapy of allergic diseases, and new clues to unravel the mechanisms of allergy development and perpetuation.

We identified the birch pollen allergen Bet v 7 and demonstrated that this allergen is a cyclophilin (Cadot et al., 2000) of the cyclophilin A subgroup. The cyclophilins A are phylogenically well-conserved proteins of about 18–20 kDa to which several intracellular functions have been attributed. They have been reported as chaperones (Freskgard et al., 1992; Gotherl and Marahiel, 1999), and were also said to exert some nuclease activity (Montague et al., 1997), or to be involved in mRNA splicing (Horowitz et al., 2002).

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Furthermore, members of the cyclophilin A family have been shown to be chemoattractants for certain immune cells (Sherry et al., 1992; Xu et al., 1992). Last but not the least, cyclophilin A is known as the intracellular ligand for the immunosuppressive drug cyclosporin A (Handschumacher et al., 1984).

In plants, cyclophilins have been demonstrated to be stress-induced proteins whose synthesis is enhanced after various stimuli, including wounding or chemical exposition (Kullertz et al., 1999; Marivet et al., 1992, 1994). This further emphasizes the “chaperone-like” function of cyclophilin, protecting proteins from degradation and preventing aggregation, two phenomena that occur during stress. The exact function of cyclophilins in pollen is not known. Most recently, it has been shown that a member of the cyclophilin A family is released from the pollen cytoplasm of higher plants into the extracellular environment under certain unfavorable conditions, which leads to irreversible inhibition of germination (Yokota et al., 2004). Interestingly, alkaline pH is one of those unfavorable conditions described by Yokota et al. (2004), and we demonstrated in a previous study that cyclophilins can be extracted from birch pollen in much higher amounts when the pH of the extraction medium is above 7.5 (Cadot et al., 1995).

Our description of Bet v 7 was also the first report of a cyclophilin as an allergen in pollen, whereas allergenic cyclophilins had already been described in the moulds *Psilocybe cubensis*, *Aspergillus fumigatus*, and *Malassezia furfur*. Those were named Pci c 2, Asp f 11, and Mal f 6, respectively (Appenzeller et al., 1999; Horner et al., 1995; Lindborg et al., 1999). The two latter cyclophilins and the human cyclophilin A have been shown to share IgE-binding epitopes, responsible for immunological cross-reactivity (Flückiger et al., 2002). Those putative cross-reactive epitopes have been determined by homology modelling and prediction of solvent-exposed amino acid residues (Roy et al., 2003). However, despite their high sequence homology, not all cyclophilins of group A seem to be immunologically cross-reactive. Indeed, the *Echinococcus granulosus* cyclophilin A was shown to share IgE epitopes with neither Mal f 6 nor the human cyclophilin A (Ortona et al., 2002).

We here report the cloning of Bet v 7, the birch cyclophilin, and its expression in a heterologous system. We also studied the immunological cross-reactivity between Bet v 7 and cyclophilins from different phylogenetic origins.

2. Materials and methods

2.1. Determination of the coding sequence of Bet v 7

The cDNA library KL-5-M is a kind gift of Dr. J. Kangasjärvi (University of Kuopio, Finland). This phage library has been constructed in lambda gt22A from ozone-exposed birch leaves (Kiiskinen et al., 1997). Eight degenerate forward primers and three degenerate reverse primers have been designed from the most consensual sequences

among various plant cyclophilins. All possible combinations of those primers have been assessed by PCR amplification of the KL-5-M library under the following conditions: 95 °C for 3 min, 35 cycles consisting in 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C, 10 min at 72 °C. Only the following combination gave rise to amplification: 5'-TTCCA(C+T)CG(A+T+G)GTGATCCC-3' forward primer with 5'-CC(C+T)TC(A+C+G)AC(A+G)AC(C+T)TGACC-3' reverse primer. Nested-PCR was carried out on the resulting PCR fragment with the same reverse primer as above and 5'-TTCATGTG(C+T)CA(A+G)GG(A+C)GG(C+T)GA-3' forward primer. The fragment was purified out of an agarose gel and cloned with pCR-Script Amp SK(+) cloning kit (Stratagene, La Jolla, CA). Positive clones were selected by blue-white screening and controlled by PCR screening using the vector-specific M13-forward and -reverse primers (Stratagene). Plasmids were purified from overnight cultures and subjected to sequencing with the M13 primers using an ABI 310 automated fluorescent sequencer (Applied Biosystems, Foster City, CA). This allowed us to determine an internal DNA sequence of Bet v 7.

In the meantime, all inserts of the cDNA library KL-5-M have been amplified using the following lambda gt22A-specific primers: 5'-ATTGGTGGCGACGATCCTGGAG-3' and 5'-CAGACCAACTGGTAATGGTAGCG-3'. To determine the 5'- and 3'-termini of the Bet v 7 coding sequence, the resulting PCR product (AAI fraction) was then subjected to PCR amplification using the same vector-specific primers as above and the following Bet v 7-specific primers designed from the previously determined internal sequence of Bet v 7: 5'-TGGCGAGTCCATCTACGGCG-3' (PC003BPCyP) and 5'-TCCACGACCTGGCCGAACAC-3' (PC004BPCyP). Nested PCR was carried out on the resulting PCR products with the vector-specific primers and the Bet v 7-specific primers 5'-GTGTTCCGGTCAGGTCGTGGA-3' (PC002BPCyP) and 5'-CGCCGTAGATGGACTCGCCA-3' (PC001BPCyP), respectively. After purification, the different DNA fragments that had been obtained were subjected to multiple sequencing reactions with the vector-specific primers and the Bet v 7-specific primers PC001BPCyP and PC002BPCyP. All results of the sequencing reactions were aligned together to determine the full coding sequence of Bet v 7. Thereafter, the sequence was confirmed by using primers covering the two extremities of the Bet v 7 coding sequence (Betv7IF and Betv7IR). Those primers also included restriction sites for further insertion of the Bet v 7 DNA sequence into a vector.

2.2. Cloning, expression and purification of rBet v 7

By using the AAI fraction as template, PCR was carried out with the primer Betv7IF, which corresponds to the beginning of the Bet v 7 coding sequence flanked with an *NdeI* restriction site, and Betv7IR, covering the end of the Bet v 7 coding sequence together with a *BamHI* site.

The product was digested with the respective restriction endonucleases, ligated into the expression vector pET-15b (Novagen- EMD Biosciences, Darmstadt, Germany), which encodes for a (His)₆-tag and a thrombin cleavage site, and amplified in DH5- α cells. Positive clones were selected by PCR screening and the Bet v 7 sequence was controlled by sequencing. After purification, the positive vectors were used for transformation of *Escherichia coli* BL21-CodonPlus strain (Stratagene). Single colony was grown at 37 °C in LB broth, and protein expression was induced by addition of isopropyl-D-thio-galactopyranoside (IPTG) to a final concentration of 1 mM. After 3 h of culture, the cells were harvested by centrifugation. The cell pellet was redissolved in phosphate buffer at pH 7.8, imidazole 50 mM, with a protease inhibitor cocktail (Roche, Basel, Switzerland) and sonicated to lyse the cells. After centrifugation, the supernatant was filtered through a 0.45- μ m membrane and loaded through a nickel-charged sepharose column (Hi-Trap Sepharose, Pharmacia Biotech). Gradient elution was carried out from 50 to 500 mM imidazole. The purity of the fusion protein was checked on Coomassie blue-stained SDS-PAGE gel. Alternatively, we obtained a non-fusion protein by enzymatic cleavage of the purified rBet v 7 with biotinylated thrombin (Novagen), according to the manufacturer's instructions. The biotinylated thrombin was later removed from the mixture by using streptavidin-agarose gel.

2.3. Cloning and production of the tomato cyclophilin

The pCG19 plasmid that contains the tomato cyclophilin (TomCyP) coding sequence (Gasser et al., 1990) is a kind gift from Prof. C. Gasser (University of California, Davis, CA). This construct, however, did not include any fusion tag to facilitate protein purification. Therefore, we decided to transfer the TomCyP coding sequence into the pET-15b expression vector. For that, plasmids were purified from overnight cultures and digested with *Nde*I and *Bam*HI restriction endonucleases before ligation into pET-15b and amplification in DH5- α cells. The positive plasmids were used for transformation of BL21-CodonPlus cells. Afterwards, the expression and purification of rTomCyP was carried out with the same procedure as that for rBet v 7.

2.4. Purification of natural Bet v 7 (nBet v 7)

Natural Bet v 7 was purified as already described (Cadot et al., 2000). Briefly, birch pollen extracts were prepared at pH 7.5. Afterwards, basic proteins in the extract were purified by ion exchange in bulk DEAE-Sephacel at pH 8.5. The resulting supernatant containing nBet v 7 was subjected to hydrophobic interaction chromatography in phenyl sepharose from which nBet v 7 could be eluted.

2.5. Rotamase assay

Rotamase activity was assayed by a modification of the method of Fischer et al. (1984). In this assay, the

trans-Ala-Pro conformation of succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (sAAPFn, Sigma, St Louis) a chromogenic artificial peptide, is cleaved by chymotrypsin. Approximately 80% of the peptide is in this conformation at equilibrium and will be almost immediately cleaved by the enzyme. The remaining 20% must undergo the relatively slow process of isomerisation from *cis* to *trans* before it can be cleaved. Cleavage can be followed by measuring absorbance at 390 nm at 2-s intervals in a spectrophotometer (Ultrospec plus 4054, Pharmacia Biotech, Sweden). The presence of a molecule with rotamase activity, which facilitates the *cis*–*trans* isomerisation, can be detected by the acceleration of the rate of peptide cleavage. Our assays were carried out in HEPES buffer 35 mM, pH 8.0, with 2 μ M chymotrypsin and 10 μ M sAAPFn. To inhibit rotamase activity, 1 μ M cyclosporin A was first added to the mixture.

2.6. Tissue distribution of Bet v 7

Fresh anthers and buds were harvested on a local silver birch tree and immediately frozen in liquid nitrogen. RNA was extracted from plant tissues ground with sand in liquid nitrogen by using an RNA extraction kit (RNeasy plant kit, Qiagen Benelux, Venlo, The Netherlands). Next, cDNA was prepared with Superscript Pre-amplification System (Invitrogen, Carlsbad, CA) before PCR amplification was carried out with the Bet v 7-specific primers PC003BPCyP and PC004BPCyP. The presence of a PCR product was then controlled in agarose gel.

2.7. Polyclonal antiserum to Bet v 7

Two white New Zealand rabbits were immunized with non-fusion rBet v 7 with a standard protocol. Blood was taken 10 d after the first and the second boosters. A pool was made of all four hyperimmune blood samples.

2.8. Allergens and allergen extracts

The mould cyclophilins rAsp f 11 and rMal f 6 were kindly given by Prof. R. Crameri (SIAF, Davos, Switzerland). The molecule rBet v 2 was purchased from Biomay (Linz, Austria).

Birch, ryegrass (*Lolium perenne*), and mugwort (*Artemisia vulgaris*) pollen extracts, *Cladosporium herbarum* and *Alternaria alternata* extracts were prepared as already described (Cadot et al., 1995). English oak (*Quercus robur*), hazel (*Coryllus avellana*), alder (*Alnus glutinosa*), English plantain (*Plantago lanceolata*), orchardgrass (*Dactylis glomerata*), Kentucky bluegrass (*Poa pratensis*) pollen extracts, cat fur, dog fur, mite (*Dermatophagoides pteronyssinus* and *D. farinae*), *Fusarium culmorum*, *Aspergillus niger* and *A. fumigatus* extracts—all were obtained from HAL (Haarlem, The Netherlands).

2.9. ELISA inhibition

To perform ELISA inhibition, two polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C. The detection plate was coated with 0.5 µg/ml rBet v 7 in 0.02 M PBS, pH 8.0. The inhibiting plate was coated with rBet v 7, rTomCyP, rAsp f 11, rMal f 6 or rBet v 2 (negative control) starting from 10 µg/ml in 0.02 M PBS pH 8.0 and then serially diluted 1/4 in the same coating buffer. After coating, free binding sites were blocked with PBS–BSA 2% for 1 h at room temperature. Rabbit polyclonal anti-Bet v 7 diluted 1/50,000 in PBS–BSA 0.5%–Tween-20 0.05% was added to the inhibiting plate and allowed to bind for 1 h at room temperature. The pre-incubated sera were then transferred to the detection plate and incubated for 1 h at room temperature. The remaining Bet v 7-specific antibodies were then detected with peroxidase-labeled swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) diluted 1/2000, and visualized after addition of the peroxidase substrate TMB in citrate buffer and reading at 450 nm after addition of sulphuric acid. All washes were made with PBS–Tween-20 0.05%. Negative controls consisted of pre-immune rabbit serum diluted 1/50,000. Percentage of inhibition was calculated from the absorbencies values of the serial dilutions of rBet v 2 arbitrarily corresponding to 0% inhibition.

2.10. SDS-PAGE immunoblotting

SDS-PAGE in reducing conditions was carried out in 13% polyacrylamide gels with 5% stacking gels. After the run, proteins were electro-transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA) in transfer buffer composed of 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was then blocked in PBS containing defatted dried milk (PBS–NFDM 0; 2%) before overnight incubation at 4 °C in rabbit anti-Bet v 7 polyclonal antiserum diluted in PBS–NFDM 0.1% (0.05 µl of rabbit antiserum/sample lane). To reveal binding, a peroxidase-labeled swine anti-rabbit immunoglobulin (DAKO) diluted in PBS–NFDM 0.1% (1 µl/sample lane) was used before incubation in TMB. Immunoblot inhibitions were carried out as follows: 50 µg per sample lane of purified rBet v 7, rTomCyP or rAsp f 11 was incubated for 1 h at room temperature with the rabbit antiserum before application onto the blotting membrane as described above.

3. Results

3.1. Full coding sequence of Bet v 7

The full coding sequence of Bet v 7 has been determined (EMBL AJ311666). It consists of 519 nucleotides that yields a protein of 173 amino acids with a deduced molecular mass

of 18294 Da. The sequence displays the classical secondary structure of cyclophilins (Galat, 1999; Roy et al., 2003). Translation of the sequence showed a match with the peptide sequences determined on purified nBet v 7 (Cadot et al., 2000). Since nBetv 7 had been purified from birch pollen where the cDNA library originated from birch leaves, this result is an indication that the same cyclophilin isoform is present in both pollen and leaves. Comparison of the protein full-length Bet v 7 with other known amino acid sequences of plant and mould cyclophilins is shown in Fig. 1. The full amino acid sequence of Bet v 7 shows up to 91%, 87%, 86% and 85% identity with the cyclophilins of Madagascar periwinkle (*Catharanthus roseus*), kidney bean (*Phaseolus vulgaris*), foxglove (*Digitalis lanata*), and tomato (*Lycopersicon esculentum*), respectively. There is much less identity with Mal f 6 and Asp f 11, 61% and 58%, respectively. It is noteworthy that the N- and C-terminal parts are more subjected to amino acid variations than the internal part of the sequences, the latter being probably more involved in the construction of the enzymatic domain. At positions 49–55, the Bet v 7 sequence contains the typical seven amino acid insert of the plant cyclophilin A family (Galat, 1999; Gasser et al., 1990; Kinoshita and Shimazaki, 1999).

3.2. Tissue distribution of Bet v 7

By PCR amplification of cDNA from mRNA of freshly collected birch anthers, we could ascertain the presence of Bet v 7 in anthers (not shown). This was expected as Bet v 7 was first purified from pollen. Furthermore, we determined the coding sequence of Bet v 7 cloned from anthers and confirmed that the entire sequence was identical to the one obtained from birch leaves, which further corroborates that the same gene is expressed in both tissues. With the same procedure, we also found expression of Bet v 7 in birch buds. This is in accordance with the findings of others, i.e. cyclophilin A is found in all tissues investigated for a given organism (Gasser et al., 1990; Ryffel et al., 1991), which adds to the universality and the importance of the role played by the cyclophilin A family.

3.3. Recombinant expression of Bet v 7 and TomCyP in *E. coli*

Recombinant Bet v 7 was found both in the cytoplasm of the bacterial cells and in inclusion bodies. For convenience, only the intra-cytoplasmic rBet v 7 was purified and used in further experiments. On Coomassie blue-stained SDS-PAGE gels (Fig. 2), highly pure rBet v 7 with His-tag appeared with a molecular mass of 21 kDa (calculated from the sequence: 20,095 Da). After removal of the tag by thrombin digestion, rBet v 7 migrated as an 18-kDa band (Fig. 2).

In a further step, we checked the enzymatic activities of the three types of Bet v 7, namely nBet v 7, rBet v 7-(His)₆, and rBet v 7 (Fig. 3). We could demonstrate that all three polypeptides exert rotamase activity with calculated specific activities

Bet v 7	1	MASNPKVFFDMEVG--C---QFVGRIVMELVADTTTPRTAENFRALCTGEK	
<i>C roseus</i> CyP	1	MP-NPRVFFDMSVG--C---QFAGRIVMELFADTTTPRTAENFRALCTGEK	
<i>P vulgaris</i> CyP	1	MP-NPKVFFDMTIG--C---QFAGRIVFELVADVTTPRTAENFRALCTGEK	
<i>D lanata</i> CyP	1	MS-LPKVFFDMSIG--C---QFCGRIVMELVADVVPKTAENFRALCTGEK	
TomCyP	1	MA-NPKVFFDLTIG--C---AFAGRIVMELFADTTTPKTAENFRALCTGEK	
Mal f 6	1	MS---NVFFDITKN--C---AELGTIKFKLFDDVVPKTAANFRALCTGEK	
Asp f 11	1	MS---QVFFDVEYAPVCTAETKVGRIVFNLFDKDVPEKTAENFRELCKRPA	
Bet v 7	46	GNCRSGKPLHYKSSSFHRVIPCFMCCGGDFTAGNGTGGESIYGAKEFADEN	
<i>C roseus</i> CyP	45	GTGRSGKPLHYKSSSFHRVIPCFMCCGGDFTAGNGTGGESIYGAKEFADEN	
<i>P vulgaris</i> CyP	45	GVCRSGKPVHFKGSIHFRVIPNFMCCGGDFTAGNGTGGESIYGAKEFADEN	
<i>D lanata</i> CyP	45	GVCKSGKPLHYKGSFHRVIPCFMCCGGDFTAGNGTGGESIYGAKEFADEN	
TomCyP	45	GVCKMGKPLHYKGSFHRVIPCFMCCGGDFTAGNGTGGESIYGAKEFADEN	
Mal f 6	43	GFC-----YAGSHFHRVIPDFMCCGGDFTAGNGTGGKSIYGAKEFADEN	
Asp f 11	48	GEC-----YRESTFHRVIPNFMCCGGDFTAGNGTGGKSIYGAKEFADEN	
Bet v 7	96	FIKKHTGFGILSMANAGPGTNGSQFFICTAKIEWLDGKHVVFQGVVEG--	
<i>C roseus</i> CyP	95	FIKKHTGFGILSMANAGPNTNGSQFFICTAKIEWLDGKHVVFQGVVEG--	
<i>P vulgaris</i> CyP	95	FVKKHTGFGILSMANAGPGTNGSQFFICTITKTEWLDGKHVVFQGVVEG--	
<i>D lanata</i> CyP	95	FVKKHTGFGILSMANAGPGTNGSQFFICTEKTAWLDGKHVVFQGVVEG--	
TomCyP	95	FVKKHTGFGILSMANAGPGTNGSQFFICTAKTEWLDGKHVVFQGVVEG--	
Mal f 6	86	FQDKHNKFGLLSMANAGPNTNGSQFFITTVVISWLDGKHVVFGEVIDG--	
Asp f 11	91	FSRKHDKKFGILSMANAGPNTNGSQFFITTAVISWLDGKHVVFGEVADEKS	
Bet v 7	144	LDIVKATEKVGSSSG--RTSKPVVVDCCQLS	173
<i>C roseus</i> CyP	143	MDVVKAIEKVGSSSG--RTAKKVVDCCQLS	172
<i>P vulgaris</i> CyP	143	LDVVKDIEKVGSSSG--KTARPVAIADCCQLS	172
<i>D lanata</i> CyP	143	MDVVRAIEKVGSSSG--KTAKPVVVDCCQLC	172
TomCyP	143	MDVIKKAEAVGSSSG--RCSKPVVIADCCQL-	171
Mal f 6	134	MNVVKAIEAGSSSG--KPRSRIEIAKCGVC-	162
Asp f 11	141	YSVVKETALGSSSGSVRSNTRPKIVNCGEL-	172

Fig. 1. Multiple sequence alignments of Bet v 7 and other cyclophilins. Amino acids conserved in all seven cyclophilins are shown on a black background; amino acids conserved in the plant cyclophilins and Mal f 6 but not in Asp f 11 appear on a dark grey background. Amino acids specific for all five plant cyclophilins are shown on a light grey background.

k_{cat}/K_M of $6.08 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $12.08 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, and $17.05 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at 20°C , respectively. Those values are lower, differing approximately by a factor 10–20, than already published data on cyclophilin kinetic parameters (Berriman and Fairlamb, 1998; Fischer et al., 1984; Harrison and Stein, 1990; Kofron et al., 1991). However, to our knowledge, our data are the first on a plant cyclophilin, and

enzymatic activities might be different between the plant and animal proteins for the substrate sAAPFn. As already observed for nBet v 7 (Cadot et al., 2000), the rotamase activity of rBet v 7 was totally inhibited by cyclosporin A but not by FK506. This latter molecule is the specific inhibitor of another class of immunophilins, the FK-binding proteins, which, conversely, are not inhibited by cyclosporin A.

Recombinant TomCyP was purified the same way as rBet v 7. Recombinant TomCyP exerts a cyclosporin A-sensitive rotamase activity with $k_{\text{cat}}/K_M = 11.15 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (for rTomCyP-(His)₆) (data not shown).

3.4. Cross-reactivity studies with other plant species

To detect cyclophilins in other plant species, an anti-Bet v 7 antiserum was raised in rabbits. In Fig. 4, we can see that this antiserum recognizes one protein only, at around 18 kDa, in a birch pollen extract. This band actually contains three polypeptides, which indicates the presence of three Bet v 7 isoforms with slightly different molecular masses in birch pollen. This is consistent with results of our previous work where we detected by IEF immunoblotting three Bet v 7 isoforms with three different isoelectric points (Cadot et al., 2000). Here as well, in addition, the rabbit antiserum detected three polypeptides with different isoelectric points in a birch pollen extract by IEF immunoblotting (not shown). Further, the antiserum reacted

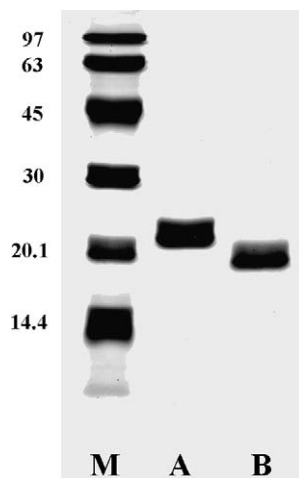


Fig. 2. Coomassie blue-stained SDS-PAGE gel showing purified rBet v 7-(His)₆ (A), and rBet v 7 after removal of the His-tag (B). Molecular mass markers are visible on lane M in kDa.

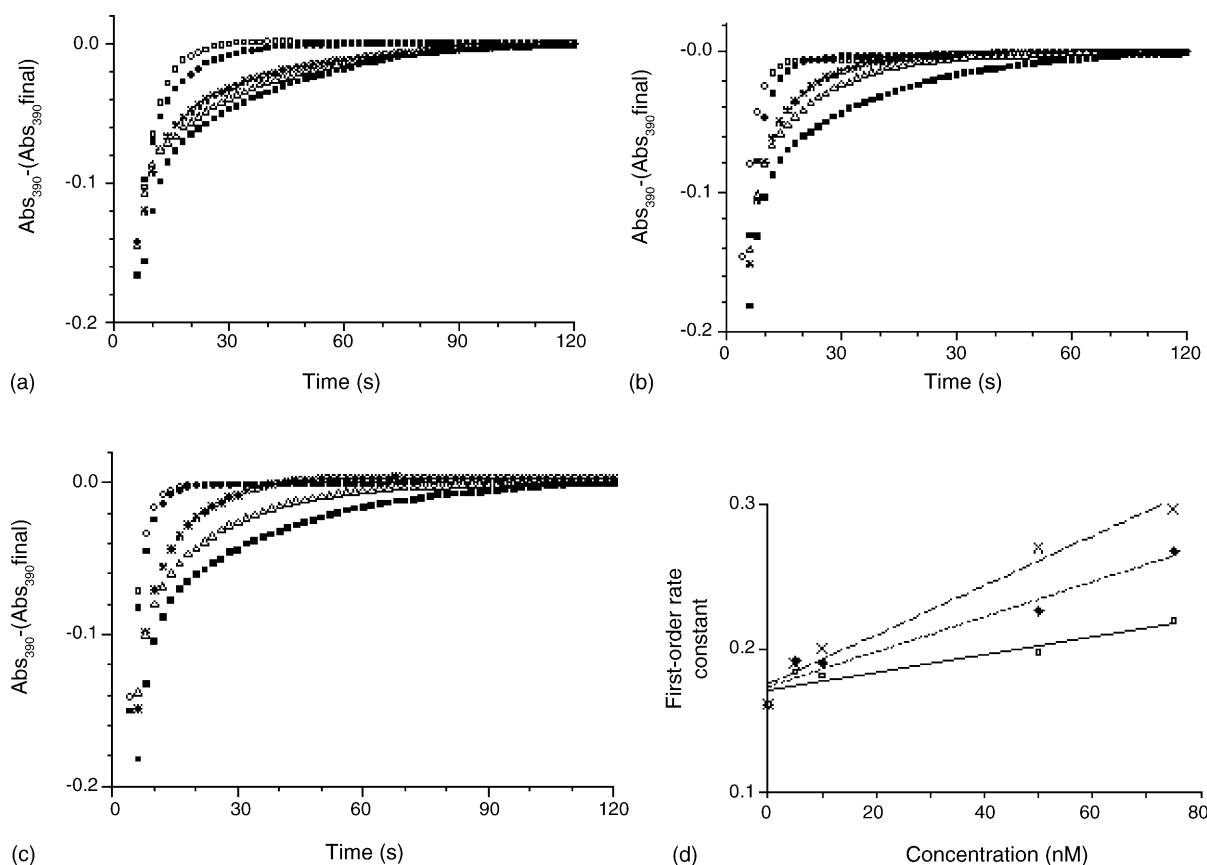


Fig. 3. Rotamase assay (see Section 2) with no (squares), 5 nM (triangles), 10 nM (asterisks), 50 nM (solid dots), and 75 nM (open circles) nBet v 7 (a), rBet v 7-(His)₆ (b), and rBet v 7 (c). In (d), the linear dependence of the enzymatic activity (first-order rate constant) as a function of enzyme concentration is depicted for nBet v 7 (open squares), rBet v 7-(His)₆ (black diamonds), and rBet v 7 (crosses).

with all three purified Bet v 7 forms, namely nBet v 7, rBet v 7-(His)₆, and rBet v 7, and with rTomCyP (Fig. 4). This cross-reactivity between the birch and the tomato cyclophilin was further confirmed by ELISA-inhibition experiments in which it is demonstrated that incubation of the antiserum with rTomCyP inhibits the reactivity to rBet v 7 (Fig. 5).

By using this rabbit anti-Bet v 7 antiserum, we next studied the presence of Bet v 7 homologues in other pollen species. As shown in Fig. 6, the presence of proteins of the cyclophilin A family was ascertained in all pollen extracts tested. Like in birch pollen, several cyclophilin bands were clearly detected in alder (*A. glutinosa* of the Betulaceae family) and hazel (*C. avellana* of the Corylaceae family) pollen. However, only one band was visible in the oak (*Q. robur*) pollen extract. This might indicate that only one Bet v 7-like isoform is present in this pollen species, or only one oak cyclophilin isoform among several cross-reacts at the immunological level with Bet v 7. It is tempting to correlate those results with the phylogenic relationships. Indeed, the genders *Alnus* and *Coryllus* are considered closer to birch than *Quercus*; hence, cyclophilins more closely resemble Bet v 7 in the former than in the latter. This, however, is not supported by the rest of our data showing more than one

cyclophilin A bands in extracts from Gramineae pollen (*D. glomerata* – cock's foot; *P. pratensis* – meadow grass), and *P. lanceolata* – English plantain (weed pollen), which are all pollen species phylogenically remoter from birch than *Quercus*.

In immunoblot inhibition experiments, after pre-incubating the rabbit antiserum with Bet v 7 or TomCyP, we could observe that the reactions to the cyclophilin bands in different pollen extracts were inhibited (Fig. 7). This further supports the existence of general immunological cross-reactivity among plant cyclophilins A. In addition, the anti-Bet v 7 antiserum allowed us to detect two cyclophilin A bands in extracts from leek (*Allium porrum*) and French bean (*P. vulgaris*) (results not shown). Such result is interesting, as it suggests that the cyclophilin A proteins can be involved in food pollen cross-allergies in patients.

3.5. Cross-reactivity studies with non-plant species

We next attempted to detect Bet v 7-like proteins in allergenic extracts from moulds (*C. herbarum*, *A. alternata*, *A. fumigatus*, *A. niger*, *F. culmorum*), invertebrates (*D. pteronyssinus*, *D. farinae*), and mammals (cat fur, and dog fur). No band could be detected with our anti-Bet v 7 antiserum in any

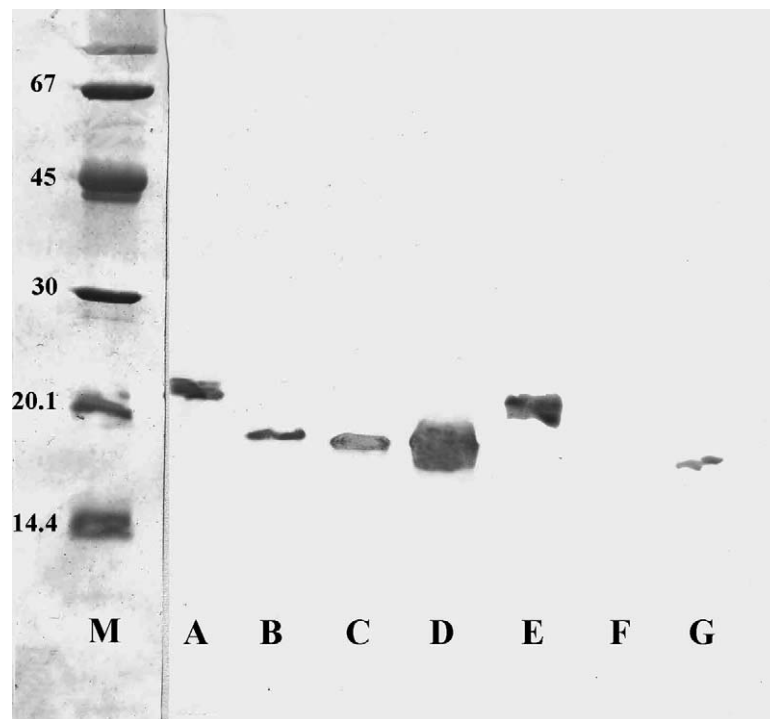


Fig. 4. SDS-PAGE immunoblot showing the reactivity of the anti-Bet v 7 rabbit anti-serum with (A) rBet v 7-(His)₆, (B) rBet v 7, (C) nBet v 7, (D) a complete birch pollen extract, (E) rTomCyP, (F) rAsp f 11, and (G) rMal f 6. Markers (M) are indicated on the left side in kDa.

of those extracts (not shown). This was rather unexpected, as some mould cyclophilins are known to be present in allergenic extracts. For example, Asp f 11 should be detected in our *A. fumigatus* extract. Although the quality of the extracts can be questioned, it cannot be excluded that non-plant cyclophilins are not immunologically cross-reactive with Bet v 7.

To confirm the different antigenic properties of the mould cyclophilins, rAsp f 11 and rMal f 6, two recombinant mould cyclophilins from *A. fumigatus* and *M. furfur*, respectively, were used. First, on immunoblot, rMal f 6 was recognized by the rabbit anti-Bet v 7 antiserum, but this was not so for rAsp f 11 (Fig. 4). Such result corroborates the absence of reactivity of the antiserum with a complete *A. fumigatus*

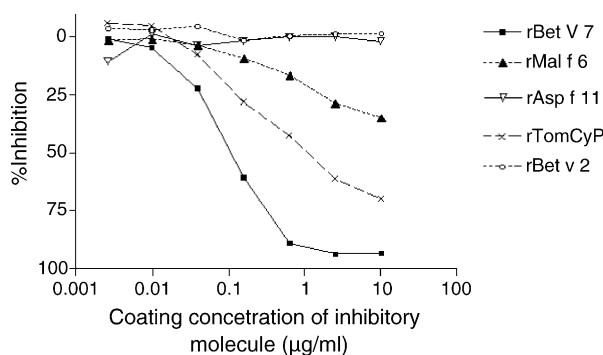


Fig. 5. Inhibition of antibody binding to rBet v 7 coated on a solid phase (ELISA inhibition). The anti-Bet v 7 rabbit antiserum was pre-incubated with increasing concentrations of the molecules indicated on the right side.

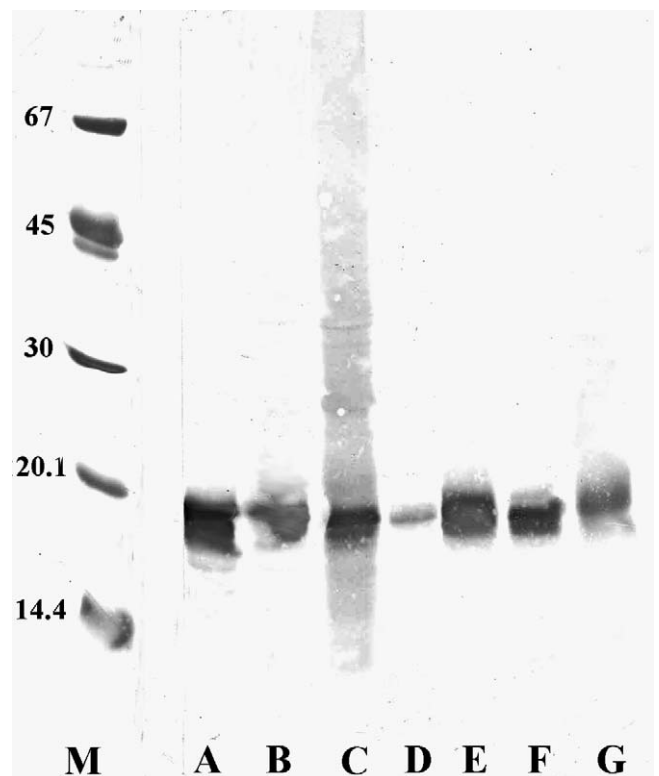


Fig. 6. SDS-PAGE immunoblot showing the reactivity of the anti-Bet v 7 rabbit antiserum with (A) a birch pollen extract, (B) a hazel pollen extract, (C) an alder pollen extract, (D) an oak pollen extract, (E) a plantain pollen extract, (F) a cock's foot pollen extract, and (G) a meadow grass pollen extract. Markers are shown on lane M in kDa.

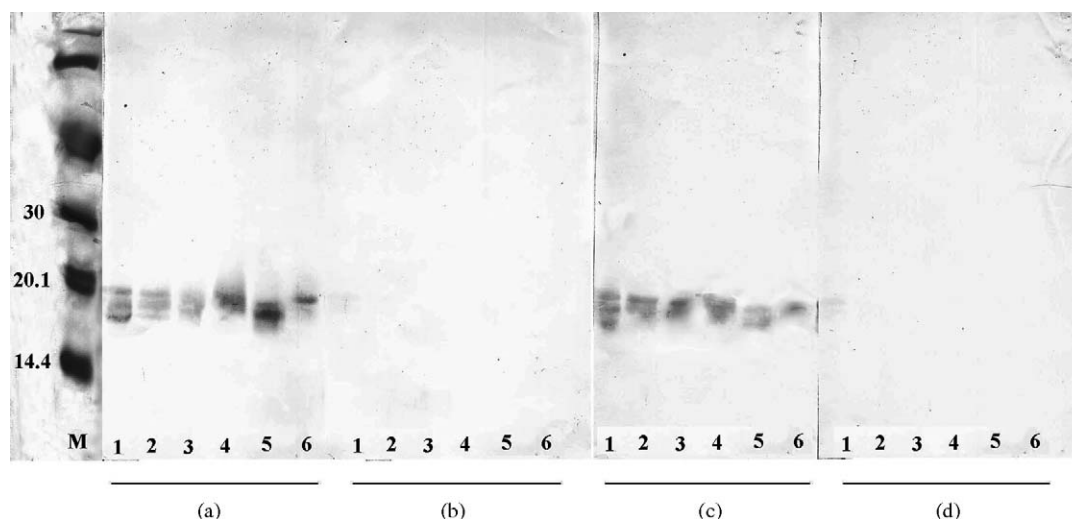


Fig. 7. SDS-PAGE immunoblot inhibition experiment. Anti-Bet v 7 rabbit antibodies were used to probe extracts of birch pollen (1), hazel pollen (2), meadow grass pollen (3), plantain pollen (4), cock's foot pollen (5), and oak pollen (6). (a) No inhibition; (b) inhibition by rBet v 7; (c) inhibition by rAsp f 11; (d) inhibition by rTom CyP. Markers (M) are indicated on the left side in kDa.

extract, as mentioned above. Next, we used ELISA inhibition to determine whether Bet v 7, Asp f 11 and Mal f 6 do share common epitopes. Fig. 5 shows that the binding of the anti-Bet v 7 polyclonal antibodies can be inhibited on the rBet v 7-coated plate by pre-incubation with rBet v 7 to a much lower extent by rMal f 6, but not at all by rAsp f 11 (similar to the negative control rBet v 2). The fact that rMal f 6 is able to slightly inhibit the binding of the polyclonal anti-Bet v 7 proves that it shares at least one epitope with the birch pollen cyclophilin, which corroborates the detection of rMal f 6 on immunoblot (Fig. 4). However, the ELISA inhibition also shows that most of the rBet v 7 epitopes seem to be absent on rMal f 6. We also performed immunoblot inhibition experiments, by pre-incubating the rabbit antiserum with rAsp f 11. We then observed that the cyclophilin bands in different pollen extracts were not inhibited by the mould cyclophilin (Fig. 7). This shows that the absence of immunological cross-reactivity of Asp f 11 is not limited to Bet v 7, but can probably be extended to all plant cyclophilins.

4. Discussion

We have cloned Bet v 7, the birch pollen cyclophilin, and produced it in a bacterial system of expression. The recombinant protein was characterized and used in cross-reactivity studies, together with other recombinant cyclophilins. From these studies, it appears that the plant cyclophilins of group A may constitute a distinct subgroup with high immunological cross-reactivity, as demonstrated with rBet v 7, rTomCyP, and all other pollen cyclophilins tested. One paper nevertheless demonstrated that a 20-kDa cyclophilin of carrot (*Daucus carota*) is not cross-reactive with Bet v 7 (Fujita et al., 2001). However, the authors used patient sera reacting with the carrot cyclophilin to probe a commercial birch pollen extract

in immunoblotting experiments and found no band. Because special extraction conditions are needed to yield Bet v 7 in a birch pollen extract (Cadot et al., 1995), most commercial extracts do not contain it, which might explain the negative results in the paper in question. Whether Bet v 7 and the carrot cyclophilin A are cross-reactive still remains to be determined.

Plant cyclophilins do not seem to show high cross-reactivity with moulds and animal cyclophilins, as observed in our immunoblots and ELISA experiments. Interestingly, mould and human cyclophilins have been shown to be highly immunologically cross-reactive (Flückiger et al., 2002; Roy et al., 2003), but no data were available on possible cross-reactivity with plant cyclophilins. Another study showed that the *E. granulosus* cyclophilin does not cross-react with either the human cyclophilin or Mal f 6 (Ortona et al., 2002). However, whether the *E. granulosus* cyclophilin is cross-reactive with any plant cyclophilin A is not known.

According to Galat (1999), a common feature of all plant cyclophilins of group A described so far is a 7–8-amino acid insert (see Fig. 1). The mould and animal cyclophilins of group A lack such insert, which might account for structural differences leading to the diminution or the absence of cross-reactivity observed with the anti-plant cyclophilin A antiserum. According to the 3D-model of the cyclophilins (Galat, 1999), the part of the sequence containing that insert would not take part in the folding of the enzymatic active site, which could explain that plant cyclophilins do exert rotamase activity despite the structural differences due to this insert. When comparing the sequences, it is obvious that Asp f 11 diverges more from the plant cyclophilins than Mal f 6 (see Fig. 1). In addition, it also contains an extra amino acid insert at position 14–18 that might perturb the hinge region between β -sheet I and β -sheet II and some extra amino acids near the C-terminal part. This might account for bigger differences in

3D-structure between Asp f 11 and the plant cyclophilins. Interestingly, the results of our ELISA inhibition experiments also point in the same direction since Mal f 6 was able to inhibit the binding to Bet v 7 slightly, which was not the case for Asp f 11. In this regard, the cyclophilin A family seems to exemplify the link between sequence, 3D-structure, and immunogenicity.

Deduced from the ribbon diagrams of the human cyclophilin A (Galat, 1999) and Mal f 6 (Roy et al., 2003), we hypothesized that the plant seven-amino acid insert is surface-exposed as a protruding loop, making it an ideal candidate as an epitope. To challenge this hypothesis, we used three overlapping oligopeptides (KGNRSRGKP, GRS-GKPLHY, PLHYKKSSF; Eurogentec, Herstal, Belgium), covering the whole insert sequence of Bet v 7 and its flanking regions in ELISA and immunoblotting inhibition experiments. For comparison, equimolar concentrations of Bet v 7 and oligopeptides were used. However, none of the oligopeptides was able to inhibit, even to a low extent, the binding of the antibodies to rBet v 7 in ELISA, or to rBet v 7 and other pollen cyclophilins in immunoblots. This suggests that the eight-amino acid insert is not an epitope. Therefore, it might be that this insert is not accessible for antibody binding, which implies that it does not protrude from the protein structure. Indeed, a plant-specific protruding loop is expected to be very immunogenic in mammals. If it does not protrude then the impact on the 3D-structure of the whole molecule probably is higher. This could be a reason why the plant cyclophilin A constitutes an immunologically; hence, a structurally distinct subgroup among the cyclophilin A family.

Our results might have implications at the clinical level in the allergy practice. Since cyclophilins of remote species, namely mould and human cyclophilin A, were shown to be cross-reactive at the antibody level (Flückiger et al., 2002), one could expect a wide pan-allergenicity of this protein family. In view of our present results, it seems that despite high sequence identity, this is not the case. At least two clusters of cyclophilin A exist, among them the plant cyclophilins with high immunological cross-reactivity within but not between the clusters. At the clinical level, this means that a patient sensitized to a given plant cyclophilin A is highly susceptible to react to all other plant cyclophilin A, leading to multi-sensitization patterns and possibly food-pollen cross-allergies. IgE-sensitization to plant cyclophilins in patients is currently under investigation.

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