

Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro

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The *Bet v 1* gene family of birch encodes the major pollen allergens as well as pathogenesis-related (PR) proteins that are induced by microbes in somatic tissues. These PR proteins belong to a group of conserved intracellular defense-related proteins that have been termed 'ribonuclease-like' PR proteins, on the basis of the partial sequence homology observed between PR1, a *Bet v 1*-homologue from parsley, and a recently characterized ginseng ribonuclease. However, this enzymatic activity has not yet been demonstrated, not for any of the members of this family of PR proteins, nor for the related pollen allergens. We have investigated the possible nuclease activity of *Bet v 1* using apparently homogeneous preparations of natural *Bet v 1* purified from birch pollen, and a recombinant non-fusion protein purified from *E. coli* extracts. We report here that *Bet v 1* proteins indeed possess an intrinsic ribonucleolytic activity as they can digest different RNA substrates in vitro, but show no activity on single or double-stranded DNA.

Key words – Allergy, *Betula verrucosa*, birch, pathogenesis-related proteins, pollen allergens, ribonuclease, self-incompatibility.

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Introduction

The term pathogenesis-related (PR) proteins is used for a heterogeneous group of host-encoded, low molecular mass polypeptides that accumulate as part of a multi-component defense response in plant tissues exposed to fungi, bacteria, viruses or viroids, or to certain chemical and biotic elicitors (for reviews see Bowles 1990, Carr and Klessig 1989, Lamb et al. 1989, van Loon 1985). In the last few years, a new family of small, acidic, intracellular defense-related proteins, different from all previously characterized PR proteins, has been identified in both dicotyledonous (Crowell et al. 1992, Fristensky et

al. 1988, Matton and Brisson 1989, Somssich et al. 1986, Walter et al. 1990) and monocotyledonous (Warner et al. 1992) plant species. Members of this ubiquitous class of conserved PR proteins are transcriptionally activated upon bacterial or fungal infection, by treatment with fungus-derived elicitors or, in some cases, by wounding or under various stress conditions.

Bet v 1 proteins, a group of highly homologous polypeptides of 17 kDa, are constitutively expressed in birch (*Betula verrucosa*) pollen and together represent the major birch pollen allergen, responsible for IgE binding in more than 95% of patients allergic to birch pollen (Jarolim et al. 1989a). A cDNA clone encoding

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the most abundant isoform, Bet v 1a, was isolated by Breiteneder et al. (1989) by immunoscreening of a pollen cDNA expression library with a patient's serum, and using PCR we have recently cloned several additional cDNAs corresponding to the minor pollen isoallergens (Swoboda et al. 1995a). Bet v 1 was included in the abovementioned class of intracellular PR proteins, initially only on the basis of its sequence homology to the other members of this family. Previously, we reported the first data on pathogen-related expression of Bet v 1 (Swoboda et al. 1994), showing that transcripts hybridizing to a pollen *Bet v 1a* cDNA probe, and proteins cross-reacting with an anti-Bet v 1 monoclonal antibody, were induced in birch cell suspension cultures in the presence of bacteria or fungi. More recently, we provided direct evidence that the *Bet v 1* gene family also encodes PR proteins. A subset of *Bet v 1* genes (EMBL Nucleotide Sequence Database accession numbers X77599 to X77601) was isolated and characterized; these genes are transcriptionally activated in suspension-cultured cells by bacteria or fungi, as well as in birch leaves infected with a natural fungal pathogen, but are not expressed constitutively in pollen (Swoboda et al. 1995b).

Similarities in amino acid sequence, protein size and total charge strongly suggest a common ancestry and an identical or related, but still unknown, function for this family of defense-related proteins. The investigation of the mechanism of action of Bet v 1 and homologous PR proteins during the defense response against pathogen attack, as well as of the biological function of the Bet v 1 pollen allergens, have been hampered by the lack of information regarding their possible enzymatic activity, since no significant homology was found to any previously characterized protein. In a recent paper, Moiseyev et al. (1994) reported the isolation of a new type of ribonuclease from a ginseng (*Panax ginseng*) cell callus. The sequence of two peptides from this enzyme showed, over 40 amino acids, ca 70 and 60% identity, respectively, to parsley PR1 and PR2, which are members of the group of Bet v 1-homologous PR proteins. However, we found that these peptides had much lower identity, 40 to 45%, to the corresponding sequences of Bet v 1 proteins from pollen and suspension cultures or of other members of the family (e.g., pI49 from pea, Daniels et al. 1987; PvPR1 from bean, Walter et al. 1990).

The availability of purified preparations of natural Bet v 1 isoforms isolated from birch pollen, and of recombinant Bet v 1a (Ferreira et al. 1993, Swoboda et al. 1995a) prompted us to investigate the possible RNase activity of these proteins. In this paper we show that Bet v 1 can digest various RNA substrates in vitro, but has no detectable activity on single or double-stranded DNA, strongly suggesting that intracellular, 'ribonuclease-like' PR proteins (van Loon et al. 1994) are indeed ribonucleases.

Abbreviations – PR, pathogenesis-related.

Materials and methods

Birch pollen washes and purification of natural and recombinant Bet v 1 proteins

Birch pollen (Allergon AB, Ängelholm, Sweden) was suspended in 10 mM potassium phosphate buffer, pH 7.0, shaken overnight at 4°C and centrifuged at 12 000 g for 5 min at 4°C in a Sorvall SS34 rotor. The supernatant, referred to as pollen wash, was concentrated by ammonium sulfate precipitation (80% saturation), dialyzed against 10 mM potassium phosphate buffer, pH 7.0, and the final sample was stored in aliquots at –80°C until used. Natural Bet v 1 (nBet v 1, a mixture of the different isoallergens) was purified from pollen washes to electrophoretic homogeneity by reversed phase HPLC on a Hypersil WP300 C₈ column, as previously described (Ferreira et al. 1993, Swoboda et al. 1995a). Recombinant non-fusion Bet v 1a (rBet v 1a) was purified from crude *Escherichia coli* lysates by chromatofocusing on a PBE-94 exchange column followed by reversed phase HPLC (Ferreira et al. 1993, Swoboda et al. 1995a). Protein concentrations were measured according to Bradford (1976), using the Bio-Rad reagent and bovine serum albumin (BSA) as standard, except for the purified nBet v 1 fraction shown in the silver-stained SDS gel (Fig. 1b) which was determined by the micro-Kjeldahl method with glycine as standard (Jacobs 1959).

SDS-PAGE and immunoblotting

Pollen washes and purified natural Bet v 1 were run on 12 or 15% SDS-polyacrylamide gels (Laemmli 1970), and proteins were visualized by staining the gels with Coomassie brilliant blue R-250 or with silver (Merril et al. 1984). For immunoblotting, polypeptides separated by SDS-PAGE were transferred to nitrocellulose membranes by semi-dry blotting (Semi-Phor TE70 transfer unit, Hoeffer, San Francisco, CA, USA) and incubated with the anti-Bet v 1 monoclonal antibody BIP 1 (Jarolim et al. 1989b). Bet v 1 was finally detected with an anti-mouse antibody coupled to alkaline phosphatase and the colorimetric substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT).

Nucleic acid substrates

RNase activity assays were carried out with total RNA prepared from a birch cell suspension culture (according to Chomczynski and Sacchi 1987) or with yeast tRNA (Boehringer Mannheim, Vienna, Austria) as non-radioactive substrates. ³²P-labelled RNA substrates were prepared by 5'-end labelling of yeast tRNA in the presence of [γ -³²P]ATP and T4 polynucleotide kinase (Maniatis et al. 1982) and by in vitro transcription from DNA fragments cloned into pGEM (Promega, Madison, WI, USA) vectors with SP6 or T7 RNA polymerase (Melton et al. 1984).

DNase activity was assayed using the pGEM7Zf(+) plasmid (Promega) as double-stranded DNA substrate, either in the super-coiled form or after linearization, and the M13mp18 vector (United States Biochemical, Cleveland, OH, USA) as a single-stranded DNA substrate.

Enzyme assays

The incubation mixtures contained, in 10 μ l final volume, a birch pollen wash (15 μ g of protein) or 1 μ g of purified nBet v 1 or rBet v 1a fractions, the different RNA or DNA substrates as indicated in the legends, and 10 mM potassium phosphate buffer, pH 7.0. The samples, as well as controls without protein, were incubated for 1 h at 37°C. In experiments performed with ^{32} P-labelled RNA probes, assays were terminated by phenol-chloroform-isoamyl alcohol (25:24:1) extraction. After ethanol precipitation, radioactive tRNA or in vitro-transcribed RNA was analyzed by electrophoresis in 6% polyacrylamide/7 M urea gels followed by autoradiography using Kodak AR X-Omat films. In the case of non-radioactive DNA or RNA substrates, the whole enzyme assays were directly loaded onto 1.2% agarose gels, and separated nucleic acids were then stained with 0.5 μ g ml⁻¹ ethidium bromide.

Results

Bet v 1 proteins accumulate at relatively high levels in mature birch pollen and are efficiently released in the presence of aqueous buffers. A protein band of 17 kDa, one of the most abundant polypeptides detected in Co-

massie-stained SDS gels of pollen washes (Fig. 1a, lane 1), was identified as Bet v 1 by its reactivity with an anti-Bet v 1 monoclonal antibody (BIP 1; Jarolim et al. 1989b) in immunoblots of the same sample (Fig. 1a, lane 3). A purified fraction of natural Bet v 1 (nBet v 1), prepared from pollen washes by reversed phase HPLC, migrated as a single band in the polyacrylamide-SDS gel, after Coomassie blue staining (Fig. 1a, lane 2). In an independent SDS/polyacrylamide gel, the more sensitive silver staining method confirmed the absence of contaminating proteins in the HPLC-purified nBet v 1 fraction (Fig. 1b).

To assay the possible RNase activity of Bet v 1 proteins, the pollen wash or the purified nBet v 1 fraction was incubated with ^{32} P-labelled RNA substrates, either a commercial yeast tRNA fraction (Fig. 2a, lanes 1–3) or an in vitro-transcribed RNA (Fig. 2a, lanes 4–6). After incubation at 37°C for 60 min, the reaction mixtures were extracted with phenol/chloroform, precipitated with ethanol and analysed by denaturing polyacrylamide gel electrophoresis, followed by autoradiography. As shown in Fig. 2a, both RNA substrates were completely degraded under these conditions. Cleavage of the nucleic acids into small fragments was indicated by the presence of very short radioactive bands, which were observed when the extraction and ethanol precipitation steps were omitted and the electrophoresis was stopped before the bromophenol blue dye, contained in the loading buffer, reached the bottom of the gel (data not shown). Degradation of the in vitro-transcribed RNA substrate was also observed in identical experiments carried out with recombinant Bet v 1a (rBet v 1a) protein,

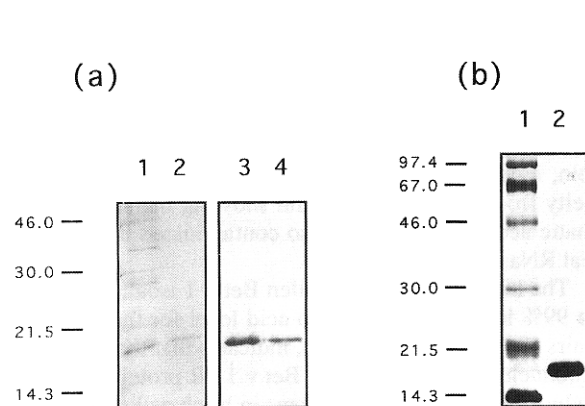


Fig. 1. SDS-PAGE and immunoblot analysis of Bet v 1 protein fractions. (a) A birch pollen wash (10 μ g protein; lanes 1 and 3) or 2 μ g of nBet v 1 purified from pollen washes by reversed-phase HPLC (lanes 2 and 4) were separated on a 15% polyacrylamide-SDS gel; polypeptides in lanes 1 and 2 were visualized by staining with Coomassie brilliant blue R-250, while samples in lanes 3 and 4 were blotted onto nitrocellulose. The membrane was developed with the anti-Bet v 1 monoclonal antibody BIP 1. Numbers on the lefthand side are molecular masses (kDa) of standard proteins. (b) Silver-stained 12% polyacrylamide-SDS gel of molecular mass markers (lane 1) and the purified nBet v 1 fraction (1 μ g protein; lane 2).

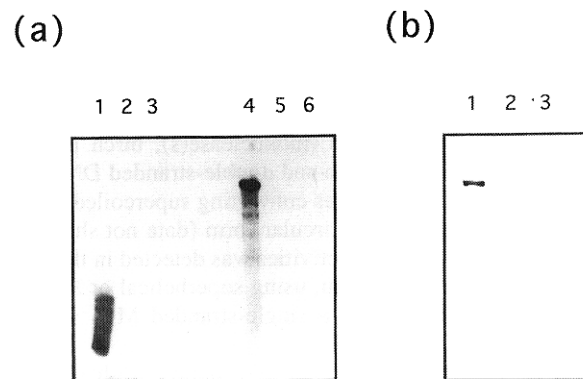


Fig. 2. RNase activity of birch pollen washes and purified nBet v 1 or rBet v 1a. (a) Commercial yeast tRNA, ^{32} P-labelled at the 5'-end (lanes 1–3), or a uniformly labelled in vitro transcript (lanes 4–6) (ca 15 000 Cerenkov cpm in all samples) were incubated for 60 min at 37°C with 10 mM potassium phosphate buffer, in the absence (lanes 1 and 4), or in the presence of either a birch pollen wash (15 μ g of protein; lanes 2 and 5) or 1 μ g of purified nBet v 1 (lanes 3 and 6). (b) 1 μ g purified nBet v 1 (lane 2) or rBet v 1a (lane 3) were incubated with the in vitro-transcribed, ^{32} P-labelled RNA as in (a). Control: RNA substrate incubated in the absence of protein fractions (lane 1). The figure shows the corresponding autoradiograms after overnight (a) or 8 h (b) exposure at -80°C.

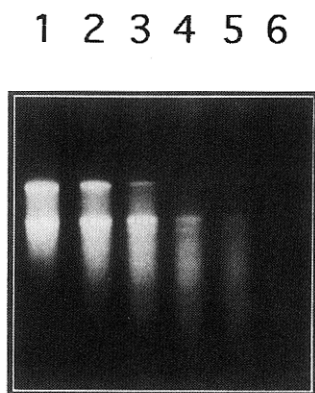


Fig. 3. Time-course of rRNA digestion in the presence of purified nBet v 1. Total RNA (2.6 µg) isolated from a birch cell suspension culture was incubated for 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (lane 4), 30 (lane 5) or 60 (lane 6) min in the presence of 1 µg of purified nBet v 1, and the samples were separated in a 1.2% agarose gel and stained with ethidium bromide.

purified from *E. coli* extracts (Fig. 2b, lane 3). These results indicate that the RNase activity detected in the pollen wash can be attributed, at least in part, to Bet v 1 proteins.

The time-course of RNA degradation was followed using the purified nBet v 1 fraction and, as a substrate, total RNA prepared from birch cell suspension cultures (Fig. 3). Partial degradation of the 28S rRNA band and accumulation of low molecular mass products was already observed after 5–10 min of incubation at 37°C (Fig. 3, lanes 2 and 3), followed by the progressive digestion of the 18S rRNA (Fig. 3, lanes 3 and 4). After 30 min, no intact RNA was detected in the ethidium bromide-stained agarose gel (Fig. 3, lane 5).

The rapid release from rehydrated mature pollen of several enzymes, including nucleases, has been reported for different plant species (Matousek and Tupy 1985). Apart from the observed ribonuclease(s), birch pollen washes also contain single and double-stranded DNases, as well as nicking activities converting supercoiled plasmid DNA into the open circular form (data not shown). However, none of these activities was detected in the purified nBet v 1 preparation, using superhelical or linearized plasmid dsDNA or a single-stranded M13 vector DNA as substrates (Fig. 4).

Relatively large amounts of the pollen wash (15 µg protein) or purified nBet v 1 and rBet v 1a fractions (1 µg) were used in our assays in order to observe the complete degradation of the different substrates. However, in preliminary experiments we have observed the release of non-precipitable radioactivity from uniformly labelled RNA transcripts in the presence of much lower protein concentrations (data not shown). This simple, quantitative assay will be used to determine the optimal conditions of the reaction (pH and ionic strength of the reaction buffer, temperature, etc.) and to characterize the RNase activity of Bet v 1 proteins further.

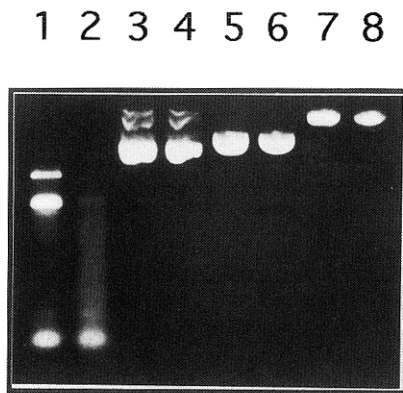


Fig. 4. Effect of purified nBet v 1 on different DNA substrates. Super-coiled double-stranded pGEM7Zf(+) plasmid (lanes 3 and 4), linearized pGEM7Zf(+) (lanes 5 and 6), single-stranded M13mp18 vector DNA (lanes 7 and 8) or, as a positive control, total RNA isolated from a birch suspension culture (lanes 1 and 2) were incubated for 60 min at 37°C in the absence (lanes 1, 3, 5 and 7) or in the presence (lanes 2, 4, 6 and 8) of 1 µg of purified nBet v 1. All samples contained 3 µg of DNA or RNA. Nucleic acids were separated in a 1.2% agarose gel and visualized by staining with ethidium bromide.

Discussion

The results reported here strongly suggest that Bet v 1 proteins, purified from birch pollen washes, possess an intrinsic nuclease activity specific for RNA, as they digest *in vitro* tRNA, rRNA or *in vitro*-transcribed RNA, but show no activity on single or double-stranded DNA substrates. Although the HPLC-purified nBet v 1 fraction used in this study appears to be homogeneous, as it migrates as a single band in silver-stained SDS gels, it cannot be completely excluded that a contaminating RNase of the same molecular mass is responsible for the observed ribonucleolytic activity. However, we consider this possibility extremely unlikely, since it would be necessary to assume that the recombinant Bet v 1a protein, independently purified to electrophoretic homogeneity from *E. coli* extracts and showing the same enzymatic activity *in vitro*, is also contaminated by a bacterial RNase of 17 kDa.

The high homology of pollen Bet v 1 isoallergens, 84 to 99% identity at the amino acid level for the different pairs (Swoboda et al. 1995a), indicates that they all have ribonucleolytic activity. The Bet v 1 PR proteins that are induced by microbial pathogens in birch cell suspension cultures (Swoboda et al. 1994) show 70 to 88% amino acid identity to the pollen allergens (Swoboda et al. 1995b). It is therefore most likely, although not yet demonstrated, that they are also RNases, as are the homologous PR proteins of the same family identified in several other plant species. Therefore, our data provide the first experimental support to the idea that this class of intracellular defense-related proteins are ribonucleases, as suggested by the relatively high identity, 60–70%, observed between two peptides of a recently isolated gin-

seng RNase and the corresponding partial amino acid sequences of the Bet v 1 homologues from parsley (Moiseyev et al. 1994). This ginseng ribonuclease obviously belongs to the same family but, since no data on its possible pathogen-related expression are available, cannot as yet be defined as a PR protein.

The ribonucleolytic activity described in this study might provide some clues on the biological function of Bet v 1 and homologous proteins during the defense reaction against pathogen attack. It is still unclear whether these PR proteins have anti-microbial activity. In situ hybridization experiments with fungus-infected parsley leaf sections demonstrated that the mRNA of PR1, a parsley Bet v 1-homologue, accumulates rapidly and massively within the area of pathogen penetration, suggesting a role for this protein in the restriction of fungal growth (Somssich et al. 1988). On the other hand, we have shown that Bet v 1 does not inhibit bacterial or fungal growth in vitro (Swoboda et al. 1994), although it might degrade microbial RNA in the infected plant in the presence of additional proteins or other factors; however, the intracellular localization of this family of PR proteins makes this possibility rather unlikely. RNAs from viruses or viroids might be other potential targets of these RNases, which could play a direct role in the defense of the plant against these types of pathogens. Unfortunately, as far as we know, it has not been tested whether Bet v 1 or its homologues are induced in the presence of viral or viroidal pathogens. Alternatively, and we favour this hypothesis, these intracellular PR proteins, once they are induced upon interaction of the pathogen with cells at the site of infection, may degrade cellular RNAs and thus contribute to the hypersensitive response, the localized cell death that blocks systemic spread of the pathogen through the plant.

The biological function of Bet v 1 proteins in pollen also remains an open question. They may play a role in the defense reaction of pollen against pathogen attack, as was initially proposed (Breiteneder et al. 1989). However, considering their presence in mature, dry pollen and their high solubility in aqueous solutions, we think it is more likely that Bet v 1 isoallergens act after pollen shedding, i.e., during pollination, as extracellular proteins. After landing on the stigma, Bet v 1 would be rapidly and efficiently released from the rehydrating pollen grains and, assuming that it indeed has some kind of anti-microbial and/or anti-viral activity, would protect the female reproductive tissues (rather than pollen itself) from infection by pathogens during the early phases of pollen tube growth through stigma and style.

In addition, a function of Bet v 1 proteins in mechanisms of self-incompatibility can be envisaged, taking into account the often proposed similarities and the hypothesized common evolutionary origin of host-pathogen interactions and self-incompatibility (Dickinson 1994, Pryor 1987). Bet v 1 proteins, originally involved in pathogen defense, may have evolved to inhibit pollen tube growth in the case of incompatible interactions,

possibly after their activation by specific proteins or other factors present in the female reproductive tissues. Indeed, in several plant species the S-alleles of gametophytic self-incompatibility encode RNases, although unrelated in primary sequence to Bet v 1 (for a review see Matton et al. 1994), and birch also has a gametophytic self-incompatibility system (Hagman 1971). However, all known self-incompatibility ribonucleases are extracellular glycoproteins expressed specifically in pistils, and Bet v 1 would represent a unique example of an extracellular S-RNase of pollen origin.

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