

The major birch pollen allergen, Bet v 1, shows ribonuclease activity

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Abstract. The major birch (*Betula alba* L.) pollen allergen, Bet v 1, has been shown to be homologous to pathogenesis-related proteins in a number of plants. Recently, it was demonstrated that a ginseng protein with high homology to an intracellular pathogenesis-related protein of parsley and to Bet v 1 is a ribonuclease (RNase). Birch pollen extract was separated in an RNase activity gel. Four major RNase bands were excised from the gel, re-separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identified by Western blotting with a specific Bet v 1 monoclonal antibody and patient's serum. Thus the monomer and the dimer of Bet v 1 showed RNase activity. Purified recombinant Bet v 1 was shown to degrade plant RNA. The RNase activity of recombinant Bet v 1 was 180 units · mg⁻¹.

Key words: Allergen – *Betula* (pollen) – Pathogenesis-related protein – Pollen – Ribonuclease

Introduction

The major birch pollen allergen, Bet v 1, is well characterized and has been shown to induce type-1 allergy in over 90% of patients allergic to tree pollen (Larsen et al. 1992). The function of this protein is still unknown. Homology of Bet v 1 to pathogenesis-related (PR) proteins in pea and parsley seems to indicate that this allergen is involved in plant resistance (Breiteneder et al. 1989). Some authors have proposed that Bet v 1 is a so-called stress-induced protein, and thus explain the increase in allergic diseases partly by environmental stress to the plants (Scheiner 1992). However, it has not yet been proven that Bet v 1 itself is a PR protein. Recently, it was demonstrated that a ginseng protein with high homology to the intracel-

lular pathogenesis-related (IPR) protein of parsley and to Bet v 1 is a ribonuclease (RNase) (Moiseyev et al. 1994).

In this communication, we demonstrate that the major allergen, Bet v 1, shows RNase activity and determine the RNase activity of birch pollen extract by an RNase activity assay.

Materials and methods

Proteins were extracted from 1 g birch (*Betula alba* L., syn. *B. verrucosa* Ehrh.) pollen (P-6770; Sigma, St. Louis, Mo., USA). Pollen was incubated in 55 ml of 0.1 M NH₄HCO₃ buffer (pH 8) for 30 min at room temperature. After centrifugation for 30 min at 17000 · g (4 °C), the supernatant was dialyzed against double-distilled RNase-free water, lyophilized and dissolved in appropriate sample buffer for SDS-PAGE. Recombinant Bet v 1 was kindly provided by ALK-Laboratories (Copenhagen, Denmark). For purification, the gene encoding Bet v 1 subcloned into the maltose-binding protein fusion vector pMAL-c (New England Biolabs, Mass., USA) was subsequently expressed in *Escherichia coli* K12 cells. The fusion protein was purified via a column of amylose resin (New England Biolabs). After enzymatic cleavage of the fusion partner, recombinant Bet v 1 was isolated by fast protein liquid chromatography (FPLC) molecular-sieve chromatography using Sephadex 75 HiLoad 16/60 (Pharmacia, Uppsala, Sweden), equilibrated with 20 mM Tris-HCl (pH 8.0).

The RNase activity gels were prepared according to the method of Yen and Green (1991), and SDS-PAGE performed as described by Bufer et al. (1995). Regions showing RNase activity were excised from the gel and gel pieces containing the RNases were applied to a vertical 12% SDS-PAGE gel (Laemmli 1970). Proteins were separated, then electroblotted onto nitrocellulose membrane on a Fastblot apparatus (Biometra, Göttingen, Germany) and finally stained in strips with india ink. The immunostaining was performed with 10 µg · ml⁻¹ of the monoclonal antibody 4B10 (Allergopharma, Reinbek, Germany) specific for Bet v 1 in a procedure described by Petersen et al. (1993).

The RNase gels were prepared and run as described by Bufer et al. (1995), and RNase activity was determined according to Barna et al. (1989) with modifications. Birch pollen proteins were incubated with 1 µg timothy grass (*Phleum pratense* L.) RNA extracted as described earlier (Bufer et al. 1994). After incubation, 1 volume of 4 M LiCl was added to the mixture, which was then stored at 4 °C for at least 3 h before centrifuging at 12 000 · g for 12 min. Supernatants were diluted and absorption was measured at 260 nm. One unit of RNase activity

Abbreviations: IPR = intracellular pathogenesis-related (protein); PR = pathogenesis related; RNasin = RNase inhibitor

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was defined as the amount of protein causing an increase in absorption of 1.0 after 30 min incubation at 56°C at pH 6.5. Alignment of sequences was done on PC/Gene (Intelligenetics, Geel, Belgium).

Results and discussion

Figure 1 demonstrates a homology of 61% between the 42 N-terminal amino acids of Bet v 1 (Breiteneder et al. 1989), Car b 1 (Larsen et al. 1992), the major allergen of hornbeam pollen, ginseng RNase (Moiseyev et al. 1994) and the IPR-1 protein of parsley (Somssich et al. 1988). Apart from this homology it has not been possible to demonstrate that Bet v 1 is an active PR protein. Likewise the homology to the ginseng RNase does not confirm that Bet v 1 is an RNase. In order to determine whether natural Bet v 1 is an RNase we separated birch pollen extract in an RNase activity gel using yeast RNA as substrate (Fig. 2). The birch pollen extract contained at least five different bands with RNase activity. In order to determine whether or not the two bands with RNase activity at 18 and 37 kDa in birch pollen extract are active forms of Bet v 1, the bands were excised from the gel, re-separated by SDS-PAGE and blotted onto nitrocellulose. Proteins were stained with india ink, auro dye and a Bet v 1-specific monoclonal antibody (Fig. 2). The upper active band at 18 kDa was precisely excised from the gel and stained by india ink and auro dye. In view of the fact that this band is bound by Bet v 1-specific monoclonal antibody and runs in the gel at the same molecular weight as the allergen in the birch pollen extract, we suggest that the excised protein is Bet v 1 and that it shows RNase activity. The

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BET_V_1      GVFNYESSTSTVIPAARLF-KAFILDGDNLFKVPQAISV 41
CAR_B_1      GVFNYESSTSTVIPAARLFKFSYVLDGDKLIPKVPQAISV 41
GINSENG      GVQKTEVEATSTVPAQKLYA-KQRIDAIDKDALTYSTIG 40
PARSLEYPR1   GVQKSEVEATSSVSAEKLFRMKQKVDKATFTYSYSIID- 41
              ** S *S*SS* SS*SS*S      S*S S S S S S* S

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Fig. 1. Alignment of N-terminal sequences of Bet v 1 (Breiteneder et al. 1989), Car b 1 (hornbeam; Larsen et al. 1992), ginseng RNase (Moiseyev et al. 1994) and the IPR-1 protein of parsley (Somssich et al. 1988). * = identity (21.4%); s = similarity (40.5%); homology = 61.9%

immunoreactivity of the excised RNase was much weaker than that of the original protein in the pollen extract, a finding which can be explained by the small amount of protein that was excised from the gel. This indicates that Bet v 1 is a weak RNase in the yeast system. Additional proof for the supposition that Bet v 1 is an RNase comes from the upper RNase-active band at 37 kDa, which was also excised from the same RNase activity gel and re-separated. This band is obviously related to the dimer of Bet v 1; because it is bound by the Bet v 1 monoclonal antibody. The fact that RNase activity is associated with both the monomer and dimer of Bet v 1 indicates that it is unlikely that the activity is attributable to contamination. Additional evidence for Bet v 1 RNase activity was sought with a different system using timothy grass RNA as substrate. Incubation of purified recombinant Bet v 1 with timothy grass RNA under defined conditions (Fig. 3, lane 5) led to the degradation of the RNA. This process could be inhibited by RNase inhibitor (RNasin; lane 6). Pancreatic RNase A completely degraded the RNA (lane 3) and, because of its high activity, was not inhibited by RNasin (lane 4). In a previous experiment it was shown that bovine serum albumin neither degraded nor complexed with timothy grass RNA (data not shown). In view of the fact that RNase contamination of the recombinant Bet v 1, after extensive purification (Fig. 4), is unlikely, there is now good evidence for this allergen to be an RNase.

The RNase activity of Bet v 1 was $180 \text{ U} \cdot \text{mg}^{-1}$ and thus more than 16 times weaker than birch pollen extract ($3000 \text{ U} \cdot \text{mg}^{-1}$) and 1600 times weaker than pancreatic RNase A (Fig. 5), as shown by an activity assay using timothy grass RNA as substrate. This disparity may be due to the different specificity of Bet v 1 as an RNase but this has still to be determined. It may also indicate that the activity assay conditions, especially the ion concentrations, were not completely optimized.

We conclude that pollen proteins with high homology to PR proteins of other plants can show RNase activity, and that these RNases can induce type-I allergy. Whether the allergenicity of Bet v 1 depends on the enzyme activity is an open question. We have recently shown that another major allergen of grass pollen is an RNase (Bufe et al.

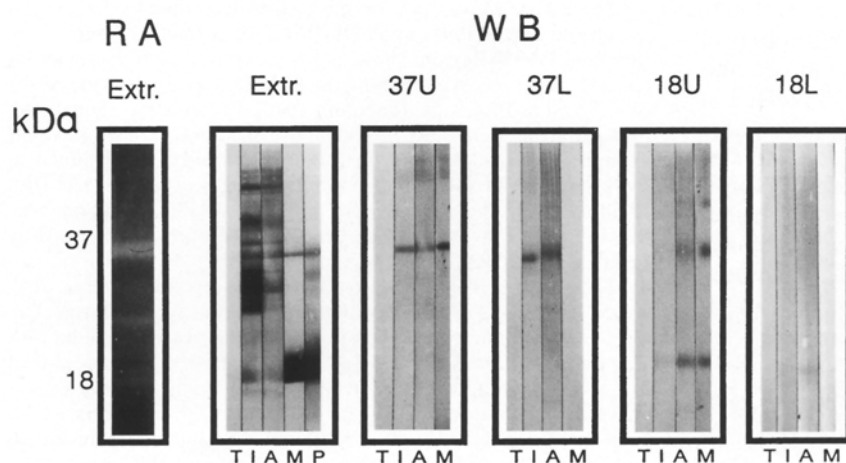


Fig. 2. Ribonuclease activity gel (RA) and Western blots (WB) of four RNase-active bands excised from the RNase activity gel of birch pollen extract and separated by SDS-PAGE. Extr., birch pollen extract; 37U, upper RNase activity band at 37 kDa; 37L, lower RNase activity band at 37 kDa; 18U, upper RNase activity band at 18 kDa; 18L, lower RNase activity band at 18 kDa. Lane T (Tris), negative control with secondary antibody; lane I, stained with india ink; lane A, stained with auro dye; lane M, probed with monoclonal antibody specific for Bet v 1; lane P, patient's serum

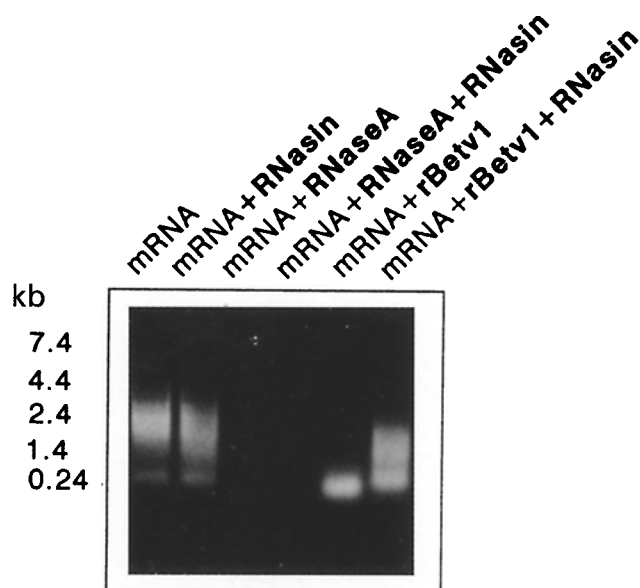


Fig. 3. Ribonuclease activity of recombinant Bet v 1. An RNase activity gel (1.2% agarose) was loaded, per lane, with 1 µg RNA from timothy grass inflorescences and incubated with, from left to right: H₂O; 38 U RNasin; 2 µg RNase A; 2 µg RNase A + RNasin; 2 µg recombinant Bet v 1; 2 µg recombinant Bet v 1 + RNasin

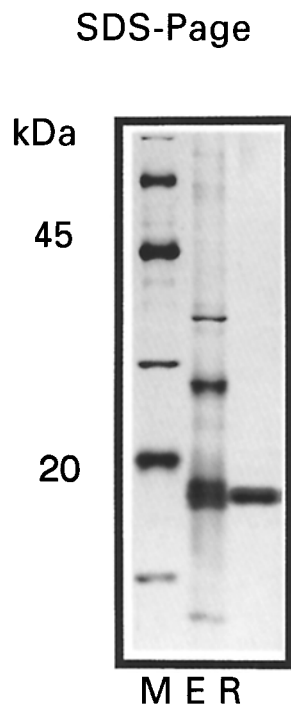


Fig. 4. Birch pollen extract (lane E) and purified recombinant Bet v 1 (lane R) on a silver-stained 12% SDS-PAGE gel. Lane M, marker (SDS-PAGE molecular weight standard low range, BioRad)

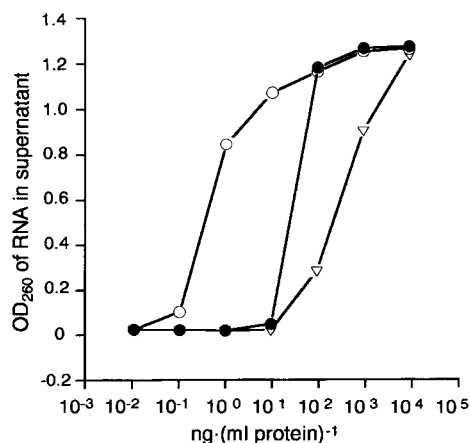


Fig. 5. Ribonuclease activity of recombinant Bet v 1, compared with birch pollen extract and pancreatic RNase A using various concentrations of timothy grass RNA as substrate. Open triangles, recombinant Bet v 1; closed circles, birch pollen extract; open circles, RNase A

1995) and that its allergenicity can be inhibited by RNasin in vitro (data not shown). The function of rapidly exported RNases in pollen has still to be elucidated.

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