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Crystallization and Preliminary X-Ray Investigation at 2.0 Å Resolution of Bet v 1, a Birch Pollen Protein Causing IgE-Mediated Allergy

Michael D. Spangfort,[1] Jørgen N. Larsen,[1] and Michael Gajhede[2]
[1]ALK A/S, Research Department, DK 2970 Hørsholm; [2]Department of Chemistry, University of Copenhagen, DK 2880 Copenhagen, Denmark

ABSTRACT **The 17 kDa protein from *Betula verrucosa* (White Birch) pollen, Bet v 1, is the clinically most important birch pollen allergen causing immediate Type I IgE-mediated allergy. The three-dimensional structure of Bet v 1 and its IgE-binding epitopes are at present not known. In addition, the biological function of Bet v 1 in birch pollen is not fully established. In this work, Bet v 1 has been expressed in *Escherichia coli* as a recombinant protein, purified and crystallized. The space group of recombinant Bet v 1 crystals is orthorhombic C2221 with unit cell parameters a = 32.13 Å, b = 74.22 Å, and c = 118.60 Å. There is one Bet v 1 molecule per asymmetric unit and the water content is 41%. Crystals diffract to 2.0 Å resolution and a complete native data set was collected from a single crystal using CuK_α X-rays from a rotating anode.** © 1996 Wiley-Liss, Inc.

Key words: allergy, Bet v 1, betula, birch, pollen, crystallization

INTRODUCTION

It is estimated that about 15–20% of the population in developed countries suffer from IgE-mediated (Type I) allergy or hypersensitivity, and there is convincing evidence that the prevalence of asthma and of other allergic disorders has steadily increased in the last decade.[1,2] In susceptible individuals, the production and binding of IgE to a foreign allergen triggers the cellular events leading to the release of inflammatory mediators followed by the appearance of clinical symptoms of allergic disease such as rhinitis, rhinoconjunctivitis, and asthma. The main sources of airborne allergens are pollens of grasses and trees, animal danders, mites, and moulds. Several major allergens from these sources have been identified as unique soluble proteins with molecular masses between 10–60 kDa.[3] Although several allergens have been cloned and their genetic structure elucidated, little information exist regarding the three-dimensional architecture of their immunoreactive structures, i.e., epitopes. Furthermore, the biological function of the majority of important allergens is unknown.

The major allergen of Birch (*Betula verrucosa*) pollen has been identified as a 17 kDa protein denoted Betv 1.[4–6] More than 95% of birch pollen allergic patient's serum IgE react with Bet v 1 when analyzed by crossed radioimmunoelectrophoresis[7] showing that it is the most clinically important allergen in birch pollen extract. Birch pollen allergic patient's IgE show cross-reactivity between Bet v 1 and the major pollen allergens Aln g 1, Car b 1, and Cor a 1 from other tree species of the *Fagales*.[8,9]

The biological function of Bet v 1 is not yet fully established although the nucleotide sequence shows homology to a pathogenesis-related protein mRNA sequence.[10] Based on in vitro measurements, it has recently also been suggested that Bet v 1 is a pollen-specific RNAse.[11] Birch pollen extracts contain more than 20 different isoforms (isoallergens) of Bet v 1[12,13] and several different Bet v 1 cDNA sequences have been described.[14] Analysis of IgE from individual birch pollen allergic patient's sera show differences in reactivity towards different Bet v 1 isoallergens indicating that they differ in epitope structure.[15–17] To study the three-dimensional epitope structure of Bet v 1, it has been produced as a recombinant protein in *Escherichia coli*.[18,19] It was found that the primary structure and immunochemical properties of recombinant Bet v 1 were indistinguishable from that of naturally occurring Bet v 1. Here we describe the crystallization and preliminary X-ray investigation of recombinant Bet v 1.

MATERIALS AND METHODS

Protein Purification

The gene encoding Bet v 1 was subcloned into the protein fusion vector pMAL-c (New England Biolabs, Beverly, MA) and expressed in fusion with the maltose-binding protein.[19] In order to generate the

Abbreviations: IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulphate; LB medium, Luria-Bertani medium; PCR, polymerase chain reaction.

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Address reprint requests to Michael D. Spangfort, ALK A/S, Research Department, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark.

authentic aminoterminal of Bet v 1 after Factor Xa cleavage, PCR was applied to position the Factor Xa protease cleavage site immediately in front of the aminoterminal glycine of Bet v 1. For purification of recombinant protein, *E. coli* strain K12 DH5α cells containing the recombinant plasmid were grown in LB medium and harvested 4 hours after IPTG-induction. Recombinant cells were broken by sonication and loaded onto an amylose affinity-column (KemEnTec, Copenhagen, Denmark) after removal of cell debris by centrifugation. The column was washed with 0.5 M NaCl, 10 mM sodium phosphate, 2 mM EDTA, and pH 7.0, and fusion protein was recovered in a single distinct fraction by elution with 10 mM maltose in the same buffer. The purified fusion construct was thereafter enzymatically cleaved into its two protein constituents by incubation for 16 hours with 0.5% (w/w) Factor Xa (Denzyme, Aarhus, Denmark) in 100 mM NaCl, 0.01% SDS, 1 mM $CaCl_2$, 20 mM TRIS, and pH 8.0. Following enzymatic cleavage, recombinant Bet v 1 was isolated by FPLC molecular sieve chromatography using Superdex 75 HiLoad 16/60 (Pharmacia, Copenhagen, Denmark) equilibrated with 20 mM TRIS-HCl pH 8.0. Purified recombinant Bet v 1 was concentrated and extensively dialysed against 1 mM sodium citrate, pH 6.5, 0.01% sodium azide. The final protein preparation was stored at 4°C.

X-Ray Crystallography

All data sets were collected in-house on a Rigaku (Molecular Structure Cooperation, USA) R-axis IIC Image Plate system with a rotating anode operating at 50 kV and 180 mA. Using a graphite monochromator and a 0.5 mm collimator, monochromatic CuK_α radiation was produced. The crystal to image plate distance was 100 mm. The temperature was 285 K during the data collections. The first dataset (dataset I) collected included 92 frames measured with 2.0° oscillations, with an exposure time of 1 hour. This data collection aimed at collecting a full P1 dataset for unambiguous determination of the spacegroup. A second dataset (dataset II) was collected from a larger crystal. Dataset II included 69 frames measured with 2.0° oscillations, each with an exposure time of 1 hour. The DENZO program[20] was used to integrate the raw data and to calculate standard deviations, whereon program SCALEPACK[20] and programs from the CCP4-package[21] were used for scaling, merging, and truncation of the data.

RESULTS AND DISCUSSION

Crystals were grown using the sitting drop diffusion technique[22] with ammonium sulphate as precipitating agent. Initial experiments at different pH and concentrations of precipitating agent produced small crystals at 2.6 M ammonium sulphate, pH 5.5 at 4°C. These crystals were used as seeds in the subsequent optimization of crystallization conditions.

TABLE I. Data Collection Statistics for Dataset II*

Number or reflections	54458
Number rejected	2
Number of unique reflections	9554
Completeness (%)	95.8%
Lowest resolution (Å)	50.0
Outermost shell (Å)	2.03–2.00
Completeness outermost shell (%)	84.2
Overall $I/\sigma(I) > 2$ (%)	86.7
Outermost shell $I/\sigma(I) > 2$ (%)	68.2
R_{MERGE} (%) overall	7.1

*The numbers are read as follows: total number of reflections with a partiality above 0.5. Number of rejections after merging. Number of unique reflections within the used resolution range. Completeness is the precentage of reflections used compared to the number of theoretically obtainable measurements. Outermost shell is the highest resolution shell included, $R_{MERGE} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle|/E_{hkl}\ \Sigma_i I(hkl)_i$. $I/\sigma(I)>2$ is the percentage of data with intensities greater than two standard deviations.

Crystals of X-ray quality were obtained by mixing drops of 10 μl containing 5 mg/ml recombinant Bet v 1 with an equal amount of 2.0 M ammonium sulphate, 100 mM sodium citrate, pH 6.3, and 0.01% sodium azide. After equilibration overnight against 1 ml of 2.0 M ammonium sulphate, 100 mM sodium citrate, pH 6.3, the drops were seeded with small Bet v 1 crystals previously obtained as described above. Seeding was performed using a rabbit whisker.[22] Rhombohedral shaped crystals appeared after about 1 week and continued to grow slowly over several weeks. The dimensions of the largest crystals were $0.2 \times 0.1 \times 0.05\ mm^3$.

The protein content of the crystals were analyzed by SDS-polyacrylamide gel electrophoresis (not shown) where the protein from washed redissolved crystals co-migrated with the non-crystallised protein and with Bet v 1 isolated from birch pollen extract. Thus, no proteolytic breakdown has occurred during crystallization.

Dataset I was collected from a crystal diffracting only to 2.6 Å. The indexing procedure suggested the crystals to be orthorhombic and C-centred with unit cell parameters a = 32.2 Å, b = 74.3 Å, and c = 118.5 Å after post-refinement. This is in agreement with the symmetry observed in the intensity weighted reciprocal lattice. The systematic absences on the c*-axis (001: k = 2n+1) lead to the assignment of the spacegroup $C222_1$. A somewhat larger crystal was found to diffract to beyond 2.0 Å resolution and was used to collect an additional dataset. From this dataset II, the cell dimensions a =32.13 Å, b = 74.22 Å, and c = 118.60 Å were determined. The mosaicity observed was 0.25° as determined from the post-refinement.

Crystals of recombinant Bev v 1 contain one molecule in the asymmetric unit and the solvent content was calculated to be 41% by use of Matthews's for-

mula.[23] The volume-to-mass ratio was 2.08 $Å^3$ Da^{-1}. Data collection statistics from dataset II are summarized in Table I. A search for heavy-atom derivatives is in progress.

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