

CRYSTALLIZATION NOTE

Crystallization and Preliminary Diffraction Data of 60-kDa Glycosylated Pollen Isoallergens from Bermuda Grass

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Crystals grown from a 60-kDa isoallergen mixture of Bermuda grass pollen have been obtained in 30% PEG 4000 and 25% isopropanol. The crystals diffract beyond 2-Å resolution and belong to a tetragonal space group with the unit cell dimensions $a = b = 86$ Å and $c = 310$ Å. The preferential crystal growth of the larger isoallergens with a blocked N-terminus indicates that crystallization can isolate proteins with compact conformation. © 1999 Academic Press

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It is estimated that 20% of the human population in industrialized countries suffer from IgE-mediated (Type I) allergic diseases such as bronchial asthma, conjunctivitis, and rinitis (Miyamoto, 1992). The major outdoor cause of allergy is airborne grass pollen. Bermuda grass (*Cynodon dactylon*) pollen is the dominant source in warm temperate and subtropical climates, such as Taiwan, Australia, and the United States. It contains a very complex mixture of at least 53 antigens, more than 12 of which have been shown to possess IgE-binding activity (Ford and Baldo, 1987; Shen *et al.*, 1988; Mattiesen *et al.*, 1989). However, only 5 of the antigens have been partly characterized: cytochrome c (Schumacher *et al.*, 1982), BG60 (Su *et al.*, 1990), Cyn d 1 (Smith *et al.*, 1996), Cyn d 7 (a Ca^{2+} -binding protein, Suphio-glu *et al.*, 1997), and Cyn d 12 (profilin; Asturias *et al.*, 1997).

BG60 (having an apparent molecular mass of 56–60 kDa) was purified as a mixture because of low yields of purified individual components. Three com-

ponents have been purified individually and shown to share immunological identities (Su *et al.*, 1991, 1996). These isoallergens are very basic with the *pI* values of 9.8–10.5. In addition, they are glycoproteins containing monosaccharides Man:GlcNAc:Fuc at a ratio of 3:2:1. The carbohydrate moiety of BG60 consists of five oligosaccharides of known structure (Ohsuga *et al.*, 1996). Two of them are novel oligosaccharides in plants. Moreover, we recently identified BG60 as a covalent flavoprotein containing nickel and copper ions (Liaw *et al.*, unpublished data).

Although many pollen allergens have been purified and their immunological properties have been characterized, only limited biochemical and structural information is available. For example, in only a few cases have protein functions been identified. Structural information on allergens would provide a good basis for understanding protein functions and allergic reactions and could lead to development of immunotherapy. As the first step in structure determination, we have obtained BG60 crystals from the isoallergen mixture isolated from Bermuda grass pollen.

BG60 was isolated from Bermuda grass pollens by a combination of ammonium sulfate precipitation, ion-exchange, gel filtration, and blue affinity chromatography as described previously (Su *et al.*, 1996). After gel filtration on an Ultrogel AcA 44 column elution by phosphate-buffered saline (pH 7.2), the green colored protein solution was dialyzed against the Tris–HCl buffer (pH 8.5) overnight and became brown in color. The protein solution was then loaded on a Cibacron blue affinity column. Three overlapping fractions were eluted by a NaCl gradient with three individual colors, green, brown (mixture of green and pink), and pink. These fractions were then dialyzed extensively against distilled water without color change. SDS–PAGE analysis by a 6% glycine gel showed two major protein bands with higher

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molecular weights (designated L forms) and one very minor band with a lower molecular weight (S forms) for the green fraction, whereas there was one major band of the S form and one minor band of the L form for the pink fraction (Fig. 1A).

The purified native proteins were digested with 1 μ g of trypsin in 20 μ l of 0.1 M pyridine corridine (pH 8.2) overnight at 37°C. Scission of the green and pink fractions yielded two major products, 22- and 33-kDa polypeptides (T22, T33). The protease-digested peptides were separated by SDS-PAGE and transferred into the PVDF membrane for subsequent sequencing by a 477A protein sequencer (Applied Biosystems). The sequences of T22 and T33 from the green

fraction were almost identical to those from the pink fraction. The N-termini of the L forms could not be sequenced, whereas those of the S forms had a sequence identical to that of T33. Therefore, these studies suggest that the L and S forms are isoforms: the L forms have a blocked N-terminus, whereas the S forms lack the N-terminus and could be a truncated form (Fig. 1A).

The three fractions described above were crystallized using the hanging-drop method of vapor diffusion in VDX plates (Hampton). The hanging drops are mixtures of 2 μ l of the protein solution and of the reservoir solution. Initial crystallization conditions were screened by the sparse matrix sampling kits

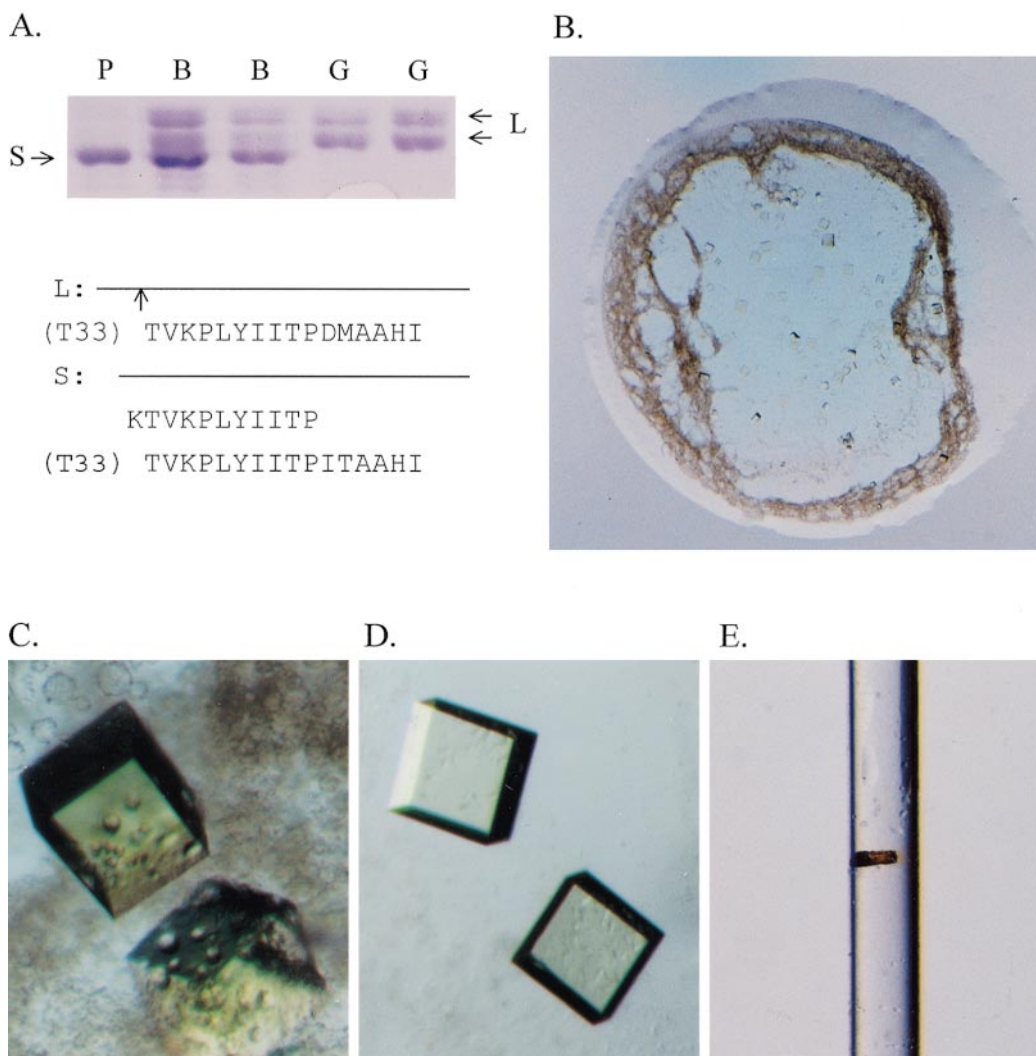


FIG. 1. (A) SDS-PAGE analysis and amino acid sequences of a trypsin-digested polypeptide T33 and the N-termini of S forms. From left to right, lane 1, 2- μ g proteins from the pink fraction (P); lanes 2 and 3: 3- and 1.5- μ g proteins from the brown fraction (B); and lanes 4 and 5, 1- and 2- μ g proteins from the green fraction (G). (B) Crystallization of BG60 isoallergens from the brown fractions. The green fraction produced nice crystals with little precipitant, whereas the pink fraction produced precipitant all over the crystal drops. Two crystal morphologies: (C) cubic crystals with dimensions of 0.15^3 mm³; and (D) thin square plates with dimensions of $0.2 \times 0.2 \times 0.05$ mm³. (E) A crystal with dimensions of $0.15 \times 0.15 \times 0.03$ mm³ mounted in a capillary with the thinnest dimension along the goniometer rotating axis during data collection in-house or at the Photon Factory.

(Hampton) at an initial protein concentration of 10 mg/ml. Crystals appeared after 3 days at room temperature, under initial conditions containing 20% polyethylene glycol (PEG) 4000, 20% isopropanol, and 0.1 M sodium citrate buffer (pH 5.6). To optimize the crystallization conditions, different protein concentrations, salts, organic polymers, divalent cations, additives, pH, and temperatures were screened. The best crystals were obtained at a reservoir solution with 30% PEG 4000, 25% isopropanol, and 0.1 M sodium citrate buffer (pH 5.6) at 22°C. The addition of nickel and copper ions at 10–50 mM concentrations enlarged the crystals.

After extensive screening, well-formed, large green crystals can be obtained from the green and brown fractions, whereas only a few tiny crystals grew from the pink fraction under the conditions mentioned above (Fig. 1B). To date, we cannot grow BG60 crystals from other reservoir solutions. Composition analysis in the BG60 crystals by SDS–PAGE and capillary electrophoresis did not identify the isoforms in crystals, perhaps due to the crystallization reagents enhancing the difficulty in separating the isoforms. Since the green fraction is composed mainly of at least two L isoforms, we suspected that these L isoforms have very similar structures and could be cocrystallized. The appearance of crystals in the brown and even in the pink fractions indicated the preferential crystal growth of L isoforms, which may suggest an important role for the N-terminus in producing a more compact conformation. Thus, we find that crystallization is not only a prerequisite for structural analysis, but it can also provide a separation technique to isolate a compact protein with definite structure.

Two crystal morphologies were commonly observed. One type of crystal is cube-shaped with typical dimensions $0.15 \times 0.15 \times 0.15 \text{ mm}^3$ (Fig. 1C), and the other type consists of a thin square-shaped plate with typical dimensions $0.2 \times 0.2 \times 0.05 \text{ mm}^3$ (Fig. 1D). The cubic crystals are generally grown within precipitants and oil drops and usually have caves on the surfaces. The thin square plate crystals appear more often, perhaps because of the flat crystallization drops. The flat crystallization drops with the appearance of oil drops are perhaps due to the high content of PEG 4000 and isopropanol. Nevertheless, all these green crystals diffract equally well.

Diffraction data were collected at room temperature on a Rigaku RAXIS IIC image plate area detector with $\text{CuK}\alpha$ radiation generated by a Rigaku RU-300 rotating anode (MSC). Three still photographs predicted the cell parameters, $a = b = 86 \text{ \AA}$ and $c = 310 \text{ \AA}$. The cell dimensions reduce to $a = b = 85 \text{ \AA}$ and $c = 306 \text{ \AA}$ at the cryo-temperature. To avoid

serious overlapping of diffraction spots, the c^* axis must be parallel to the vertical ϕ axis in the RAXIS IIC area detector during data collection. The long c^* axis was found parallel to the normal of the thin square-plate crystal (Fig. 1E). The crystal-to-detector distance was 160 mm. Diffraction data at the cryo-temperature were improved by addition of 30% glycerol into the reservoir, but still worse than at room temperature. Therefore, diffraction data were always collected at room temperature. Another advantage for data collection at room temperature was an easy alignment of crystal orientations (Fig. 1G). All the intensity data were processed by DENZO (Otwinowski and Minor, 1997). Approximately 27 000 unique reflections ($F_0 > 2\sigma$) were measured to 2.8-Å resolution with a R_{sym} (I) of 7.5%, representing 90% completeness. A pseudo-processing photograph derived from the diffraction data and the systematic absences suggested that the space group is either $P4_12_12$ or its enantiomer $P4_32_12$.

In addition, a complete diffraction data set at high resolution has been collected at the Photon Factory (KEK, Japan) using X rays with wavelengths of 1.00 Å and a Weissenberg camera installed on beamline BL-6B (Sakabe, 1991). The crystal-to-detector distance is 573 mm, and the image plate size is 400 mm $\times$ 800 mm. The crystals diffract strongly to beyond 2-Å resolution at room temperature and are very resistant to radiation damage. Approximately 63 750 unique reflections were collected at 2-Å resolution with a R_{sym} (I) of 5.9%, representing 80% completeness, if one image plate was used. In order to collect more complete data, especially at high resolution, two image plates must be used simultaneously (2 $\times$ 400 mm $\times$ 800 mm; Fig. 2). The crystal oscillation was 2.5° with an exposure time of 150 s for each image. Approximately 75 500 unique reflections were measured to 2-Å resolution with a R_{sym} (I) of 6.3%, representing 96% completeness.

Since BG60 retards in size-exclusive columns during protein purification, perhaps due to interactions of its carbohydrates with the bead media, cross-linking was performed to determine its oligomeric state. For cross-linking studies, a few micrograms of protein in a PBS buffer was incubated with various amounts of glutaraldehyde (Merck) or with dithiobis-(succinimidylpropionate) (DSP, Pierce) with a total volume of 10–200 μl . After 30 to 240 min, 50 mM Tris–HCl (pH 7.5) was added to stop the DSP reaction, and the SDS–PAGE sample buffer was added to stop the glutaraldehyde reaction. The reaction samples were then applied to a 7.5% tricine gel. The protein bands in SDS–PAGE were visualized with Coomassie brilliant blue R-250 or with silver staining.

The dimeric *Schistosoma japonicum* glutathione

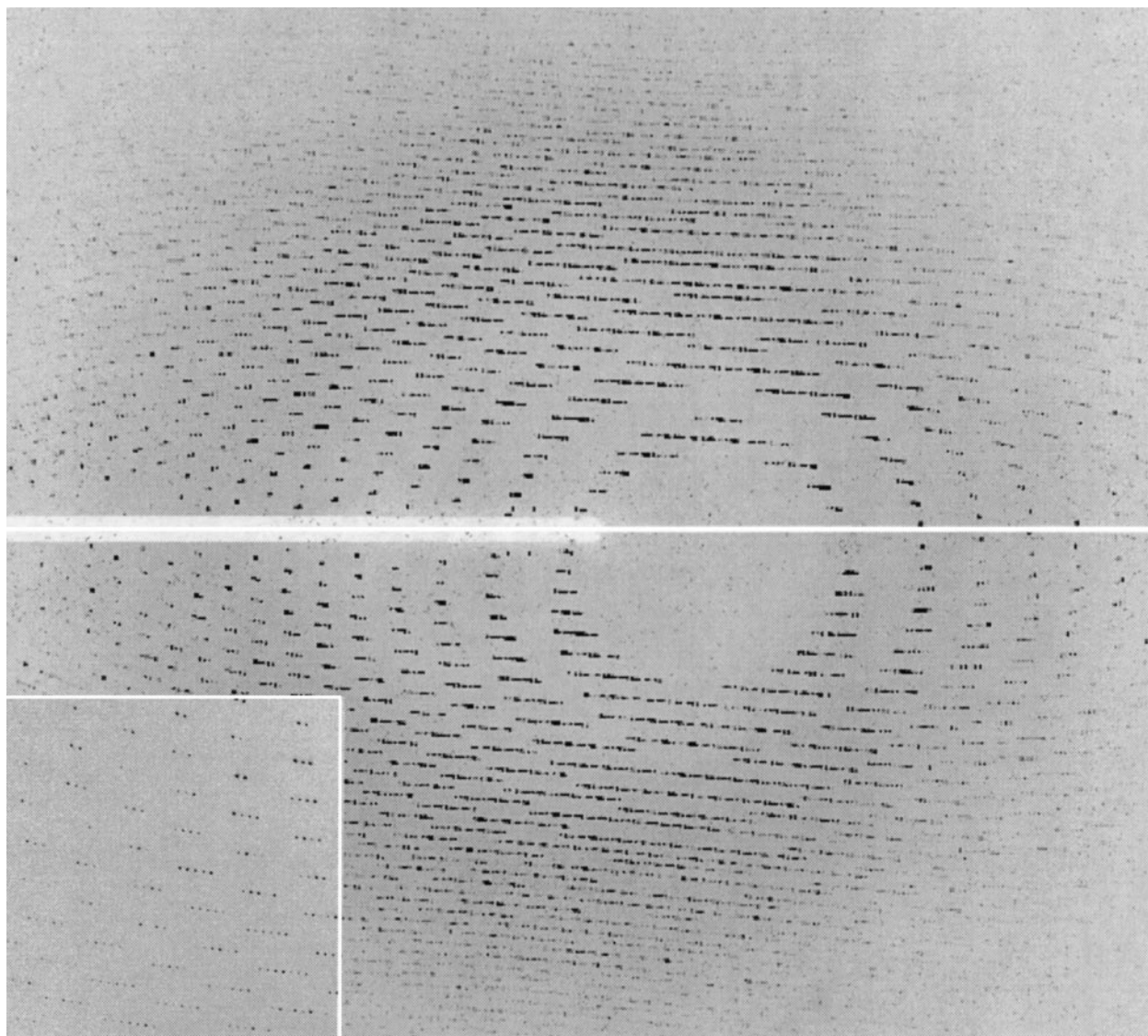


FIG. 2. A 2.5° oscillation photography for BG60 crystals using the synchrotron beamline BL-6B (Photon Factory, KEK, Japan). Two image plates must be used simultaneously to collect more complete data. The higher magnification inset illustrates the fine spacing of reflections resulting from the long c axis of 310 Å.

S-transferase (GST) and the monomeric bovine DNase were used here as internal controls (Fig. 3). All GST molecules were cross-linked into dimers, tetramers, and higher oligomers in the presence of 0.2–5 mM DSP with a reaction time of 30 min. Some GST molecules were not cross-linked in the presence of glutaraldehyde unless higher concentrations and longer reaction times were used, for example, 0.5% glutaraldehyde for 2 h. On the other hand, most DNase and BG60 molecules were not cross-linked by DSP or by glutaraldehyde. Very minor bands of dimeric DNase and BG60 may be due to random cross-linkage. The cross-linking rate of GST increased and some DNase and BG60 were cross-linked into dimers and higher oligomers when an old

glutaraldehyde solution was used, perhaps due to polymeric glutaraldehyde. Moreover, the cross-linking reagents could affect the mobilities and widths of the protein bands on SDS-PAGE. For example, DSP smeared the protein bands and made them more mobile than glutaraldehyde. The cross-linking studies suggested that BG60 is monomeric in solution at neutral pH.

Self-rotation function studies were carried out using the POLARRFN program in the CCP4 suite and the MERLOT program to locate possible noncrystallographic symmetry axes. The data used in self-rotation searches were varied in resolution shells between 2.0 and 30 Å, and the outer radius of integration varied between 10 and 40 Å. All these

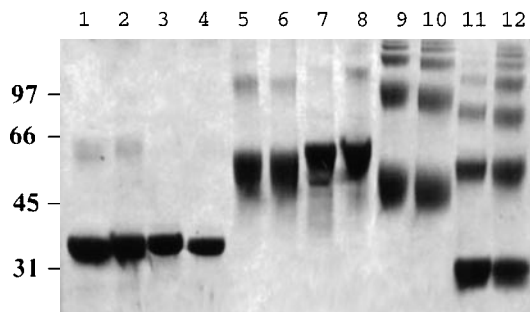


FIG. 3. Cross-linking studies of BG60 with DSP and with glutaraldehyde. The positions of molecular size standards (in kDa) are indicated. The protein bands were visualized with Coomassie blue. Lanes 1–4, 5–8, 9–12: 3 μ g DNase, 4 μ g BG60, and 8 μ g GST reacted with 0.5 mM, 2 mM DSP, and 0.02%, 0.05% glutaraldehyde, respectively. Under the same conditions, the dimeric GST was cross-linked, whereas BG60 and monomeric DNase were not.

gave consistent results that revealed no additional significant peaks besides the crystallographic axes. Thus if there is any noncrystallographic symmetry axis, it must be parallel to one of the crystallographic axes. Consideration of the Matthews' (1968) parameter, V_m , with the cross-linking studies suggested that each asymmetric unit probably consists of two BG60 monomers ($V_m = 2.4 \text{ \AA}^3/\text{Da}$; 48% solvent content). The V_m and the solvent content would be unusual for one molecule ($4.8 \text{ \AA}^3/\text{Da}$; 74%) or for three molecules ($1.6 \text{ \AA}^3/\text{Da}$; 23%) in one asymmetric unit. The search for suitable heavy atom derivatives is under way in order to determine the crystal structures of BG60 isoallergens.

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