

Structural Characterization of the 60-kDa Bermuda Grass Pollen Isoallergens, a Covalent Flavoprotein

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Our studies suggest a tripartite structure for the 60-kDa allergen of Bermuda grass pollen (BG60) including a short N-terminal segment, a FAD-binding domain, and a C-terminal domain. The lower molecular weight isoallergens lack the N-terminal segment. The higher protease susceptibility and the lower melting temperature of approximately 20°C of the lower molecular weight isoforms suggest that the N-terminal segment is essential for a compact structure. Database screening reveals that the protease-digested peptide sequences (~180 residues in total) share 40% identity with the plant berberine bridge enzymes. In particular, a 24-residue peptide sequence displays high similarity to a conserved FAD-binding motif. The spectroscopic and SDS-PAGE analyses suggest that the cofactor FAD is covalently linked to the central domain. Therefore, we conclude that BG60 is identified as the first flavinylated allergen. © 2001 Academic Press

Key Words: bermuda grass allergen BG60; domain structure; structural stabilization; berberine bridge enzyme; flavoprotein.

Approximately 20% of the human population in industrialized countries suffer from IgE-mediated (Type I) allergic diseases such as bronchial asthma, conjunctivitis, and rinitis. The major cause of outdoor allergy is from airborne grass pollen. In warm temperate and sub-tropical climates, Bermuda grass (*Cynodon dactylon*) is the dominant source of allergen. Bermuda grass contains a very complex mixture of antigens, in which more than 12 protein molecules have been demonstrated to bind specific human IgE and IgG molecules by immunoblot techniques (1). How-

ever, only some of the antigens have been partly characterized. BG60, with an apparent molecular weight of 56–60 kDa, was purified as a mixture because of low yields of purified individual components (2, 3). Three components have been further purified individually and shown to share immunological identities (4). These isoallergens are very basic with the pI values of 9.8–10.5. In addition, they are glycoproteins containing some unusual novel plant oligosaccharides (5). Moreover, crystals suitable for x-ray diffraction analysis have been obtained from the BG60 isoallergen mixture (6).

Although many pollen allergens have been purified and their immunological properties have been characterized, only limited biochemical and structural information is available. For example, only in a few cases have protein functions been identified. Information on the biochemical functions of important human allergens such as the enzymatic activities is vital to the effort of making immunotherapy safe and effective. We report here the identification of BG60 as the first flavinylated allergen.

MATERIALS AND METHODS

Protease digestion. BG60 was isolated from Bermuda grass pollens as described previously (4). Limited proteolysis reaction mixtures (50 µl), containing 100 mM pyridine collidine-acetate (pH 8.2 or pH 6.5), 10–50 µg BG60, and proteases, were incubated at 37°C for various lengths of time. The reactions were stopped by addition of SDS sample buffer and the proteolysis products were resolved by the Tricine-SDS-PAGE and transferred to PVDF membranes. Some protein was also digested with proteases in 20 µl of 25 mM Tris-HCl (pH 8.5), 1 mM EDTA and 0.01% SDS at 37°C overnight. After digestion, the solution was injected into a C-18 reverse phase-HPLC column and eluted with a linear gradient of acetonitrile with 0.1% trifluoroacetic acid (P1, P2, and P3 in Fig. 2). Automatic sequencing of peptides was performed using a model 494 microsequencer (Applied Biosystems). Screening of the sequence databases at the National Center for Biotechnology Information (NIH) with the peptide sequences was performed using the BLASTP program through the world wide web.

Abbreviations used: BG60, the 60-kDa bermuda grass pollen allergen; BBE, berberine bridge enzyme.

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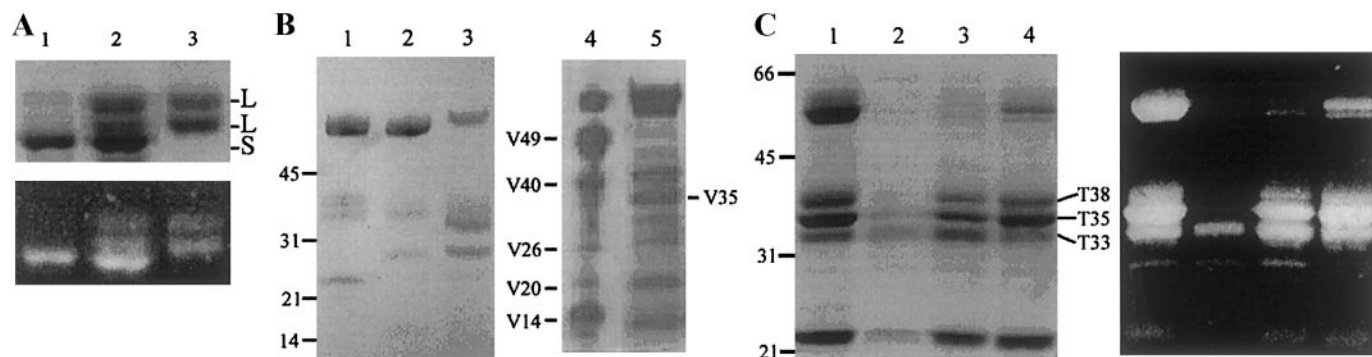


FIG. 1. SDS-PAGE analysis. (A) Lanes 1–3: 2- μ g, 3- μ g, and 2- μ g proteins from the pink, brown, and green fractions were loaded, respectively. The gel was visualized by transillumination at 312 nm (bottom) and then stained with Coomassie Brilliant Blue R-250 (top). (B) Lanes 1–3: Each contained the digests of 13 μ g of the green fraction digested with 1 μ g trypsin, chymotrypsin, or Lys-C overnight, respectively. Lanes 4–5: Each contained 40- μ g of the green and pink fractions, digested with V8 for 5 days and 45 seconds, respectively. (C) Lanes 1–4: 15- μ g, 10- μ g, 10- μ g, or 10- μ g proteins from the green, pink, brown, and green fractions were digested by 1 μ g trypsin for 24 hours. The gel was visualized as in Fig. 1A. The apparent molecular weight of each peptide was estimated by the relative mobility.

Spectroscopic analysis. The purified protein for spectroscopic measurements was dialyzed against distilled water. The protein concentrations were determined by a convention protein-dye binding method using bovine serum albumin as the standard. A molecular weight of 60,000 was assumed for BG60. All experiments were repeated at least three times. All spectra were corrected by subtracting the buffer baseline. The UV spectra of BG60 (~ 0.3 mg/ml) and the VIS spectra of BG60 (~ 1.0 – 10.0 mg/ml) were carried out with a Hitachi mode S3200 spectrophotometer. The fluorescence of proteins (~ 0.1 – 10.0 mg/ml) was analyzed in a Hitachi luminescence spectrometer S7500. Total fluorescence emission was monitored between 250 and 800 nm. Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter equipped with constant nitrogen flush. The absorption spectra were measured over the range of 190–270 nm, each for three scans, with a scan rate of 20 nm/min. Results were expressed in term of mean molecular ellipticity $[\Theta]$ in degree $\text{cm}^2 \text{dmole}^{-1}$. The protein secondary structure was estimated from spectral simulations based on reference CD spectra. The metal contents were detected by a Hitachi model Z-8200 atomic absorption spectrophotometer. Analytical grade metal salts were used as standards. Proteins of mass 0.3–0.4 mg were dissolved in deionized water for each analysis. Each protein sample was measured four to six times separately.

Thermal stability. Thermal stability was examined by exposing BG60 to various temperatures with monitoring the fluorescence emission and circular dichroism absorption. The protein solution was heated by circulated water through the sample chamber. The spectra were recorded at various temperatures, and the melting temperature, T_m , half of the protein fluorescence or absorption to be lost, was calculated. Analyses of the unfolding curves were performed according to the procedure of Pace *et al.* (7), assuming a two-state or a three-state folding-unfolding mechanism.

RESULTS AND DISCUSSION

Domain Structure of BG60

Limited proteolysis of BG60 was used to investigate whether BG60 has a multidomain structure (Fig. 1). Three overlapping fractions of BG60 were eluted from the blue affinity column with three individual colors: green, brown, and pink, respectively. SDS-PAGE analysis showed that the green fraction contained two ma-

jor isoforms with molecular weights greater than that of the pink fraction, which consisted of mainly one isoform. The brown fraction was composed of the mixture of the green and pink fractions (Fig. 1A). Limited proteolysis of the green fraction (L isoforms) by trypsin, endoprotease Lys-C, and chymotrypsin demonstrated two similar proteolytically resistant regions, however *S. aureus* V8 cleaved in a different pattern (Fig. 1B). Scission of L isoforms by trypsin yielded a triplet of 33, 35, and 38 kDa polypeptides (T33, T35, T38) and a polypeptide of 22 kDa (T22) (Fig. 1C), whereas the pink fraction (S isoforms) was cleaved into a 33 kDa (T33) and a 22 kDa (T22) polypeptides. The polypeptides of 22-kDa and 35-kDa were resistant to trypsin digestion as observed after a longer digestion time. Instead of the 22 kDa polypeptide, a polypeptide of 25 kDa (L25) was observed when endoprotease Lys-C was used. However, cleavage of L isoforms by *S. aureus* V8 produced two major polypeptides of 49 kDa (V49), and 14 kDa (V14), and two minor polypeptides of 26 kDa (V26), and 20 kDa (V20). The prominent 40 kDa polypeptide (V40) was due to that of *S. aureus* V8. The polypeptides of V49 and V14 were very resistant to V8 digestion.

N-terminal sequencing of these proteolytic products by automated Edman chemistry revealed that approximately 180 residues in total share 40% identity to the plant berberine bridge enzymes (BBE) (Fig. 2). The BBEs ((S)-reticuline:oxygen oxidoreductase (methylene-bridge-forming)) from *Eschscholtzia californica* (California poppy) and *Berberis stolonifera* catalyze the regio- and stereospecific conversion of the N-methyl moiety of (S)-reticuline into the berberine bridge carbon of (S)-scoulerine in the biosynthesis of antimicrobial alkaloids in response to pathogenic attack (8, 9). Based on the known BBE sequences one can construct a representative sequence structure for BG60 as shown

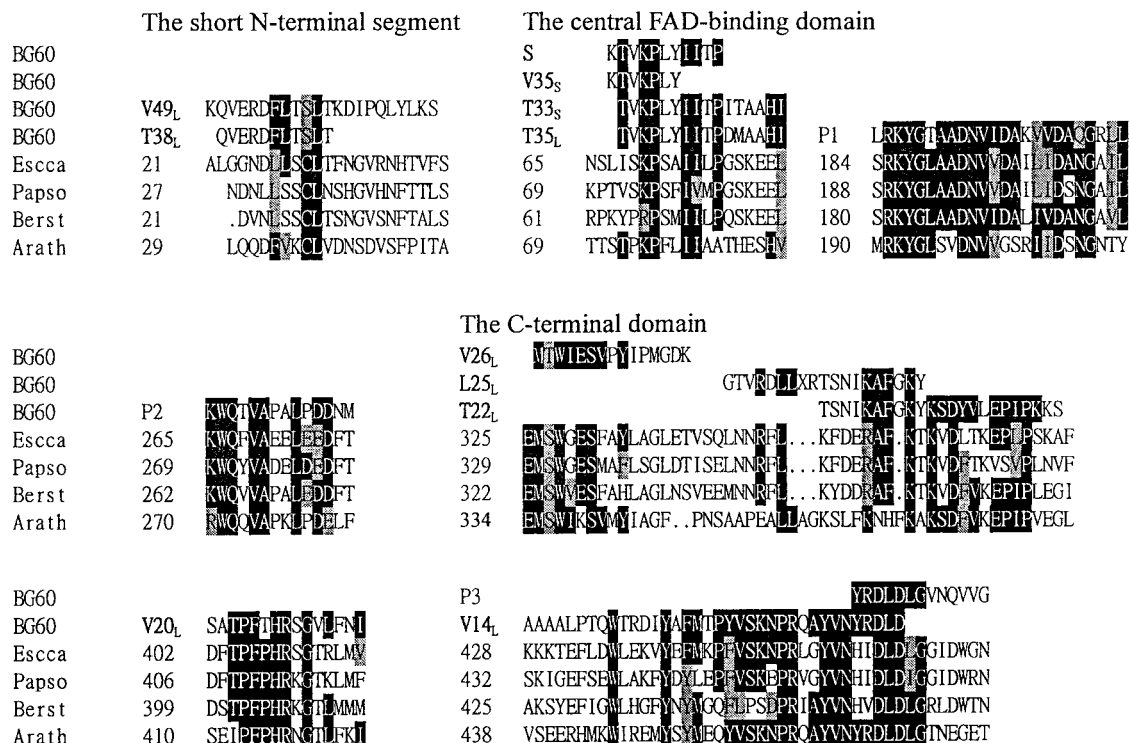


FIG. 2. The N-terminal sequences of the proteolytic peptides of BG60 and alignment with the putative plant BBE from *Eschscholtzia californica* (Escca, S65550), *Papaver somniferum* (Papso, U59232), *Berberis stolonifera* (Berst, AF049347), and *Arabidopsis thaliana* (AC007060_15).

in Fig. 3. As the apparent molecular weights of L38 and L25 were added up to those of the full-length L forms, we presumed that they were the products of a single proteolytic cut. L forms were cleaved by Lys-C (trypsin) first into L38 (T38) and L25 (T22), and L38 (T38) was further cleaved into L35 (T35) and a short N-terminal segment. For V8 digestion, the L forms were cleaved mainly into V49 and V14, with the minor products of V38 and V26, V26 was further cleaved into V20. The V38 was not easily observed because of the 40-kDa V8

band. Therefore, limited proteolytic analysis suggested a tripartite structure for BG60 including a short N-terminal segment, a 35-kDa central domain, and a 25-kDa C-terminal domain. The N-terminal sequence of the S isoform was identical to those of T33 and T35. However, the N-termini of L isoforms could not be sequenced. T38, L38, and V49 were observed only in digestion of L isoforms but not in S isoforms. These suggested that L forms are larger than S forms by the short N-terminal segment.

Structural Stabilization by the Short N-Terminal Segment

It has been shown previously that the L isoforms but not the S isoforms produced nice crystals for x-ray diffraction analysis (6). Limited proteolysis showed that it takes at least hours for proteases to cleave the L forms, but only minutes for the S forms. These suggest the short N-terminal segment may be involved in the protein structural stability. Circular dichroism measurement was then used to monitor the protein thermal stability. Both L and S isoforms consist of approximately 15% α -helix and 40% β -sheet. The higher absorption at 208 nm than at 222 nm revealed an $\alpha + \beta$ structure (Fig. 4A). Exposure to high temperature unfolded the protein and decreased the circular dichroism ellipticity and the fluorescence intensity without

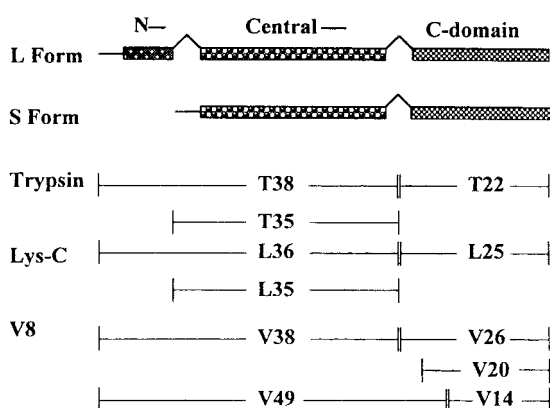


FIG. 3. Diagram of the representative structure for BG60 and the identification of the major proteolytic fragments.

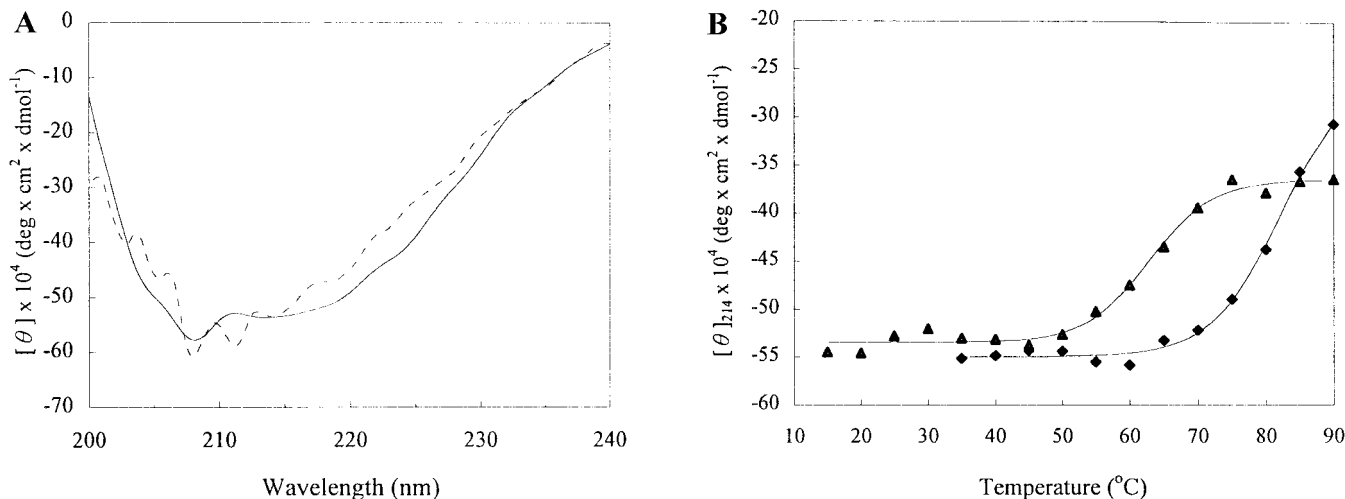


FIG. 4. (A) Circular dichroism spectra of the purified BG60. The green fraction is shown in solid lines, and the pink fraction in dotted lines. Both protein concentrations are 0.1 mg/ml. (B) Thermal unfolding curves for BG60 (0.1 mg/ml). The ellipticity was measured at 214 nm; squares for the green fraction and triangles for the pink fraction.

significant wavelength shifts. Results from decreased circular dichroism ellipticity were adequately described by a two-state model (Fig. 4B). The unfolding process was irreversible. The melting temperature, T_m , of the L forms was estimated between 78 $^{\circ}\text{C}$ and 85 $^{\circ}\text{C}$, in contrast to 55–62 $^{\circ}\text{C}$ for the S forms for wavelengths between 210–222 nm. The fluorescence emission curves also showed similar melting temperatures (data not shown). Therefore, L isoforms are much more stable than the S isoforms, suggesting that the short N-terminal segment is indeed involved in the structural stabilization. Protection of cleavages at the inter-domain linkers by the short N-terminal segment implies that the N-terminal segment makes the linkers not readily exposed to protease, or stabilizes the linkers through interactions with the other two domains. The thermal denaturation curve also suggested that the central- and C-terminal domains were cooperatively folded.

Putative FAD Linkage in the Central Domain

Since BBE is FAD covalently linked (9), spectroscopic measurements were performed to detect the possible flavin group of BG60. Both the green and pink fractions displayed two absorption maxima at wavelengths of 280 nm and 330 nm, and emitted at a fluorescence maximum of 345 nm, and 450 nm, respectively. The UV spectra of all BG60 fractions also showed high absorbance at 260 nm, confirming a possible nucleotide cofactor. The UV-VIS spectrum of the green fraction is very similar to that of nitroalkane oxidase from the plant fungus *Fusarium oxysporum* (10), with a single peak at 330 nm, and a pronounced shoulder at 410 nm (Fig. 5A). The extinction coefficient of 7.4 $\text{mM}^{-1} \text{cm}^{-1}$ has been determined at 336 nm for

the protein-bound flavin. If the BG60-bound flavin has a similar coefficient as nitroalkane oxidase, a stoichiometry of FAD to BG60 was 0.98.

The fluorescence emission spectrum of the green and pink fractions showed the emission maximum at 450 nm with excitation wavelength of 310–375 nm, which may result from the cofactor FAD (Fig. 5B). One additional fluorescent peak was observed at 590 nm for the pink fraction. The pink fraction may contain some unknown compounds such as pollen pigments resulting in the pink color and resulting in the unusual UV-VIS absorbance and emission spectra. Denaturation of the green fraction by heat also displayed a similar fluorescence emission spectrum with decreased fluorescence intensity. Analysis for autofluorescence of BG60 by SDS-PAGE yielded a yellow-green protein band visualized in the presence of 7% acetic acid by transillumination at 312 nm (Fig. 1A). The polypeptides T33, T35, and T38 but not T22 were fluorescent in SDS-PAGE analysis (Fig. 1C), showing that the putative cofactor FAD is covalently linked to the central domain.

The Possible Function of BG60

To date, more than 20 covalent flavoenzymes, each possessing one of five modes of FAD or FMN linkage, have been shown to catalyze a wide range of biochemical reactions in the biosynthesis/dissimilation of organic materials and to display a variety of folding topologies (11). The BG60 peptides show high sequence similarity to the plant BBEs. However, Bermuda grass does not contain alkaloids, BG60 should be not BBE. Instead they may belong to one of covalent flavoprotein superfamilies. The BBE from *Eschscholtzia californica* shares 65–70% sequence identity to those from *Papaver somniferum* and *Berberis stolonifera*, 40% iden-

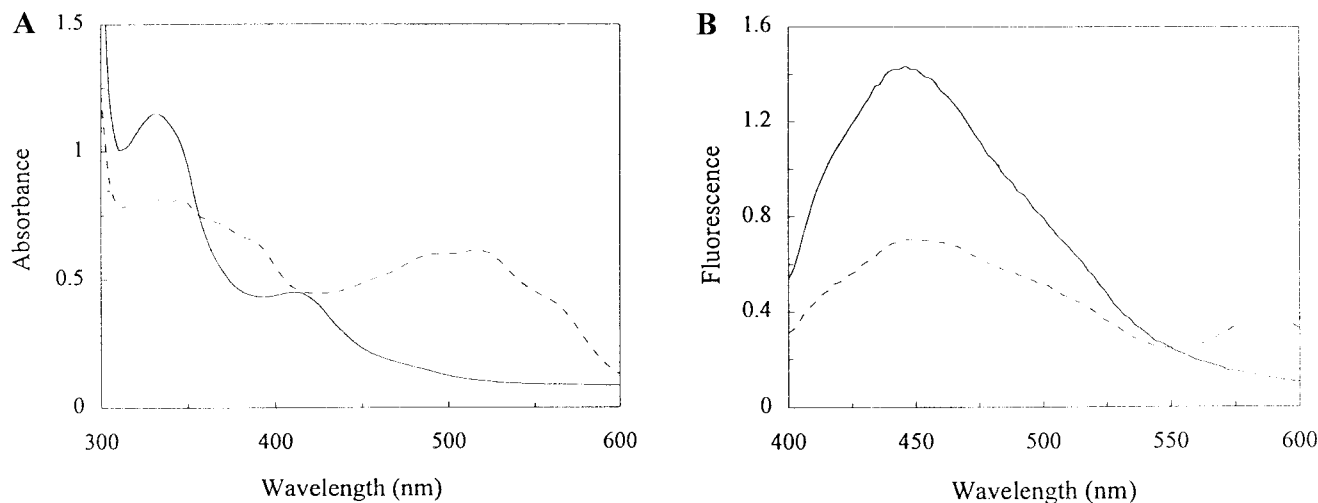


FIG. 5. Spectral measurements of BG60: the green fraction is shown in solid lines; the pink fraction in dashed lines. (A) UV-Vis spectra. The spectra were obtained at a concentration of 10 mg/ml for the green fraction, 3.7 mg/ml for the pink fraction. (B) Fluorescence emission spectra. BG60 (1 mg/ml) was irradiated at 330 nm, resulting the fluorescence emission maxima at 450 nm.

tity to eight putative genes on chromosome 1 from *Arabidopsis thaliana*, 30% identity to two uncharacterized genes from *Bacillus subtilis*, and 20–25% identity to a hexose oxidase from *Chondrus crispus*, a mitomycin radical oxidase from *Streptomyces lavendulae*, and a 6-hydroxy-D-nicotine oxidase from *Arthrobacter oxidans*. There are three conserved regions found among these FAD covalently linked oxidases: a FAD-attachment site at a histidine with a consensus sequence of RSGGH, and a 70-residue segment in the central region, and a 35-residue segment near the C-terminus. The 70-residue segment is found in a distinct group of flavoproteins including some noncovalent flavoproteins, and has been identified as a putative FAD-binding motif (12). One of the BG60 peptides (P1 in Fig. 2) shares high similarity to this FAD-binding motif. The central domain includes not only the FAD-attachment site but also the putative FAD-binding motif, thus could be referred as the FAD-binding domain. On the other hand, the C-terminal domain may involve in the substrate binding as some other flavoproteins.

Metal Analysis

Previous characterization of the BBE from *Berberis* species was shown to be sensitive to metal chelators such as EDTA (8). However, no metal of Cu, Fe, Mn, Mo, and Zn was detected from the purified recombinant enzyme from California poppy by atomic absorption spectrophotometry (9). Metal analysis of the purified BG60 revealed that the green fraction contained the nickel of $1.42 \text{ g/mole} \pm 0.28$ and copper of $5.11 \text{ g/mole} \pm 0.73$, whereas the brown fraction contained the nickel of $1.00 \text{ g/mole} \pm 0.13$ and copper of $9.78 \text{ g/mole} \pm 0.87$. However, Fe, Zn, Mg or Mn were not

detected. Addition of copper or nickel ions could enlarge the BG60 crystals (6). The presence of these metal ions may be due to their adsorption to the contaminated pollen pigments or their involvement in the protein function.

Implications for Allergenicity

The sensitization mechanisms by which allergens influence immunogenicity are still poorly understood. Allergens might contain common functional and/or structural motifs for the allergenicity. For example, many environmental allergens are found to possess hydrolytic activity. However, only few oxidoreductases are found to be allergens, for instance Bet v 5, a isoflavone reductase-like protein, from birch pollen (13), aldehyde dehydrogenase in fungi *A. alternata*, and *C. herbarum* (14) and superoxide dismutase from olive (sw:sodc_oleu). Here we identify BG60 as the first covalently flavinylated allergen. Therefore, our structural and functional studies of BG60 may provide some new insights on characterization of allergens.

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