Molecular Immunology 56 (2013) 794–803

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Structural and bioinformatic analysis of the kiwifruit allergen Act d 11, a member of the family of ripening-related proteins

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ARTICLE INFO

Article history:
Received 14 February 2013
Received in revised form 28 June 2013
Accepted 4 July 2013
Available online 23 August 2013

Keywords:
Act d 11
Kirola
Allergen
Food allergy
Kiwifruit
Bet v 1

ABSTRACT

The allergen Act d 11, also known as kirola, is a 17 kDa protein expressed in large amounts in ripe green and yellow-fleshed kiwifruit. Ten percent of all kiwifruit-allergic individuals produce IgE specific for the protein. Using X-ray crystallography, we determined the first three-dimensional structures of Act d 11, produced from both recombinant expression in *Escherichia coli* and from the natural source (kiwifruit). While Act d 11 is immunologically correlated with the birch pollen allergen Bet v 1 and other members of the pathogenesis-related protein family 10 (PR-10), it has low sequence similarity to PR-10 proteins. By sequence Act d 11 appears instead to belong to the major latex/ripening-related (MLP/RRP) family, but analysis of the crystal structures shows that Act d 11 has a fold very similar to that of Bet v 1 and other PR-10 related allergens regardless of the low sequence identity. The structures of both the natural and recombinant protein include an unidentified ligand, which is relatively small (about 250 Da by mass spectrometry experiments) and most likely contains an aromatic ring. The ligand-binding cavity in Act d 11 is also significantly smaller than those in PR-10 proteins. The binding of the ligand, which we were not able to unambiguously identify, results in conformational changes in the protein that may have physiological and immunological implications. Interestingly, the residue corresponding to Glu45 in Bet v 1 (Glu46), which is important for IgE binding to the birch pollen allergen, is conserved in Act d 11, even though it is not in other allergens with significantly higher sequence identity to Bet v 1. We suggest that the so-called Gly-rich loop (or P-loop), which is conserved in all PR-10 allergens, may be responsible for IgE cross-reactivity between Bet v 1 and Act d 11.

1. Introduction

Food allergies affect 2–3% of adults and 8% of children and their prevalence is influenced by dietary habits and other factors (Rona et al., 2007; Sicherer and Sampson, 2010; Zuidmeer et al., 2008). Frequently, food allergies are caused by cow's milk, eggs, peanuts, wheat, soy, tree nuts, fish, shellfish, and fruits. The first reports on allergic reactions to kiwifruit appeared three decades ago (Cumplido-Laso et al., 2012). Clinical reports show that kiwi allergy causes mostly oral symptoms, but in some cases the reactions are more severe and may include life-threatening anaphylaxis (Fine, 1981; Miyawaki et al., 2012). Currently, there are fourteen kiwifruit allergens that are registered by WHO/IUIS Allergen Nomenclature Sub-committee. Eleven allergens were identified in green kiwifruit (*Actinidia deliciosa*) and three in yellow-fleshed kiwifruit (*Actinidia chinensis*).

Kirola, according to the official allergen nomenclature termed Act d 11, is a 17-kDa protein found in high amounts in ripe green and yellow-fleshed kiwifruit (Ciardiello et al., 2009). Ten percent of kiwifruit allergic individuals have IgE that recognizes Act d 11

Abbreviations: MLP, major latex protein; PDB, Protein Data Bank; PR-10, pathogenesis-related proteins family 10; RMSD, root mean square deviation; RRP, ripening related protein.

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http://dx.doi.org/10.1016/j.molimm.2013.07.004

(D'Avino et al., 2011; Bublin et al., 2011). This protein belongs to the major latex protein/ripening-related protein family (MLP/RRP), and is the first protein from this family identified as an allergen (D'Avino et al., 2011). Act d 11 is immunologically related to Bet v 1-like allergens that are members of the PR-10 protein family. MLP/RRP and PR-10 families both belong to the Bet v 1 superfamily, but the sequence identity between the members of the two protein groups is rather low (<25%) (Osmark et al., 1998). However, it was shown that despite the low sequence identity, Act d 11 is able to inhibit, at least partially, binding of IgE to Bet v 1, Cor a 1, Dau c 1 and Mal d 1, suggesting that these allergens share some IgE epitopes (D'Avino et al., 2011).

Allergens belonging to the Bet v 1 allergen family are the main cause of pollen-related food allergies (so called pollen-food allergy syndrome) (Cano, 1991). In general, Bet v 1 related allergens are characterized as labile proteins, in contrast to other food allergens, which are more resistant to heating and proteolysis (Bollen et al., 2010). The physiological role of these proteins in plants is not well understood. They may function as ligand carriers, as one of the most distinctive features of the Bet v 1 fold is a large hydrophobic cavity (Cano, 1991). In the case of the archetypal Bet v 1 protein, its high degree of structural similarity with the START domain of the human MLN64 protein (Radauer et al., 2008), which binds steroids, led to the hypothesis that Bet v 1 may be involved in steroid binding. The ability of Bet v 1 to bind these kinds of molecules was later confirmed and it was suggested that Bet v 1 may serve as a plant steroid carrier (Markovic-Housley et al., 2003). However, it was also shown that the protein is able to bind many other structurally and chemically divergent compounds, and it is possible that Bet v 1 is involved in many different biological processes (Kofler et al., 2012; Mogensen et al., 2002).

Here we present a thorough structural analysis of Act d 11 that was purified from both its natural source and as a recombinant protein from *Escherichia coli*. Results of the structural analysis are discussed within the context of other Bet v 1-like proteins including allergens belonging to this group.

2. Materials and methods

2.1. Cloning, expression and purification

A synthetic Act d 11 gene was ordered from GenScript and re-cloned from the pUC57 vector into the pMCSG7 vector, a derivative of pET21a (Novagen), using a ligation-independent cloning protocol. The pMCSG7 vector encodes a hexahistidyl-tag followed by a spacer and a tobacco etch virus (TEV) protease cleavage site at the N-terminus of the expressed protein. The amino acid triplet Ser-Asn-Ala remains at the N-terminus of the protein after cleavage of the tag by the TEV protease. Purified plasmid was transformed into the *E. coli* strain BL21-CodonPlus(DE3) RIL which harbors an extra plasmid encoding three rare tRNAs (AGG and AGA for Arg, AUA for Ile, and CUA for Leu; Stratagene Inc.).

The cells were grown in lysogeny broth (LB) medium at 37 °C to an optical density (at 600 nm) of approximately 1.2 then induced with IPTG to a final concentration of 1 mM. After induction, the cells were incubated overnight with shaking at 16 °C. Cells expressing Act d 11 were harvested, resuspended in binding buffer [500 mM NaCl, 50 mM Tris (pH = 7.5), 5% glycerol, and 5 mM imidazole] and lysed by sonication after the addition of 'complete' EDTA-free protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation (30 min at 17,000 × *g*), and the liquid fraction was applied to a Ni-NTA resin (Qiagen) pre-equilibrated with binding buffer. The resin with bound protein was washed with wash buffer [500 mM NaCl, 50 mM Tris (pH = 7.5), 5% glycerol, and 30 mM imidazole] to remove weakly binding contaminants and then eluted from the column with elution buffer [500 mM NaCl, 5% glycerol, 50 mM Tris (pH 7.5), and 250 mM imidazole]. The metal affinity tag was cleaved from the protein by treatment with recombinant His-tagged TEV protease. The cleavage reaction was conducted during the dialysis [500 mM NaCl, 50 mM Tris (pH = 7.5), 5% glycerol, 0.5 mM EDTA and 1 mM TCEP] to remove the imidazole. The cleaved protein was then separated from the cleaved His-tag and the His-tagged protease by passing the mixture through a second Ni^{2+}-chelate affinity column, followed by gel filtration in crystallization buffer [150 mM NaCl, 10 mM Tris (pH = 7.5)]. Pure protein fractions were pooled together and concentrated to about 3.4 mg/mL for use in crystallization.

Natural Act d 11 was purified according to a previously described protocol (D'Avino et al., 2011).

2.2. Crystallization and data collection

Crystallization experiments were performed at 293 K using the hanging drop vapor diffusion method and NeXtal plates (Qiagen). A solution of the natural protein (9 mg/mL; in 10 mM Tris–HCl, 250 mM NaCl, pH 7.5) was mixed with the well solution in a 1:1 ratio. The Index (Hampton Research) screen was used to find initial crystallization conditions. Crystallization experiments were tracked and analyzed with the XTALDB crystallization system (Cymborowski et al., 2010; Zimmerman et al., 2005). Crystallization and cryocooling conditions for the recombinant protein are summarized in Supplementary Table 1. For crystallization, the recombinant protein was used at a concentration of 3.4 mg/mL. Data collection was performed at the 19-ID beamline of the Structural Biology Center (Rosenbaum et al., 2006) at the Advanced Photon Source (APS). Data collection from a $HgCl_2$-soaked crystal was performed at 19-BM beamline. Data were collected at 100 K and processed with HKL-2000 (Otwinowski and Minor, 1997). Data collection statistics are reported in Table 1.

During the search of possible Act d 11 ligands compounds used for crystal soaking were added directly to the drop in solid form.

2.3. Structure determination, refinement and validation

Single-wavelength anomalous diffraction (SAD) was used for structure determination. Structure determination was performed with HKL-3000 (Minor et al., 2006) which integrates SHELXC/D/E (Sheldrick, 2008), MLPHARE (Otwinowski, 1991), DM (Cowtan and Main, 1993), RESOLVE (Terwilliger, 2004), ARP/wARP (Perrakis et al., 1999) and selected programs from the CCP4 package (Winn et al., 2011). Single Hg-site was used to generate an initial map. The partial model, which was built with RESOLVE using the 2.4 Å data collected from the $HgCl_2$ soaked crystal, was later used as a starting model for ARP/wARP. The high-resolution native ($P6_322$) data set was used for model building with ARP/wARP. The model was later updated with COOT (Emsley and Cowtan, 2004) and refined with REFMAC (Murshudov et al., 2011). TLS was used in the final stages of the refinement and TLS groups were determined using TLSMD server (Painter and Merritt, 2006). MOLPROBITY (Davis et al., 2007) and ADIT (Yang et al., 2004) were used for structure validation. The high-resolution model was used later as a starting model in determination of other Act d 11 crystal forms. Molecular replacement was performed with HKL-3000 and MOLREP (Vagin and Teplyakov, 1997). Refinement and validation for other Act d 11 structures were done using the same methodology as in case of the high-resolution structure. Refinement statistics are summarized in Table 1. Coordinates and structure factors have been deposited to the PDB with the following accession codes: 4IGV, 4IGW, 4IGX, 4IGY, 4IH0, 4IH2 and 4IHR.