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Crystal Structure of Peanut (*Arachis hypogaea*) Allergen Ara h 5

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ABSTRACT: Profilins from numerous species are known to be allergens, including food allergens, such as peanut (*Arachis hypogaea*) allergen Ara h 5, and pollen allergens, such as birch allergen Bet v 2. Patients with pollen allergy can also cross-react to peanut. Structural characterization of allergens will allow a better understanding of the allergenicity of food allergens and their cross-reactivities. The three-dimensional structures of most known food allergens remain to be elucidated. Here, we report the first crystallographic study of a food allergen in the profilin family. The structure of peanut allergen Ara h 5 was determined, and the resolution of the final refined structure was 1.1 Å. Structure alignment revealed that Ara h 5 is more similar to Bet v 2 than to Hev b 8, although sequence alignment suggested that Ara h 5 is more closely related to Hev b 8 than to Bet v 2, indicating that homology-model-based prediction of immunoglobulin E epitopes needs to be interpreted with caution.

KEYWORDS: *Peanut allergy, allergen stability, X-ray crystallography, Ara h 5, profilin*

1. INTRODUCTION

Food allergy has become a major health concern in industrialized countries in recent years.[1−3] Most food allergies are triggered by the immunoglobulin E (IgE) recognition of allergenic proteins.[4−6] To date, there is no cure for food allergies, although immunotherapy using engineered hypoallergenic proteins is considered promising for treating the disease.[1,7] Peanut and tree nuts account for most of the fatal and near-fatal food allergy cases in the United States.[8,9] Cross-reactions of peanut-allergic individuals to other tree nuts, legumes, and oil seeds pose additional risks to these patients.[10,11]

Extensive research has been devoted to peanut allergy and peanut allergens in the past 2 decades.[1,12] To date, 12 peanut allergens (Ara h 1−3 and Ara h 5−13) have been officially recognized by the Allergen Nomenclature Subcommittee of the International Union of Immunological Societies. Ara h 2 and Ara h 3 have been reported to have trypsin inhibitor activities,[13,14] indicating that resistance to protease digestion may be one of the characteristics of food allergens. Recombinant peanut allergens have been expressed, and a number of linear IgE-binding epitopes on Ara h 1, Ara h 2, and Ara h 3 have been reported.[15−17] Structural information of peanut allergens Ara h 1, Ara h 2, Ara h 3/4, and Ara h 6 has been reported recently.[18−22]

Peanut allergen Ara h 5 is a member of the profilin family. Profilins are ubiquitously present in all eukaryotic cells. In mammalian cells, profilins regulate various cellular processes, including cytoskeletal dynamics, membrane trafficking, and nuclear transport.[23] In plants, specific cellular functions may require distinct profilin isoforms.[24] Profilins in many species have been identified as pollen allergens or food allergens, and profilin is thus referred as a panallergen.[25,26] Structural characterization of profilins may provide critical information necessary to understand the allergenicity of this group of proteins, and structural comparison may provide insights into cross-reactivities caused by profilins. Here, we report the crystal structure of recombinant Ara h 5 determined at 1.10 Å resolution.

2. MATERIALS AND METHODS

2.1. Isolation of Genomic DNA and Total RNA and Reverse Transcription (RT). Isolation of genomic DNA from peanut embryos was carried out as described.[21] mRNA was prepared from unripe peanut kernels with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the protocols of the manufacturer. Briefly, 100 mg of peanut embryos was placed in a mortar containing enough liquid nitrogen to keep the sample submerged and ground with a pestle. After grinding, the powder and liquid nitrogen were transferred to a RNase-free, DNase-free, and liquid-nitrogen-cooled microcentrifuge tube (2 mL). As soon as the liquid nitrogen was evaporated, 450 μL of buffer RLT (composition not known, a proprietary component of the kit) was added to the sample. The sample was then vigorously vortexed, and the amount of the sample corresponding to 50 mg of peanut was transferred to a QIAshredder spin column and centrifuged for 2 min at 13 000 rpm in a microcentrifuge. The supernatant of the filtrate was

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transferred to an RNAeasy spin column placed in a 2 mL collection tube and spun for 2 min at 13 000 rpm. Washing of the column and RNA elution were carried out following the kit protocol, including the usage of the optional steps. A total of 50 mL of Milli-Q water was used to elute the RNA.

An Omniscript Reverse Transcription Kit (Qiagen) was used to synthesize cDNAs from the total RNA. The RT reaction was carried out following the protocols of the manufacturer using 5 μM of a 20-nucleotide poly dT primer and 2 μL of the fresh total RNA as a template in a 20 μL RT mixture. The RT reaction was allowed to proceed for 60 min at 37 °C in the presence of a recombinant ribonuclease inhibitor (RNaseOUT from Invitrogen) at a concentration of 2 units/μL. The reverse transcriptase was inactivated by incubating the reaction mixture at 95 °C for 5 min. The RT product was either used immediately as a template in polymerase chain reaction (PCR) or stored at −80 °C for future use.

2.2. Plasmid Construction and Protein Expression. Two primers (ctcgaaggagatctataacatgtcgtggcaaacctacgtcg, forward, and tgggtctcgagggcccgcgcgaagcctcgtataggagcc, reverse) were designed on the basis of a published Ara h 5 sequence (GI: 5902967)[27] to amplify the coding sequence of Ara h 5. The underlined portions of the primers were added as the priming site for a second PCR. The template of the PCR was either the genomic DNA or the RT product. The PCR product was separated on a 1% agarose gel and purified to serve as a template in a second PCR with primers ggaatggggacaagtttg-tacaaaaaagcaggctcgaaggagatctataacatg (forward) and ggaatgggggac-cactttgtacaagaaagctgggtctcgagggcccgc (reverse) to add the attachment sites for Gateway cloning (Invitrogen, Carlsbad, CA). After separation and purification, the PCR product was used in an *in vitro* site-specific recombination with the pDONR/221 donor vector using BP enzyme mix (Invitrogen) to create pD-arah5. After *Escherichia coli* transformation, colonies originating from both genomic DNA and total RNA were used to prepare pD-arah5 for sequencing to determine the coding sequence for mature Ara h 5.

pD-arah5 with the Ara h 5 coding sequence confirmed by DNA sequencing was digested with *Afl*III and *Xho*I. This released the Ara h 5 coding sequence plus the 63 base pair of its 3′ UTR, and the release sequence was ligated with the pRSFdeut-1 vector prepared by *Nco*I and *Xho*I double digestion to generate pRSF-arah5. The ligation product was transformed to *E. coli* strain dH5α, and the plasmid prepared from the isolated colonies was sequenced to confirm correct insertion of the Ara h 5 coding sequence. pRSF-arah5 was then transformed to the *E. coli* strain BL21 Star (DE3) (Invitrogen) for protein expression. The transformed bacteria were grown in 1 L of Luria-Bertani (LB) medium containing 50 mg/L kanamycin (Gold Biotechnology, St. Louis, MO) at 37 °C until the OD_{600} of the culture reached 1.0. Then, the cell culture was incubated in a shaking incubator at 25 °C for 0.5 h. Isopropyl-β-D-thiogalactopyranoside (IPTG, Gold Biotechnology) was added to the medium to a final concentration of 1 mM, and the expression of Ara h 5 was allowed for 6 h. The cells were collected by centrifugation, and the cell pellet was stored at −80 °C for future use.

2.3. Protein Purification and Identification. *2.3.1. Ion-Exchange Chromatography (IEC).* The cell pellet from a 1 L culture was thawed on ice and resuspended in 50 mL of low ionic strength anion-exchange-column binding buffer (buffer AB, 10 mM Tris-HCl at pH 7.9) plus a protease-inhibitor cocktail. The cocktail contained aprotinin, antipain, leupeptin, and pepstatin, with final concentrations of 100 nM, 50 μM, 50 μM, and 0.5 μg mL^{-1}, respectively. All inhibitors were purchased from Sigma (St. Louis, MO). Cells were sonicated for 10 min in a beaker surrounded by a mixture of ice and water, followed by centrifugation at 25000*g* for 30 min at 4 °C. The supernatant was filtered with 0.20 μm syringe filters, and the sample was then loaded onto an 8 mL Source15 Q column (GE Healthcare, Piscataway, NJ). The columns were washed with 7 bed volumes of buffer AB. Ara h 5 was then eluted with a linear NaCl gradient of 0−1 M (10 bed volumes).

2.3.2. Size-Exclusion Chromatography (SEC). IEC fractions containing the overexpressed protein (based on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis) were pooled together and concentrated to a final volume of 15 mL using an Amicon Ultra centrifugal filter device with kDa molecular weight cut off for proteins (Millipore, Bedford, MA). The concentrated sample was loaded onto a XK 26/70 Superdex-75 column (GE Healthcare), pre-equilibrated with 100 mM NaCl and 10 mM Tris-HCl at pH 7.9, and eluted with the same buffer. All chromatographic steps were carried out at 4 °C using a fast protein liquid chromatography (FPLC) system (GE Healthcare).

Samples of the target protein after each step of purification were boiled in 1× SDS sample buffer (50 mM Tris-HCl at pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM β-mercaptoethanol) for 5 min, separated by electrophoresis on 4−20% gels, and stained by Coomassie Brilliant Blue.

The purified protein was subjected to N-terminal amino acid sequencing by Edman degradation using a Procise model 491 protein sequencer (Applied Biosystems, Foster City, CA) as described.[28]

2.4. Crystallization and X-ray Data Collection. The purified protein was desalted by repeated dilution and concentration using an Amicon Ultra centrifugal filter device and concentrated to 10 mg/mL at the end of the desalting process. The crystallization experiments were performed at 298 K in Linbro plates (Hampton Research, Aliso Viejo, CA) with the hanging drop vapor diffusion technique. An initial crystallization screen was performed using Hampton Research Crystal Screen and Crystal Screen Lite Kits. A total of 1 μL of the protein sample was mixed with 1 μL of reservoir solution and sealed against 0.5 mL of reservoir solution. All reservoir solutions contained 5 mM dithiothreitol (DTT) and 0.02% (w/v) sodium azide. Numerous crystals appeared in wells containing solution 8 of the Crystal Screen Lite Kit in 1 week, and efforts to optimize the crystallization conditions by varying the precipitant concentration in the reservoir solution did not result in improvement of the visual quality of the crystals or the number of crystals in a typical drop. To harvest the crystals, single crystals were picked, briefly soaked in a drop of a mixture of 80% mother solution and 20% ethylene glycol, flash-cooled in liquid nitrogen, and stored in a liquid nitrogen tank. X-ray data collection was performed using a MAR-300 charge-coupled device (CCD) detector at the SER-CAT 22-ID beamline at the Advanced Photon Source (APS), Argonne National Laboratories. The diffraction data were processed using the HKL2000 suite of programs.[29]

2.5. Structure Determination and Refinement. A structural model was derived by molecular replacement calculations using PHASER[30,31] and a template of a homology model of Ara h 5, which was constructed with SCRWL[32] and the structure of latex profilin Hev b 8 (PDB ID code 1G5U). Structure refinement was carried out with REFMAC5[33] implemented in CCP4i.[34] The refinement was alternated with model building and model improvement using Coot.[35]

The final structure was refined with diffraction data to a 1.10 Å resolution, and the final model was checked by PROCHECK[36] and MolProbity validation.[37] The structure model was also checked with a shake and omit protocol by introducing random errors up to 0.3 Å to the coordinates of the final refined structure using PDBSET distributed with CCP4.[34] For each region to be checked, the concerned region of the shaken structure was manually omitted and 20 cycles of restrained refinements were carried out using REFMAC5.[33] This was followed by inspecting the $F_o - F_c$ map together with the final refined structure. The structure of Ara h 5 has been submitted to the Protein Data Bank with a PDB ID code 4ESP.

Structure-based sequence alignment and the corresponding superposition of structures were carried out using MUSTANG,[38] and root-mean-standard deviations (rmsd) were calculated using FAST.[39] Molecular graphics were prepared using RASMOL,[40] MOLSCRIPT,[41] Raster3D,[42] and PyMOL (http://pymol.org/).

3. RESULTS AND DISCUSSION

3.1. Isolation of Ara h 5 Coding Sequences from Peanuts. An mRNA sequence of Ara h 5, GI: 5902967, could be found in the GenBank that also contained the 5′ and 3′ untranslated regions (UTRs). There was another entry for Ara h 5 (GI: 284810528) in the GenBank without the UTRs. The

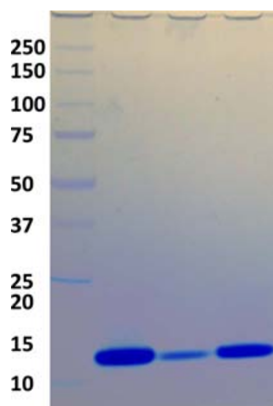

Figure 1. SDS−PAGE analysis of Ara h 5. Lanes 2, 3, and 4 from the left were loaded with different amounts of the purified protein (100, 20, and 50 μg, respectively). The first lane from left shows the protein standards with the molecular masses of the individual bands (in kilodaltons) shown on the left side of the gel image.

Table 1. X-ray Crystallographic Statistics and Refinement

Data Collection	
wavelength (Å)	1.0
temperature (K)	110
space group	$P4_12_12$
$a = b$, c (Å)	58.57, 101.72
resolution limits (Å)^a	50.76−1.10 (1.13−1.10)
number of observed reflections	1896089
number of unique reflections^a	71959 (3510)
completeness (%)^a	99.6 (98.8)
mean $I(\sigma)^a$	14.4 (6.3)
$R_{sym}{}^{a,b}$	17.2 (47.1)
Refinement	
resolution range (Å)	38.36−1.10
number of reflections	
working set	68220
test set^c	3629
number of atoms	
protein	1084
water	174
ligands and ion	40
R/R_{free} (%)	16.8/18.3
rmsd from Ideal Geometry	
bond lengths (Å)	0.034
bond angles (deg)	2.5

^{a}Numbers in parentheses are in the outer shell. $^bR_{sym} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl. ^{c}A total of 5% of the total reflections were randomly chosen.

first 22 bases in the open reading frames (ORFs) of GI: 5902967 and GI: 284810528 were identical and were used to design the forward primer of this study. The reverse primer used to isolate the Ara h 5 sequence in this study matched a region in the 3′ UTR of GI: 5902967. Sequencing results indicated that the coding sequence isolated in this study has 96% sequence identity with that in GI: 5902967 but has 99% sequence identity with that in GI: 284810528. For the entire protein sequence, the Ara h 5 cloned in this study has 92% identity with the Ara h 5 encoded by GI: 5902967 and 98% identity with the Ara h 5 encoded by GI: 284810528. The sequence of the mRNA isolated in this study has been deposited in the GenBank (GI: JQ974980).

3.2. Purification of Ara h 5. The expressed Ara h 5 was highly water-soluble, and its theoretical pI is 4.58. In this study, anion-exchange chromatography was used as the first step for Ara h 5 purification. The first broad peak contained the overexpressed protein as indicated by SDS−PAGE analysis (data not shown). Fractions of this peak were pooled and loaded onto a Superdex 75 column for purification by size exclusion. The major peak from the Superdex 75 column was shown to contain the overexpressed protein by SDS−PAGE analysis (data not shown). To increase the purity of the purified protein, the sample was subjected to a second round of IEC and SEC (data not shown). The final sample obtained after these four steps of purification was analyzed by SDS−PAGE with a 4−20% polyacrylamide gel, and the Coomassie Brilliant Blue staining of the gel is shown in Figure 1. This procedure routinely resulted in the putative recombinant Ara h 5, with its purity greater than 95%. To confirm that the expressed protein was Ara h 5, the purified protein was subjected to Edman degradation. The results of 11 cycles of N-terminal amino acid sequencing showed that the first 11 residues of the purified protein are SXQTYVDDHLL, indicating that the first methionine of Ara h 5 was removed during expression in *E. coli*.

3.3. Crystallization and Structure Determination. Diffraction quality crystals were obtained with the screening condition of solution number 8 of the Crystal Screen Lite Kit [0.2 M sodium citrate tribasic dihydrate, 0.1 M sodium cacodylate trihydrate at pH 6.5, and 15% (v/v) 2-propanol] in 3 weeks. The best crystals diffract to 1.10 Å, and a complete diffraction data set was collected using one crystal (Table 1). The sequence of Ara h 5 isolated in this study and that of latex allergen Hev b 8 have 79% identity. Using a homology model of Ara h 5 constructed with Hev b 8 as a template, a structural solution was obtained with molecular replacement. The structure of the initial solution contained most of the backbone fitted in the electron density. Larger movements of the residues in the N-terminal helix were required to fit the electron density map during refinements.

The final refined structure of Ara h 5 was a typical profilin-like fold. It consisted of a seven-stranded antiparallel β-sheet with two α-helixes on one side and one helix on the other side. It gave R/R_{free} values of 16.8/18.3% for all data to 1.10 Å (Figure 2 and Table 1). The rmsd values from ideal empirical values were 0.034 Å for bond lengths and 2.5° for bond angles, with no main-chain bond length deviating more than 0.05 Å or main-chain bond angles deviating more than 10° from the "ideal" small-molecule values. On the Ramachandran plot calculated with MolProbity validation,[37] all residues were in allowed regions and 98.4% of all residues were in favored regions. In the final refined structure, there was one Ara h 5 molecule in the asymmetric unit. It included 130 amino acids, 84 water molecules, and 10 solvent molecules (8 ethylene glycol molecules and 2 isopropyl alcohol molecules).

3.4. Comparison to Latex Allergen Hev b 8 and Pollen Allergen Bet v 2. Searching the PDB database with BLAST and using the sequence of Ara h 5 as a query, latex allergen Hev b 8 was returned as the protein with the highest percentage of sequence identity (79%). Thus, the structure of Hev b 8 (PDB ID code 1G5U) was used in this study as a template in building the starting homology model for molecular replacement calculations. After refinement, the structure of Ara h 5 was aligned with that of Hev b 8 using MUSTANG[38] (Figure 3A).

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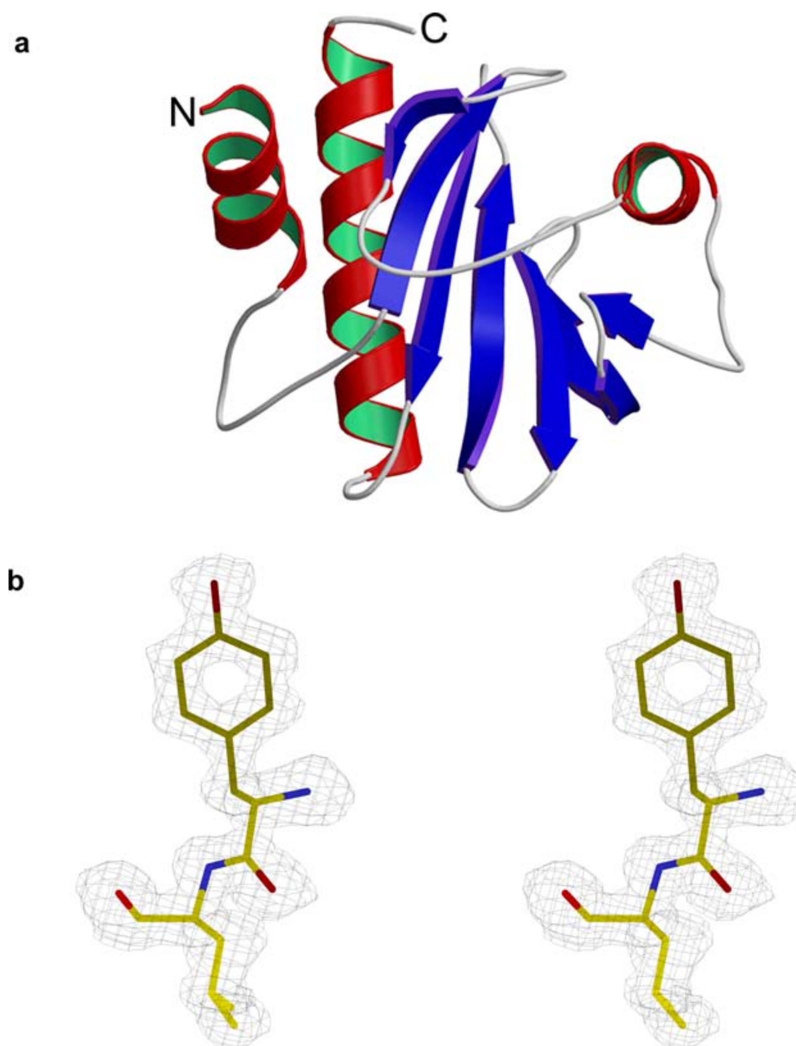

Figure 2. Structure of Ara h 5. (a) Ribbon diagram of Ara h 5. α-Helixes are shown in red and green; β-strands are shown in blue; and turns and random coils are shown in gray. (b) Stereoview of a F_o-F_c shake and omit map at a 1.1 Å resolution calculated with Tyr66 and Leu67 omitted using Refmac 5.5.0072. A stick representation of Tyr66 and Leu67 in the final structure is also shown.

In Figure 3B, the structural alignment of Ara h 5 and a pollen allergen Bet v 2 is shown. The structural alignment between Ara h 5 and Bet v 2 (rmsd = 0.735) is apparently better than the alignment between Ara h 5 and Hev b 8 (rmsd = 1.659), although the sequence alignment between Ara h 5 and Hev b 8 is better than that between Ara h 5 and Bet v 2 (75% sequence identity).

In this study, we determined the three-dimensional structure of peanut profilin. As a panallergen, profilin may significantly contribute to allergic cross-reaction. For example, in a study that involved 1521 allergic patients whose sera contained IgE antibodies that recognized at least 1 profilin of 9 profilin allergens (Mer a 1, Hev b 8, Bet v 2, Ole e 2, Hel a 2, Pho d 2, Cyn d 12, Par j 3, and Phl p 12) tested, the average number of the profilin allergens that a patient's serum recognized was 5 or 55.6% of the profilins obtained from different species and tested.[25] It is likely that a large number of the patients developed IgE antibodies to a profilin from one species but reacted to a broad range of profilins because of cross-reactivity. Although all of these proteins tested were not food allergens and most of the patients were pollen-allergy sufferers, profilins from many species have been identified as food allergens.[25,26] The allergenicity of profilins as food allergens has not been well-studied, and little is known about the IgE epitopes of profilin allergens in foods. A recent homology modeling study attempted to predict IgE epitopes of peanut allergen Ara h 5 based on a homology model.[43] However, it is known that homology models are rarely better than the starting template structures in resembling the native structures.[44] We do not have access to the coordinates of the Ara h 5 homology model used for the IgE epitope prediction.[43] It was noticed that the N-terminal helix of the homology model developed by Cabanos et al.[43] was quite different from what was determined by X-ray crystallography in this study (Figure 3). The N-terminal region is also among the regions that contain the epitopes predicted by Cabanos et al.[43] Prediction of IgE epitopes is not the intent of the current study, but the high-resolution structure can be used in a future attempt to map IgE epitopes when experimental data of IgE epitopes are available.

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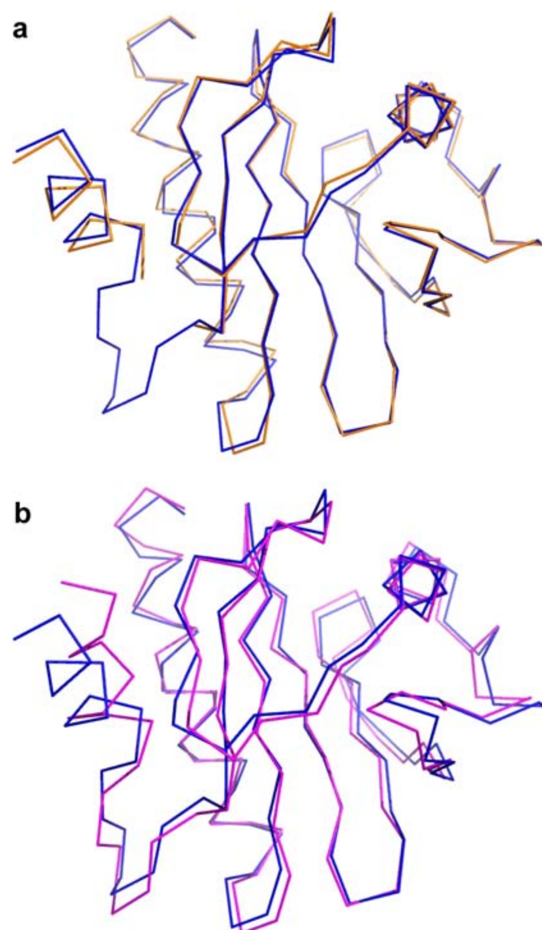

Figure 3. Comparisons of Ara h 5 with Bet v 2 and Hev b 8. (a) Structural alignment of Ara h 5 (blue) and Bet v 2 (yellow). (b) Structural alignment of Ara h 5 (blue) and Hev b 8 (magenta).

Notes

Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

The authors declare no competing financial interest.

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