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RESEARCH ARTICLE

Expression of recombinant Ara h 6 in *Pichia pastoris* but not in *Escherichia coli* preserves allergic effector function and allows assessment of specific mutations

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Scope: Ara h 6 has recently been recognized as an important peanut allergen. Recombinant allergens have been used for analysis of IgE binding, but have not been used to analyze the allergic effector activity that is more relevant to allergic reactions.
Methods and results: Ara h 6 was expressed as a recombinant protein in both *Escherichia coli* and *Pichia pastoris* (rAra h 6-*E. coli* and rAra h 6-*Pichia*, respectively). Effector activity was assayed by measuring degranulation of RBL SX-38 cells sensitized with IgE from patients with severe peanut allergy. Compared to native Ara h 6 (nAra h 6), rAra h 6-*Pichia* had intact effector function whereas rAra h 6-*E. coli* had significantly reduced function. The lower effector activity in rAra h 6-*E. coli* compared to nAra h 6 and rAra h 6-*Pichia* did not appear to be due to differences in posttranslational modificatons (analyzed by mass spectrometry and staining for carbohydrates) and may be due to subtle alteration(s) of folding seen on CD analysis and on nonreduced gels. Finally, we introduced point mutations in four important IgE-binding linear epitopes of Ara h 6 and found dramatically reduced allergic effector activity.
Conclusion: Our studies demonstrate the utility of fully functional rAra h 6-*Pichia* as a starting point for analysis of specific mutations that adversely affect allergic effector function.

Keywords:
Ara h 6 / Basophils / Peanut allergy / Recombinant allergen

1 Introduction

Peanut allergy is a prevalent, severe, and long-lasting food allergy occurring primarily in industrialized countries [1–3]. Although strict avoidance can be effective, peanuts are used in many dishes leading to frequent accidental ingestion [4]. Recently, oral desensitization has been shown to be a useful approach for treatment but this is difficult and not universally successful [5, 6]. Eleven peanut allergens have been described (Ara h 1–11) (http://www.allergen.org) [7]. Of these, the 2S albumins, Ara h 2 and Ara h 6, account for most of the allergic effector activity defined as the ability to cross-link IgE/FcεRI (high-affinity receptor for IgE) complexes leading to activation of mast cells and basophils [8–12].

Ara h 6 has recently been recognized as an important peanut allergen and, along with Ara h 2 and Ara h 7, belongs to the 2S albumin family of plant seed storage proteins [13–16]. The presence of IgE that binds Ara h 2 has been found to be particularly useful in component-specific diagnostics [17]. Ara h 2 and Ara h 6 are ~60% homologous at the amino acid level, are characterized by a motif of 4–5 disulfide bridges with cysteine residues in a conserved pattern, are immunologically cross-reactive, are heat stable, and are resistant to digestive enzymes such as trypsin, chymotrypsin, and pepsin [8, 14, 18, 19]. Reduction and alkylation of disulfide bonds leads to a change in secondary structure, loss of IgE binding, and loss of allergic effector activity, demonstrating the importance of 3-dimensional epitopes [14, 20, 21].

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Abbreviations: CPE, crude peanut extract; **Ek**, enterokinase; **HIC**, hydrophobic interaction chromatography; **PAS**, periodic acid-Schiff; **PTMs**, posttranslational modifications

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Recombinant allergens are useful tools for the characterization of allergens and for diagnostic studies. Furthermore, recombinant allergens have the potential to be useful starting points for dissection of the molecular details whereby allergens effectively cross-link IgE/FcεRI complexes [22–25]. The common hosts for recombinant protein expression are bacteria (such as *Escherichia coli*), yeast (such as *Pichia pastoris*), insects, or eukaryotic cell lines. *Escherichia coli* is the most widely used organism for production of recombinant proteins due to its fast growth rate, high protein production efficiency, and undemanding growth conditions. However, it is not unusual that expression of recombinant proteins in *E. coli* produces misfolding especially of those proteins that are dependent upon disulfide bridges for structure [26]. This is often overcome by using specific bacteria with a reducing milleu and/or adding thioredoxin as a fusion protein [27]. This system has been used to produce biologically active Ara h 2 and Ara h 6 although the biologic activity of these proteins was demonstrated for only a few sera (from peanut allergic subjects) and at limited dose ranges [8]. The only report to date of rAra h 6 showed that it had activity but was less potent than rAra h 2. Of note, its activity was not directly compared to that of nAra h 6 [8]. The major advantage of *P. pastoris* over *E. coli* is that *P. pastoris* is capable of producing more properly formed disulfide bonds and able to glycosylate target proteins [28].

The aim of this study was to prepare completely functional recombinant wild-type Ara h 6 and test the hypothesis that alterations of a limited repertoire of amino acids could significantly reduce its allergic effector activity. We chose to study Ara h 6 rather than Ara h 2 because it is smaller (15 kD vs. 17 and 19 kD), it exists as a single gene product although with multiple isoforms, and because fully functional rAra h 6 has not been previously described [16]. rAra h 6 was expressed as a soluble protein first in *E. coli* and then in *P. pastoris*. We found that the rAra h 6-*Pichia* has allergic effector activity that is identical to that of nAra h 6 for cells sensitized with peanut-specific IgE from a pool of ten sera and with peanut-specific IgE from four of five individual sera examined. Furthermore, rAra h 6-*Pichia* was greatly superior to rAra h 6-*E. coli*. The molecular basis for this dramatic difference appears to be due to minor differences in folding seen on CD and on native gel electrophoresis as there were no significant differences in posttranslational modifications (PTMs) seen on mass spectrometry and no evidence of glycosylation in either the native or rAra h 6-*Pichia* proteins. Finally, we introduced point mutations in four important IgE-binding sites in the sequence of Ara h 6 and found substantially reduced effector activity demonstrating that the allergic effector activity of Ara h 6 can be disrupted by altering a limited number of amino acids.

2 Materials and methods

2.1 Human sera

Individuals were selected on the basis of a strong history of systemic allergic reactions to peanuts and having peanut-specific IgE ≥14 IU/mL on the Pharmacia ImmunoCap assay (Phadia AB, Uppsala, Sweden), a level associated with a 95% confidence level of having a clinical reactivity to peanut [29]. Ten individual patient sera were combined proportionally based on the concentration of anti-peanut IgE to generate a serum pool with 501 kU/L of total IgE and 68 kU/L of antipeanut IgE, values similar to what we have used previously [30]. In addition, sera from five of these subjects were studied individually. The total and peanut-specific IgE values (kU/L) of these sera were D44 (1015, 65), D63 (3263, 79), D65 (2421, 180), D80 (545, 164), and D81 (268, 68). This study was approved by the Institutional Review Board of the University of Colorado Denver (no. 00–802). All subjects or their parents or guardians signed informed consent and for minors, informed assent.

2.2 Crude peanut extract (CPE) and purification of nAra h 6

CPE and the 20 kD fraction containing Ara h 2 and Ara h 6 were prepared and characterized as previously described [10, 11]. Native Ara h 6 was further purified from the 20 kD fraction by hydrophobic interaction chromatography (HIC) using HiLoad 16/13 Phenylsepharose column (Amersham Biosciences, Piscataway, NJ, USA). Elution was performed using linear and step-wise gradients of ammonium sulfate in 0.1 M phosphate, pH 7.0 at 2 mL/min.

2.3 Expression and purification of recombinant Ara h 6 fusion protein in *E. coli*

Total RNA was extracted from Georgia Green peanuts (a gift from Golden Peanut Company, Alpharetta, GA, USA) using TRIzol (Invitrogen, Grand Island, NY, USA). RNA was reverse transcribed into cDNA with Multiscribe RT and random hexamers (Bio-rad, Hercules, CA, USA). Ara h 6 was amplified by PCR from cDNA with high-fidelity *Pfu* polymerase (Stratagene, La Jolla, CA, USA). The strategy to express soluble rAra h 6 was very similar to that described by Lehmann et al. [8, 31]. The only difference was that we used an enterokinase (Ek) recognition protease site instead of a PreScission protease recognition site. The PCR product encoding the mature Ara h 6 without the leader sequence was cloned in frame into pET-32 Ek/LIC following the manufacturer's instruction (Novagen, Madison, WI, USA). This generated a sequence that contained only the native sequence plus an N-terminal thioredoxin fusion (Trx) and a hexahistidine tag that could be specifically removed enzymatically. The cloned Ara h 6 sequence corresponded to the GeneBank accession number AY849314 (~14.8 kDa). The PCR primers used for cloning were sense 5′ atg agg cgc gag agg ggg aga cag ggg gac and antisense 5′ tta gca tct gcc gcc act cac gtc caa atc.

Plasmids were transformed into *E. coli* strain BL21 (DE3) Codon plus-RIL (Novagen). The expression of the Trx-Ara

h 6 fusion protein was induced by isopropylthiogalactoside and the soluble protein was purified with ProBond Purification System under native conditions (Invitrogen). Purified Trx-Ara h 6 was incubated with Enterokinase (Novagen) for 8 h at room temperature. This material was then dialyzed against 20 mM Tris, pH 8.0 buffer applied to Mono Q 5/50 GL anion exchange column (Amersham Biosciences) on an AKTA FPLC system (GE Healthcare, Piscataway, NJ, USA), and eluted with a linear gradient of 20 mM Tris to 20 mM Tris, 0.5 M NaCl, pH 8.0 buffer at 1 mL/min to separate rAra h 6-*E. coli* from the Trx-tag, the $(His)_6$-tag, and the residual undigested fusion protein.

2.4 Expression of recombinant Ara h 6 protein and its mutant, m-rAra h 6, in *P. pastoris*

The Ara h 6 gene was synthesized with yeast-optimized codons (Genscript, Piscataway, NJ, USA) and cloned into the pPink-HC vector. The construct pPink-HC-Ara h 6 was linearized with PmeI and transformed into electrocompetent *Pichiapink* cells (*P. pastoris* strain 4) (Invitrogen, Grand Island, NY, USA). The white recombinant clones were selected and restreaked on fresh Phenylalanine (PAD) selection plates. Small-scale expression was performed to test expression and secretion of Ara h 6 in different clones during methanol induction. The chosen clone was cultured in 1-L baffled flasks and then induced with 1% methanol to express rAra h 6 (rAra h 6-*Pichia*). Time course studies revealed that the highest yield of rAra h 6 expression was achieved after an induction period of 3 days (data not shown).

Mutated-rAra h 6-*Pichia* (m-rAra h 6-*Pichia*) was constructed with the following 8 point mutations: amrrergrqg dssscerqvd rvnlk(K35A)pce(E38A)qh imqrimgeqe qyd(D53A)syd(D56A)irst rssdqqqrcc delnementq rcmce (E85A)alqqi me(E92A)nqcdrlqd rqmvqqfk(K108A) re(E110A) lmnlpqqcnf rapqrcdldv sggrcs. The mutant gene was cloned in pPink-HC vector and expressed in yeast as discussed above.

2.5 Purification of rAra h 6-*Pichia* and m-rAra h 6-*Pichia*

Culture supernatants from yeast were dialyzed against 0.1 M phosphate, 1.5 M ammonium sulphate, pH 7.0 buffer, and purified by HIC and gel filtration chromatography as described for nAra h 6.

2.6 Electrophoresis, Coomassie staining, glycoprotein staining, silver staining, and immunoblots

Proteins were separated by SDS-PAGE and analyzed by Coomassie blue staining (Bio-Rad), glycoprotein staining (Pierce, Rockford, IL, USA), or silver staining (Pierce) according to manufacturer's instructions. Immunoblotts were performed as previously described, with a combination of three rabbit peptide-specific anti-Ara h 6 antibodies (aa 7–19, 41–59, 129–144) and goat antirabbit IgG conjugated to HRP (Bio-Rad) as previously described [11]. Native-PAGE was performed on Tris-HCL ready gel (Bio-Rad) with native running buffer: 25 mM Tris and 192 mM glycine.

2.7 RBL SX-38 cell release assay

The RBL SX-38 cell assay has been previously described [11, 32, 33]. Briefly, the cells were cultured in MEM medium containing 3% of human AB serum (Gemini Bio-products, West Sacramento, CA, USA) and 7% of FBS. A total of 2×10^5 cells/mL were labeled with 1 μCi/mL of tritiated-5-hydroxytryptamine (5-HT, serotonin; PerkinElmer Life Sci, USA) and cultured overnight with a 1:15 dilution of the serum pool or a 1:10–1:20 dilution of individual sera to passively sensitize the cells with IgE. The sensitized cells were then washed and challenged with CPE (200 ng/mL; positive control) or varying doses of nAra h 6, rAra h 6-*E. coli*, rAra h 6-*Pichia*, or mutated m-rAra h 6-*Pichia*. Antigen-specific release was expressed as a percentage of release with an optimal dose of CPE (200 ng/mL). Potency of these proteins were compared by determining the concentration (EC_{50}) of each that gave 50% of the maximal degranulation seen with CPE as previously described [10].

2.8 Electrospray ionization mass spectrometry

To determine if nAra h 6, rAra h 6-*Pichia*, and rAra h 6-*E. coli* have significant differences in PTM, the respective purified proteins were excised from gels and digested with sequencing grade trypsin. Samples were subjected to a microcapillary HPLC tandem mass spectrometry (μLC-MS/MS) on LTQ-Orbitrap Velos mass spectrometer coupled to an Eksigent2D Ultra Nano-HPLC system. Two μLC-MS/MS experiments were performed on each sample using two different fragmentation methods, collision-induced dissociation and electron transfer dissociation, to optimize results for PTMs. Results of each μLC-MS/MS experiment were searched against Ara h 6 dataset using MASCOT database (http://hsc-mascot2/mascot/) searching program. This program compares experimentally acquired spectra with "in silico" digests of proteins. Protein results were then re-searched against the database using an "Error Tolerant" setting. The peptide data were compared for unique modifications that only occur in nAra h 6 or in both nAra h 6 and rAra h 6-*Pichia*, but not in rAra h 6-*E. coli*.

2.9 Circular dichroism (CD)

Samples were dialyzed against 1× PBS and their protein concentrations adjusted to 0.2–0.25 mg/mL. Far UV spectra (190–260 nm) were recorded with 200 μL of sample in a 1 mm path-length quartz cuvettes (Jasco, Easton, MD, USA).

CD spectra were recorded on a Jasco 816 spectropolarimeter (Easton, MD, USA) equipped with a Peltier temperature control system and Lambda E100 waterbath. Six scans were recorded and averaged (buffer spectra subtracted) with a scanning speed of 50 nm/min at 20°C and 4°C. The results were expressed as Molar Ellipticity [Θ]: $[\Theta] = \theta/(10 \times n \times c \times l)$; θ = rotation in mdeg; n = number of amino residues; c = concentration in molarity; and l = path length in cm. Secondary structural modes were estimated from ellipticities by multiple protein secondary structure prediction [34].

2.10 Statistical analysis

One-way analysis of variance with Tukey's multiple comparison test was used to compare rAra h 6-*E. coli* and rAra h 6-*Pichia* with nAra h 6. If only two materials were compared, a Student's *t*-test was used. All statistical comparisons were two tailed. Data were considered to be significantly different when the *p*-value was < 0.05. All statistical comparisons, the best-fit lines, and EC_{50} values were generated with GraphPad Prism 5.0c for the MacIntosh (GraphPad Software, La Jolla, CA, USA).

3 Results

3.1 Purification of recombinant Ara h 6 derived from *E. coli* (rAra h 6-*E. coli*)

The fusion protein, Trx-rAra h 6, expressed as a soluble protein in *E. coli* (BL21-CodonPlus, DE3-RIL) was purified with a nickel-chelating resin under native conditions (Fig. 1A), cut with Ek, and applied to Mono Q column (Fig. 1B) as described in Section 2. Purified rAra h 6-*E. coli* eluted in a single peak of ~15 kD distinct from the fusion protein (Fig. 1C).

3.2 Purification of recombinant Ara h 6 produced by yeast (rAra h 6-*Pichia*)

Pichiapink cells transformed with the construct of pPink-HC-Ara h 6 were used to express rAra h 6-*Pichia*. SDS-PAGE revealed a ~15 kD protein secreted from yeast that co-migrated with native Ara h 6 (Fig. 2A). Immuno-blots further confirmed the production of rAra h 6 (Fig. 2B). Following HIC, rAra h 6-*Pichia* was purified (Fig. 2C). As shown by silver staining (Fig. 2D), the purity of nAra h 6 is estimated to be >95%.

3.3 Effector activity of rAra h 6-*E. coli*, and rAra h 6-*Pichia* compared with nAra h 6

RBL SX-38 cells were sensitized with IgE from the serum pool and triggered with rAra h 6-*E. coli*, rAra h 6-*Pichia*, or nAra h 6 at different concentrations (0.001–100 ng/mL) as described in Section 2. The results of these degranulation assays are shown in Fig. 3. Using IgE from the pool of ten sera, the EC_{50} of rAra h 6-*E. coli*, rAra h 6-*Pichia*, and native protein were 50 ± 2.5 ng/mL, 0.75 ± 0.2 ng/mL, and 0.65 ± 0.3 ng/mL (n = 4), respectively. Clearly, rAra h 6-*E. coli* is much less potent than either nAra h 6 or rAra h 6-*pichia* in effector function ($p < 0.001$) whereas nAra h 6 and rAra h 6-*Pichia* had similar effector activity (p = ns).

3.4 Electrospray ionization and periodic acid-Schiff (PAS) staining

It is known that recombinant proteins expressed in *E. coli* lack PTMs [35]. For this reason, we considered that the reduced effector activity of rAra h 6-*E. coli* might be due to lack of PTMs. To assess this, we first performed tryptic digests followed by mass spectrometry. As shown in Supporting Information Tables S1 and S2, we found no significant differences in PTMs among these three proteins. However, given the variability in glycosylation products that are found in glycoproteins, mass spectrometry is limited in its ability to detect glycosylation [36]. To specifically test whether nAra h 6 is a glycoprotein, we performed glycoprotein staining with PAS. As shown in Supporting Information Fig. S1, nAra h 6 did not stain with PAS. Furthermore, nAra h 6 migrates as a crisp band on protein gel electrophoresis, a characteristic of proteins without extensive glycosylation (Fig. 2D). Taken together, these observations are evidence that lack of glycosylation of rAra h 6-*E. coli* is not a viable explanation for its poor effector activity.

3.5 CD analysis of rAra h 6-*E. coli*, rAra h 6-*Pichia*, and nAra h 6

Circular dichroism was used to determine structural properties of rAra h 6-*E. coli*, rAra h 6-*Pichia*, and nAra h 6 (Fig. 4A and B). The CD spectra of both rAra h 6-*Pichia* and nAra h 6 showed two minima at 208 and 222 nm, suggesting that rAra h 6-*Pichia* has predominantly alpha-helical secondary structure similar to the nAra h 6. The spectrum of rAra h 6-*E. coli* also revealed alpha-helical secondary structure, but it also exhibited a slight difference in the lower wavelength range (195–225 nm) reflecting a decrease in α-helices in favor of β-strands and random coil or loop formation. These results suggested that the rAra h 6-*E. coli* might not have the same folding properties as nAra h 6. Since the near-UV CD spectrum can be sensitive to certain changes in tertiary structure region due to aromatic amino acids and disulfide bonds, we measured the CD spectrum of rAra h 6-*E. coli*, rArah 6-*Pichia*, and nAra h 6 in the "near-UV" region (250–350 nm). We did not see significant difference among these proteins (data not shown).

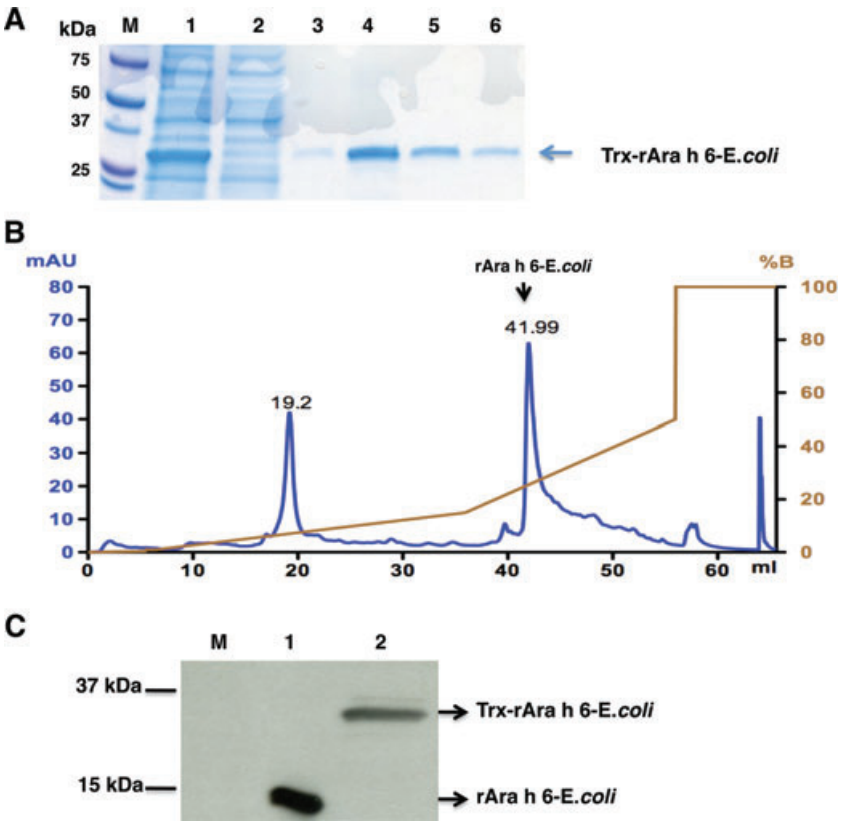

Figure 1. Expression and purification of recombinant Ara h 6-*E. coli*. (A) Coomassie blue staining of recombinant Ara h 6 fusion protein (Trx-rAra h 6-*E. coli*) derived from *E. coli* (lane 1), empty vector control (lane 2), purified Trx-rAra h 6-*E. coli* by ProBond purification column (lanes 4–6). (B) Purification of rAra h 6-*E. coli* with MonoQ chromatography after removing Trx fusion partner. (C) Immunoblots of rAra h 6-*E. coli* (lane 1) and Trx-rAra h 6-*E. coli* fusion protein (lane 2). Probes were a combination of three rabbit anti-Ara h 6 peptide antibodies. The data are representative of three independent experiments with similar results.

3.6 Native PAGE analysis

It is not unusual to have aggregation of certain disulfide bond-rich proteins in *E. coli* expression system [26]. To test whether aggregation occurs in rAra h-*E. coli*, we performed native-PAGE and silver staining. The result revealed two bands with greater mobility compared with monomeric nAra h 6 (Supporting Information Fig. S2). This indicates that it is unlikely that protein aggregation is the cause for the dramatically reduced effector activity of rAra h 6-*E. coli* and is compatible with an abnormality in folding that can alter electrophoretic mobility on native gel.

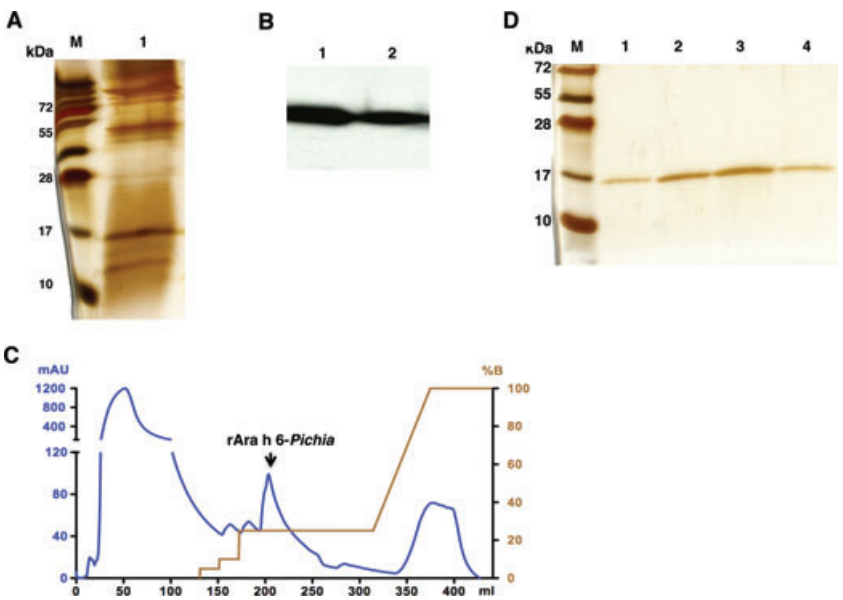

Figure 2. Expression and purification of recombinant Ara h 6-*Pichia*. (A) Silver staining of rAra h 6-*Pichia* expressed in culture medium of yeast (lane 1). (B) Immunoblots of rAra h 6-*Pichia* (lane 1) and nAra h 6 control (lane 2), performed as in Fig. 1C. (C) Purification of rAra h 6-*Pichia* with hydrophobic interaction chromatography (HIC). (D) Silver staining of increasing amounts of rAra h 6-*Pichia* after purification by HIC and gel filtration chromatography (lanes 1–3), and nAra h 6 control (lane 4). The data are representative of three independent experiments with similar results.

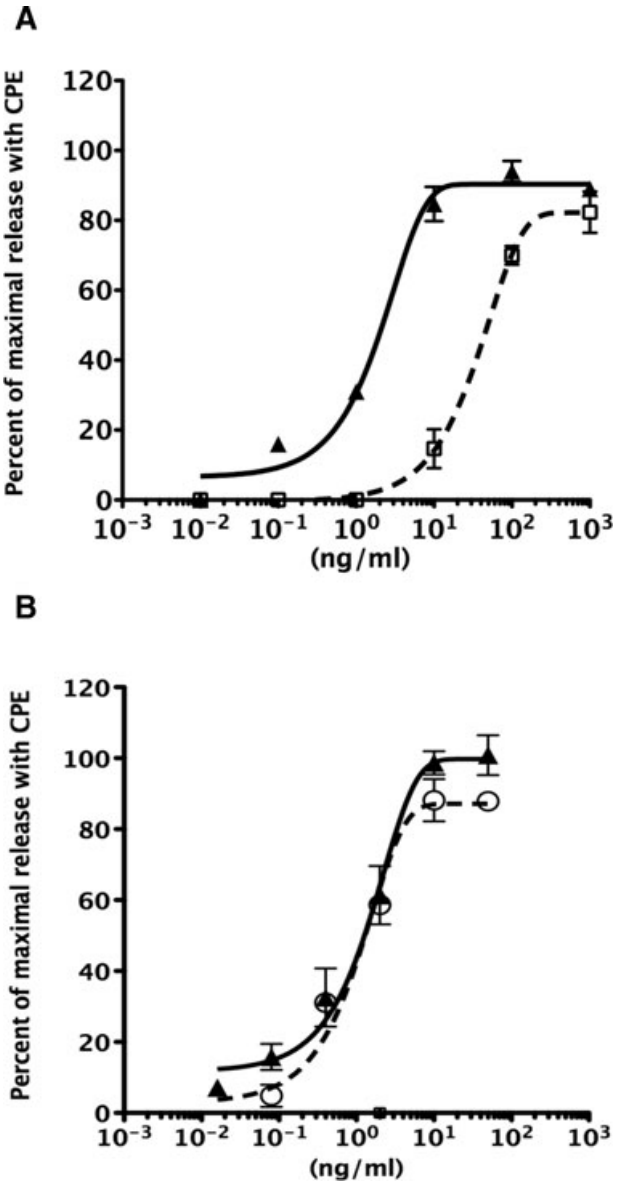

Figure 3. Effector activity of rAra h 6-*E. coli*, rAra h 6-*Pichia*, and nAra h 6. RBL SX-38 cells were sensitized with IgE from a pool of sera from highly peanut-allergic subjects and stimulated with rAra h 6-*E. coli* (A), rAra h 6-*Pichia* (B), and nAra h 6. Solid triangles (▲) are nAra h 6; open squares (□) are rAra h 6-*E. coli* ($n =$ 5 separate experiments); and open circles (o) are rAra h 6-*Pichia* ($n = 5$ separate experiments). In each experiment, cells were stimulated in triplicate with a maximal amount of CPE (200 ng/mL) as positive control. In order to pool the experimental results, the data are expressed as a percent of the maximal net (total minus background) degranulation in each assay. The background ranged from 2 to 9%, the total degranulation ranged from 28 to 48% and the net release ranged from 25 to 40%. Best-fit lines were generated using a one-phase decay model (Prism 5.0c; GraphPad Software). The data are representative of five independent experiments with similar results.

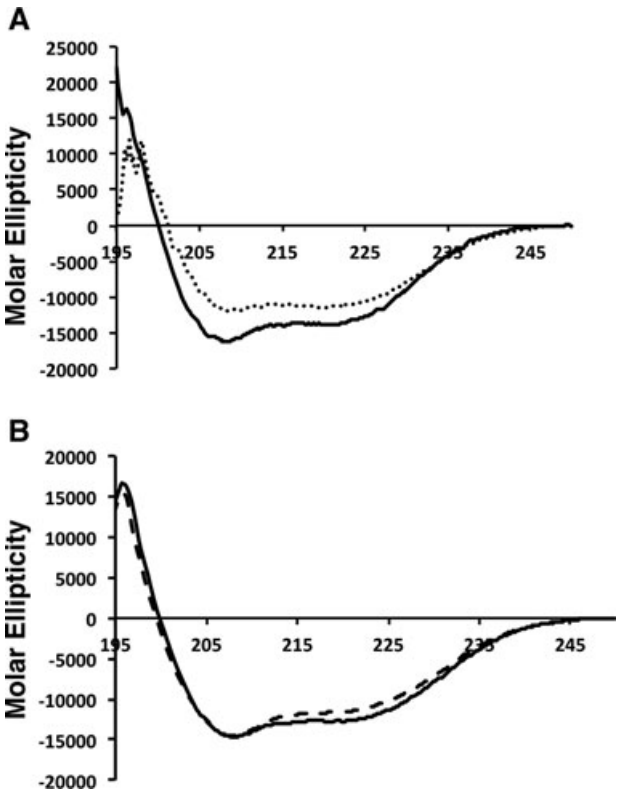

Figure 4. CD spectrum of rAra h 6-*E. coli*, rAra h 6-*Pichia*, and nAra h 6. The CD spectra of rAra h 6-*E. coli* (A), rAra h 6-*Pichia* (B), and nAra h 6 were collected on a Jasco 816 spectropolarimeter in PBS. The solid lines are the data for nAra h 6 (A and B); the dotted line is the data for rAra h 6-*E. coli* (A), and the dashed line is the data for rAra h 6-*Pichia* (B). The scanning spectra ranged from 190–260 nm. Six scans were recorded and averaged with a scanning speed of 50 nm/min at 20°C. The results were expressed as Molar Ellipticity [Θ] as described in Section 2. The data are representative of two independent experiments with similar results.

3.7 Effector activity of mutated rAra h 6-*Pichia*

Recently, our laboratory performed epitope mapping of Ara h 6 by microarray and identified four potentially important IgE-binding linear epitopes [37]. To test whether these epitopes are important for the effector activity of rAra h 6, we expressed a mutant rAra h 6-*Pichia* with 2 point mutations in each of the four epitopes (m-rAra h 6-*Pichia*). The specific amino acids that were changed to alanine were chosen based on hydrophilicity values and prediction of antigenicity [38, 39]. The effector activity of the m-rAra h 6-*Pichia* was first compared to the rAra h 6-*Pichia* by mediator release from RBL SX-38 cells sensitized with IgE from the serum pool (Fig. 5). As can be seen, the dose–response curve with the m-rAra h 6-*Pichia* is shifted >3 logs to the right demonstrating that this protein has $<0.1\%$ the effector activity seen with rAra h 6-*Pichia*.

Mediator-release assays were then performed with five individual sera (Fig. 6A–E). For four of the individual sera tested (Fig. 6A–D), the data seen with the serum pool (Fig. 5)

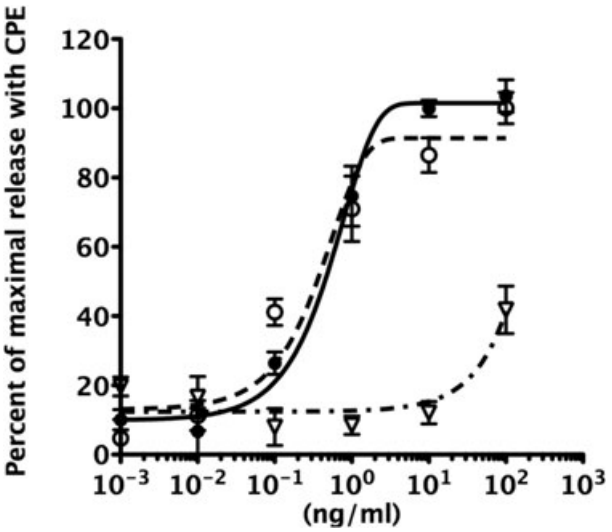

Figure 5. Effector activity of epitope mutant of recombinant rAra h 6 from yeast in pooled serum. RBL SX-38 cells were sensitized with IgE from a pool of sera from highly peanut-allergic subjects and stimulated with nAra h 6, rAra h 6-*Pichia*, and m-rAra h 6-*Pichia* as indicated. Solid circles (●) are nAra h 6, opens circles (o) are rAra h 6-*Pichia* and open inverted triangles (▽) represent rAra h 6 mutant. The background, total and net degranulation values are same as Fig. 3. The data are representative of three independent experiments with similar results.

were recapitulated such that rAra h 6-*Pichia* had similar IgE cross-linking activity as nAra h 6 sera and m-rAra h 6-*Pichia* was much less active. Unexpectedly, rAra h 6-*Pichia* was ineffective in cross-linking IgE from one of the individual sera even though nAra h 6 was fully effective.

CD analysis of the mutant Ara h 6 revealed a significant alteration in the secondary structure of the m-Ara h 6 variant characterized by loss of α-helices and the presence of random coils (Fig. 7).

4 Discussion

Ara h 6 is an important peanut allergen based on specific IgE reactivity, skin testing, and ex vivo basophil activation [13, 14]. To fully understand the molecular details of how Ara h 6 cross-links IgE/FcεRI complexes to activate mast cells (allergic effector activity), it is important to have a recombinant allergen with effector activity resembling its natural counterpart. In this study, we expressed recombinant Ara h 6 in *E. coli* and *P. pastori* (Fig. 1 and 2). Our results demonstrate that properly folded rAra h 6-*Pichia* preserves effector function for most peanut allergic subjects and has much higher biological potency than rAra h 6-*E. coli* (Fig. 3). Finally, we have used this system to demonstrate that mutation of key amino acids in four important IgE-binding linear epitopes of Ara h 6 is sufficient to dramatically reduce its allergic effector activity.

Our inability to generate rAra h 6-*E. coli* of potency similar to that of nAra h 6 is of interest. The minor difference in our approach compared to that of Lehmann (different excision site) is unlikely to account for this difference so we examined our purified rAra h 6 proteins in detail.

We first considered that a possible explanation for the discrepancy between rAra h 6-*Pichia* and rAra h 6-*E. coli* could be the lack of PTM in *E. coli*. It has been reported that post-translational processing of allergens has important consequences for their IgE-binding and effector properties [40–43]. Although the results of mass spectrometry analysis did not show significant alteration of PTM between rAra h 6-*Pichia* and rAra h 6-*E. coli*, we could not exclude the possibility that glycosylation may contribute to the disparity in the effector activity of native and rAra h 6-*Pichia* compared to that of rAra h 6-*E. coli*. However, the observation that nAra h 6 runs as a crisp band on protein gel electrophoresis and the lack of carbohydrate staining (Supporting Information Fig. S1) suggests that nAra h 6 is not a glycoprotein. So, it is unlikely that glycosylation is involved in IgE cross-linking on Ara h 6 and unlikely that lack of glycosylation in rAra h 6-*E. coli* explains its poor effector activity. Finally, it is important to point out that our evaluation of PTMs has some limits. Specifically, some PTMs, such as phosphorylation, are often lost during sample preparation; and labile modifications, such as sulfation and some forms of glycosylation, are difficult to detect on mass spectrometry [36].

We then examined the native and recombinant proteins by circular dichroism (CD). The CD spectra showed that rAra h 6-*Pichia* displayed a curve almost identical to that of the nAra h 6, suggesting that it is folded properly with a predominantly alpha-helical secondary structure. Although the CD-spectra of rAra h 6-*E. coli* also have a predominantly alpha-helical secondary structure, subtle difference were observed in the secondary structure (Fig. 4). In that, Ara h 6 contains ten cysteine residues that are capable of forming five disulfide bonds and are required for correct folding and allergic reactivity, it is likely that the disulfide bonds in rAra h 6-*E. coli* are not in their native configuration. In that, rAra h 6-*E. coli* has increased mobility on native gel analysis (Supporting Information Fig. S2) and ran appropriately (~15 kD) on gel filtration chromatography during purification (data not shown), it is unlikely that protein aggregation is the cause for its dramatically reduced effector activity. Rather, based on the subtle differences from the native protein in CD spectra (Fig 4A) and the marked differences seen on native gel (Supporting Information Fig. S2), we speculate that the functional disparity between the two recombinant proteins is more likely due to alterations in folding.

Once we established that the *Pichia* expression system was suitable for expression of fully functional recombinant Ara h 6, we used this platform to test the role of specific epitopes of Ara h 6 for their importance in allergic effector function. For this purpose, we generated an Ara h 6 mutant carrying amino acid substitutions in four major IgE-binding epitopes. The m-rAra h 6-*Pichia* exhibited extremely low IgE

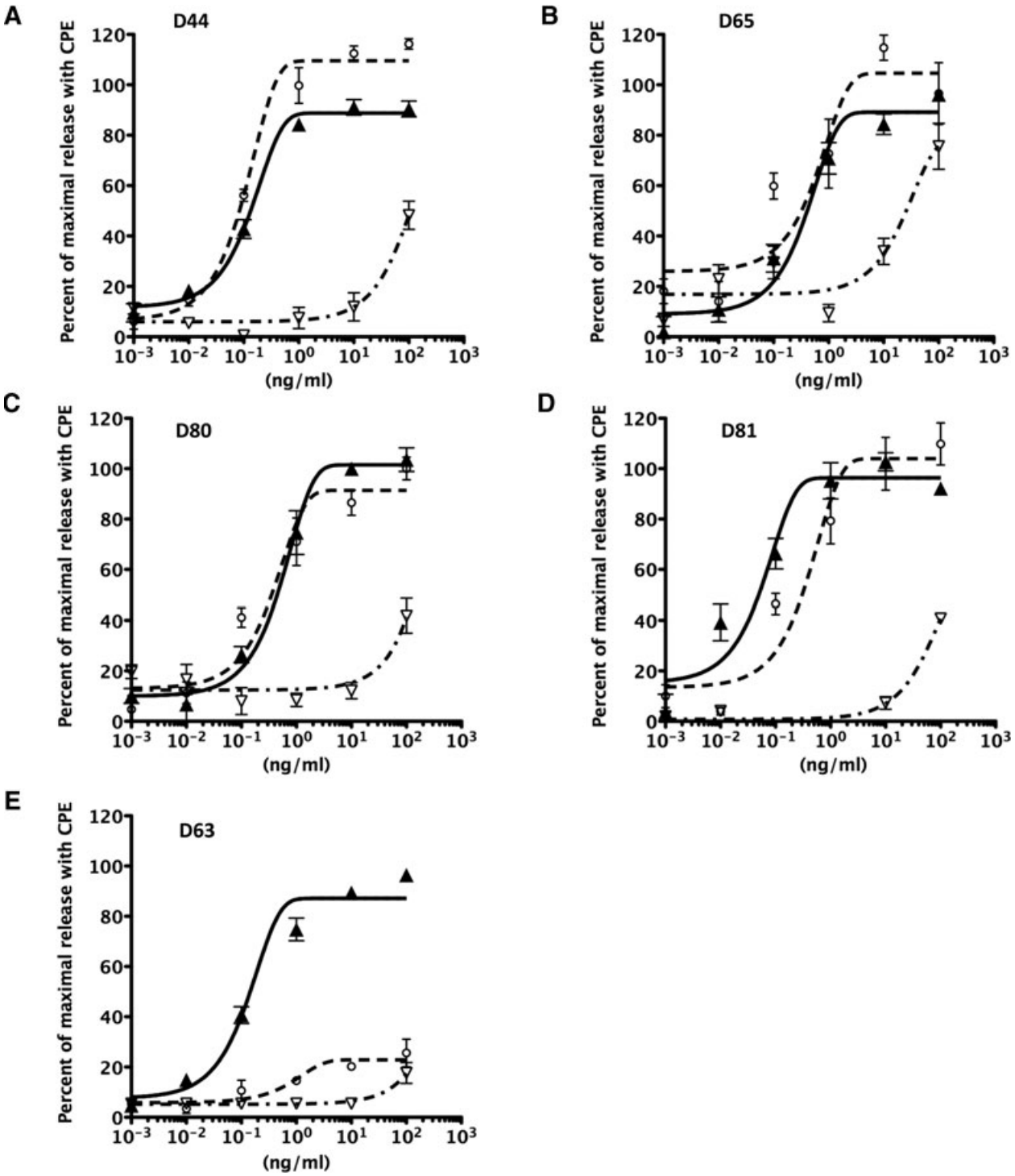

Figure 6. Effector activity of epitope mutant of recombinant rAra h 6 from yeast in individual serum. RBL SX-38 cells were sensitized with IgE from individual serum from highly peanut-allergic subjects (D44, D65, D80, D81, and D63) and stimulated with nAra h 6, rAra h 6-*Pichia*, and m-rAra h 6-*Pichia* as indicated. Solid triangles (▲) are nAra h 6, open circles (o) are rAra h 6-*Pichia*, and open inverted triangles (▽) represent rAra h 6 mutant. The background, total and net degranulation values are same as Fig. 3. The data are representative of two or three independent experiments with similar results.

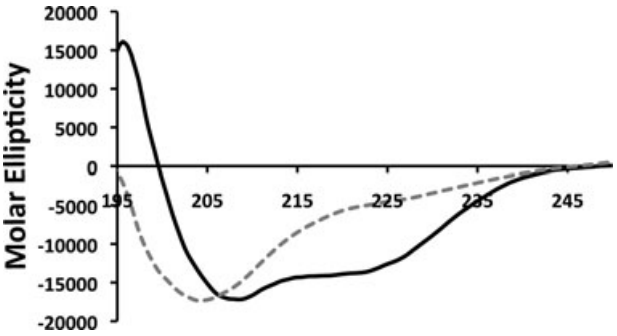

Figure 7. CD spectrum of rAra h 6-*Pichia* and m-rAra h 6-*Pichia*. The CD spectra of rAra h 6-*Pichia* and rAra h 6-*Pichia*-mutant were collected on a Jasco 816 spectropolarimeter in PBS. The solid line is the data for rAra h 6-*Pichia*; the dashed line is the data for m-rAra h 6-*Pichia*. The data are representative of two independent experiments with similar results.

cross-linking activity (0.1% compared with rAra h 6-*pichia*) in the RBL SX-38 cell assay and a disturbed secondary structure seen on CD analysis but not on native gel (Figs. 6, 7, and S2). Thus, the reduction in allergic effector activity of m-rAra h 6 is due to either the amino acid changes, to disturbed secondary structure, or to a combination of these changes.

In conclusion, *P. pastoris* is superior to *E. coli* for generation of properly folded and biologically active rAra h 6. Our data indicated that nAra h 6 is not a glycoprotein and has only minor PTMs. Furthermore, rAra h 6-*Pichia* is fully potent when assessed with IgE from most patients and this is likely due to correct folding as evidenced by its CD spectrum. Finally, we have demonstrated that a mutant of Ara h 6, m-rAra h 6-*Pichia*, with two mutations in each of 4 IgE-binding domains, is inactive confirming that a limited number of amino acid changes in Ara h 6 can adversely affect the allergic effector function.

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