Development and Expression of Recombinant Ara h 1 Fragment Proteins

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Background **: Peanut allergy is a significant health problem because of the potential severity of the allergic reaction, the chronicity of the allergic sensitivity, and the ubiquity of peanut products.**

Objective **: We set out to clone and characterize the Ara h 1 fragment peanut allergens and the expressed fragments of Ara h 1 appeared to bind IgE from the peanut allergic patient serum very efficiently.**

Methods **: We have used PCR amplification, DNA sequencing and analysis and bacterial expression and purification of recombinant Ara h 1 fragments.**

Results **: The recombinant form of this protein was expressed in a bacterial system and was recognized by serum IgE from peanut hypersensitivity patients.**

Conclusion **: Ara h 1, a major allergen of peanuts appears to bind significant amounts of IgE through linear epitopes. (Ann Dermatol 16(3) 91~98, 2004)**

Key Words: Ara h 1, Peanut allergy

INTRODUCTION

Peanuts are a major cause of serious allergic reaction in both children and adults. The hypersensitivity to peanuts often starts early in childhood and continues throughout life[1]. It is less likely to resolve spontaneously and more likely to result in fatal anaphylaxis[2]. Avoidance is the only effective means of dealing with this food allergy. The current use of peanuts and peanut by products as supplements in many different types of foods makes accidental ingestion almost inevitable[3].

Recombinant methodology to clone an allergen provides an efficient means of producing pure polypeptides which, in their native source, form complex mixtures and are often represented in only very small amounts. Several inhaled allergens have been cloned, including the allergens of house dust mites and pollen grains, however, in comparison little work has been directed toward producing recombinant food allergens[4].

Various studies have shown that the most allergenic portion of the peanut is the protein fraction of the cotyledon[5]. A major allergen found in the cotyledon is the peanut protein, Ara h 1. This protein is recognized by greater than 90% of peanut-sensitive patients, thus establishing it as an important allergen[6]. Previous results have demonstrated similarity between the level of IgE binding to recombinant Ara h 1 protein and the native form of this allergen when individual patient serum was tested[4]. These results indicated that the recombinant protein could be considered for use in both diagnostic and immunotherapeutic approaches to peanut hypersensitivity[7]. In order for this, the recombinant allergen should be soluble. Currently, the full-length recom-

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binant Ara h 1 expressed in *E. coli* is known to be insoluble due to incorrect folding of the monomer leading to extensive polymerization. Because of this problem, studies using the recombinant Ara h 1 remain hardly progressed world-widely now. So we performed this study to produce a recombinant Ara h 1 considered soluble.

MATERIALS AND METHODS

1. PCR amplification of the Ara h 1 cDNA fragment sequences (1-9, 10-15, 16-23)

The PCR (polymerase chain reaction) was carried out in a buffer containing 2 mM $MgCl_2$, 500 mM KCl, and 100 mM Tris-HCl, pH 7.5. Each cycle of the PCR consisted of 30seconds at 95℃, followed by 1 minute at 56℃, and 2 minutes at 72℃. Thirty cycles were performed with both primers present in all cycles. The primers above were used to PCR amplify the three fragments of Ara h 1. Each forward (f) primer contains a Sac I restriction site which is underlined in the sequence. Each reverse (r) primer contains a Hind III restriction site which is underlined in the sequence. The position recognized by each primer in the full length Ara h 1 sequence is listed to the right of the primer. Table 1 lists the primers used to amplify the three separate fragments of Ara h 1. The portion of the primer homologous with the Ara h 1 sequence are grouped into the three base codons they represent while the restriction sites are underlined. In addition, each primer contains a two G or C bases to insure efficient restriction of the final PCR product. The position at which each primer hybridizes to the full length Ara h 1 sequence is given to the right of the primer sequence. The orientation of each primer is denoted with an 'f' for those primers creating the amino terminal end of each fragment and an 'r' for those primers creating the carboxyl terminal ends.

2. Subcloning P41b PCR product into pET 24b expression vector

PCR amplification was used to amplify a portion of the Ara h 1 sequence. This PCR product and the expression vector pET 24b were then cut with the restriction enzymes Sac I and Hind III so that the fragment of Ara h 1 could be cloned into the expression vector in the proper orientation and in the correct reading frame for expression. The Ara h 1 fragment was then ligated into the pET 24b expression vector and used to transform the Nova Blue strain of *Escherichia coli*.

3. DNA sequencing and analysis

Plasmid DNA was prepared from the transformed cells and sequenced to confirm that the cloned fragment was correct and inserted properly. Sequencing was done according to the methods of Sanger et al[8].

Table 1. The Primers Above were Used to PCR Amplify the Three Fragments of Ara h 1.

Primers	Orientation	Sequence	Position
P41b1-9 Sac I	f	5' GGGAGCTCT ACG CAT GCC AAG TCA TCA CCT 3'	116-136
P41b9-1 Hind III	r	5' GGAAGCTT CCT TGA CGG GAA GTA GAA AGG 3'	569-589
P41b10-15 Sac I	f	5' CCGAGCTCT ATC TTG AAC CGC CAT GAC AAC 3'	869-889
P41b15-10 Hind III	r	5' CCAAGCTT GAG CAT CAA AGC TCC TTC TTT G 3'	1363-1384
P41b16-23 Sac I	f	5' CCGAGCTCT CAC TTC AAC TCA AAG GCC 3'	1385-1405
P41b23-16 Hind III	r	5' GGAAGCTT GTT AAA AGC CTT CAA AAT TGA AAG G 3'	1904-1927

Each forward (f) primer contains a Sac I restriction site which is underlined in the sequence. Each reverse (r) primer contains a Hind III restriction site which is underlined in the sequence. The position recognized by each primer in the full length Ara h 1 sequence is listed to the right of the primer.

4. Production of recombinant Ara h 1 fragment protein

The plasmid DNA was used to transform the BL21 (DE3) strain of *E. coli* for optimal expression of the cloned fragments of Ara h 1. Protein expression in the *E. coli* strain BL21 (DE3) was induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM once the culture reached A600=0.6. The cells were harvested at 1-h intervals, resuspended in SDS sample buffer containing DTT, and boiled at 100℃ for 5 min. Samples were either used immediately for immunoblot analysis, or samples were pelleted, washed with 50 mM Tris-HCL, and stored for later use as a frozen pellet at -70℃.

Recombinant Ara h 1 fragment was purified from bacterial lysates under denaturing conditions using the His-Bind Purification Kit (Novagen Inc., Madison, wisconsin, USA). Cell extracts were resuspended in 4 ml of cold Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea; supplied with Novagen kit), sonicated to shear DNA, and incubated on ice for 1 h. Next, the lysate was centrifugated at 12,000 g for 45 minutes to remove cellular debris. The postcentrifugation supernatant was prepared for loading onto the column by passing it through a 0.45 um membrane using a syringe-end filter. A His-Bind Quick Column (Novagen) was packed with His-Bind metal chelation resin, washed with deionized H_2O, and charged until saturation with Charge Buffer (50 mM $NiSO_4$; Novagen). After equilibration of the column with Binding Buffer, 2 volumes of supernatant were loaded onto the column. The column was washed with 10 volumes of Binding Buffer and 6 volumes of Wash Buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea;Novagen). After that, the bound proteins were eluded with wash buffer containing 1 ml imidazole. Fractions collected over the course of the experiment containing recombinant Ara h 1 fragment were lyophilized and stored in 1XPBS.

5. IgE immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli[9]. All gels were composed of a 10% acrylamide resolving gel and 4% acrylamide stacking gel. Electrophoretic transfer and immunoblotting on nitrocellulose paper were performed by the procedure of Towbin et al[10]. The blots were incubated with antibodies diluted in a solution containing TBS and 1% bovine serum albumin for at least 12 hours at 4℃ for 2 hours at room temperature. Detection of the primary antibody was done with ^{125}I-labelled anti-IgE antibody.

RESULTS

1. PCR results of the Ara h 1 cDNA fragments (1-9, 10-15, 16-23)

The primers used to amplify the fragments of Ara h 1 were designed with a Sac I restriction site on the forward primers and a Hind III site on the reverse primers. These restriction sites were chosen because they would not cut the fragments internally and would allow the fragments to be placed into pET 24b in the correct orientation. The cloned Ara h 1 fragments (1-9), (10-15), and (16-23) were examined by PCR (Fig. 1). The entire multiple cloning

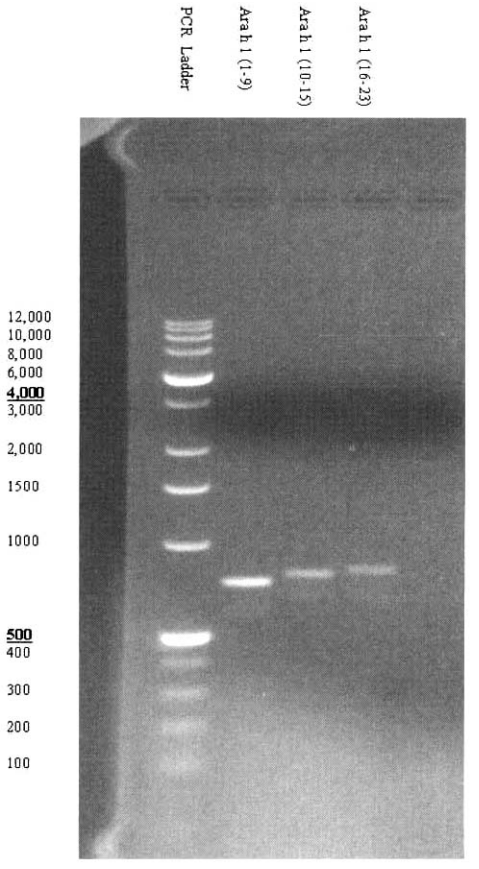

Fig. 1. PCR results of the Ara h 1 cDNA fragment (1-9, 10-15, 16-23). Three demarcated bands showed between 500 and 1000 base pair.

site was amplified and the product sizes determined. The vector specific primers T7 promoter and T7 terminator were used in our standard PCR reaction. The PCR products were examined on a 1.5% agarose gel. containing ethidium bromide. Ara h 1 fragment (1-9), (10-15), and (16-23) gave only 473, 515 and 542 base pair bands, respectively. The PCR products were each approximately 275 bp larger than the insert size alone due to the additional sequence amplified from the pET-24b vector.

2. Nucleotide sequence of an Ara h 1 cDNA fragments (1-9, 10-15, 16-23)

The primary DNA sequence of the largest cDNA clone (P41b) was determined by Sanger dideoxy sequencing using oligonucleotide primers directed to different regions on the insert or a series of subclones constructed by Sac I and Hind III digestion of the inserts. Clone P41b carried a 2,032 base insert. The first ATG protein synthesis start codon was located at nucleotide position 50-53. The sequence around this codon agrees with the translation initiation sequence found in most eukaryotic mRNAs. The insert contained a large open reading frame stating with this codon and ending with a TGA stop codon at nucleotide 1928-1930. A search of the GenBank database revealed significant sequence homology between the Ara h 1 cDNA clones and a class of seed storage proteins called vicilins (Fig. 2).

3. Expression and purification of recombinant Ara h 1 fragments (1-9, 10-15, 16-23)

The Ara h 1 cDNA was cloned into a pET 24 plasmid and expressed in a bacterial system. Optimal expression was obtained following a three-hour induction by IPTG. The immunoblot was performed using serum IgE from a pool of patients with peanut hypersensitivity to determine the molecular weight and specificity of IgE binding (Fig. 3). The 0 time point shows the level of expression before the addition of IPTG and the 3hr time point shows the level of expression after 3hr in the present of 1mM IPTG. Clone Ara h (1-9) had same background expression at 0 hr but was induced to much higher levels by IPTG at 3 hr. Clone Ara h 1 (16-23) produces a smaller frequent probably due to the incomplete synthesis or enzymatic breakdown of the full length peptide. From the blot, the estimated size

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  1  AATAATCATA TATATTCATC AATCATCTAT ATAAGTAGTA GCAGGAGCAA
 51  TGAGAGGGAG GGTTTCTCCA CTGATGCTGT TGCTAGGGAT CCTTGTCCTG
                P41b(1  9) →
101  GCTTCAGTTT CTGCAACGCA TGCCAAGTCA TCACCTTACC AGAAGAAAAC
151  AGAGAACCCC TGCGCCCAGA GGTGCCTCCA GAGTTGTCAA CAGGAACCGG
201  ATGACTTGAA GCAAAAGGCA TGCGAGTCTC GCTGCACCAA GCTCGAGTAT
251  GATCCTCGTT GTGTCTATGA TCCTCGAGGA CACACTGGCA CCACCAACCA
301  ACGTTCCCCT CCAGGGGAGC GGACACGTGG CCGCCAACCC GGAGACTACG
351  ATGATGACCG CCGTCAACCC CGAAGAGAGG AAGGAGGCCG ATGGGGACCA
401  GCTGGACCGA GGGAGCGTGA AAGAGAAGAA GACTGGAGAC AACCAAGAGA
451  AGATTGGAGG CGACCAAGTC ATCAGCAGCC ACGGAAAATA AGGCCCGAAG
501  GAAGAGAAGG AGAACAAGAG TGGGGAACAC CAGGTAGCCA TGTGAGGGAA
                                ← P41b(9  1) P41b(9  10)→
551  GAAACATCTC GGAACAACCC TTTCTACTTC CCGTCAAGGC GGTTTAGCAC
601  CCGCTACGGG AACCAAAACG GTAGGATCCG GGTCCTGCAG AGGTTTGACC
651  AAAGGTCAAG GCAGTTTCAG AATCTCCAGA ATCACCGTAT TGTGCAGATC
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701  GAGGCCAAAC CTAACACTCT TGTTCTTCCC AAGCACGCTG ATGCTGATAA
751  CATCCTTGTT ATCCAGCAAG GGCAAGCCAC CGTGACCGTA GCAAATGGCA
801  ATAACAGAAA GAGCTTTAAT CTTGACGAGG GCCATGCACT CAGAATCCCA
                ← P41b(10 - 9)  P41b(10 - 15) →
851  TCCGGTTTCA TTTCCTAC|AT CTTGAACCGC CATGACAACC AGAACCTCAG
901  AGTAGCTAAA ATCTCCATGC CCGTTAACAC ACCCGGCCAG TTTGAGGATT
951  TCTTCCCGGC GAGCAGCCGA GACCAATCAT CCTACTTGCA GGGCTTCAGC
1001 AGGAATACGT TGGAGGCCGC CTTCAATGCG GAATTCAATG AGATACGGAG
1051 GGTGCTGTTA GAAGAGAATG CAGGAGGTGA GCAAGAGGAG AGAGGGCAGA
1101 GGCGATGGAG TACTCGGAGT AGTGAGAACA ATGAAGGAGT GATAGTCAAA
1151 GTGTCAAAGG AGCACGTTGA AGAACTTACT AAGCACGCTA AATCCGTCTC
1201 AAAGAAAGGC TCCGAAGAAG AGGGAGATAT CACCAACCCA ATCAACTTGA
1251 GAGAAGGCGA GCCCGATCTT TCTAACAACT TTGGGAAGTT ATTTGAGGTG
1301 AAGCCAGACA AGAAGAACCC CCAGCTTCAG GACCTGGACA TGATGCTCAC
                                  ← P41b(15 - 10) P41b(16 - 23) →
1351 CTGTGTAGAG ATCAAAGAAG GAGCTTTGAT GCTC|CCACAC TTCAACTCAA
1401 AGGCCATGGT TATCGTCGTC GTCAACAAAG GAACTGGAAA CCTTGAACTC
1451 GTGGCTGTAA GAAAAGAGCA ACAACAGAGG GGACGGCGGG AAGAAGAGGA
1501 GGACGAAGAC GAAGAAGAGG AGGGAAGTAA CAGAGAGGTG CGTAGGTACA
1551 CAGCGAGGTT GAAGGAAGGC GATGTGTTCA TCATGCCAGC AGCTCATCCA
1601 GTAGCCATCA ACGCTTCCTC CGAACTCCAT CTGCTTGGCT TCGGTATCAA
1651 CGCTGAAAAC AACCACAGAA TCTTCCTTGC AGGTGATAAG GACAATGTGA
1701 TAGACCAGAT AGAGAAGCAA GCGAAGGATT TAGCATTCCC TGGGTCGGGT
1751 GAACAAGTTG AGAAGCTCAT CAAAAACCAG AAGGAATCTC ACTTTGTGAG
1801 TGCTCGTCCT CAATCTCAAT CTCAATCTCC GTCGTCTCCT GAGAAAGAGT
1851 CTCCTGAGAA AGAGGATCAA GAGGAGGAAA ACCAAGGAGG GAAGGGTCCA
                                  ← P41b(23 - 16) Stop
1901 CTCCTTTCAA TTTTGAAGGC TTTTAACTGA GAATGGAGGC AACTTGTTAT
1951 GTATCGATAA TAAGATCACG CTTTTGTACT CTACTATCCA AAAACTTATC
2001 AATAAATAAA AACGTTTGTG CGTTGTTTCT CC
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Fig. 2. Nucleotide sequence of an Ara h 1 cDNA fragment clones. The primer sequences that begin and end each fragment are shown highlighted and underlined in the full length Ara h 1 sequence. The protein synthesis start (ATG) and stop (TGA) sites are boxed.

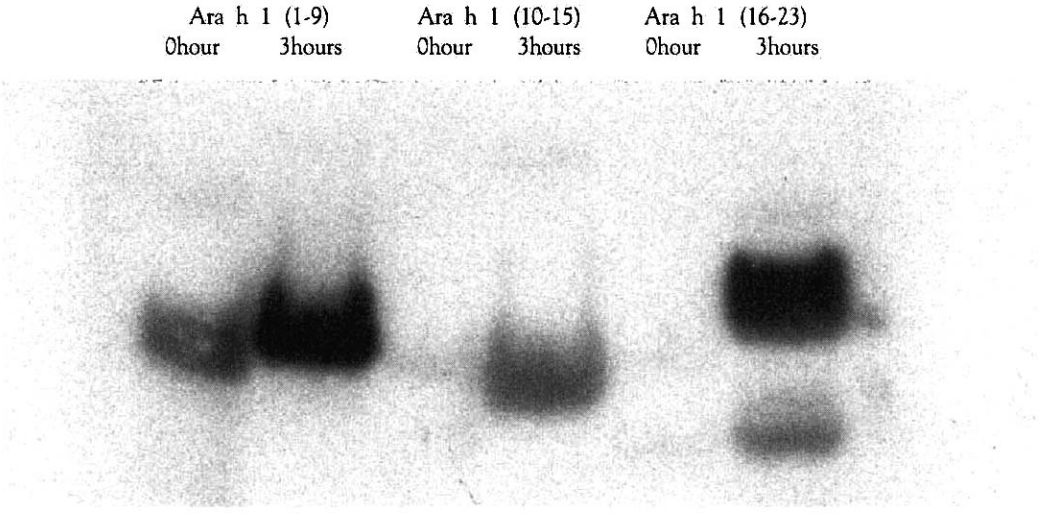

Fig. 3. Induction of protein expression from Ara h 1 clones. The immunoblot was performed using serum IgE from a patients with peanut allergy. The 0 time point shows the level of expression before the addition of IPTG and the 3 hours time point shows the level of expression after 3 hours in the presence of IPTG.

of the recombinant peptides produced by bacterial cells is about 20-23 kDa, which correspondes to the predicted molecular mass encoded by the clones.

DISCUSSION

Peanut allergy is a significant IgE-mediated health problem because of the increased prevalence, potential severity, and chronicity of the reaction[11]. Sensitive individuals may experience symptoms ranging from urticaria to anaphylaxis[12]. Because of the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, the risk to the peanut-sensitive individual is increasing[4].

Identification and purification of allergens are crucial to the understanding of IgE-mediated disease. Several allergens have been identified that stimulate IgE production and cause IgE-mediated disease in humans. In comparison with the body of work done to identify and purify inhaled allergens, significantly less work has been done on the food allergens[13-15]. Because peanuts are a relatively common and often fatal cause of food hypersensitivity reactions, we chose to use this model to study IgE-mediated reactions. Cloning of respective gene is a very important part of an allergen characterization. First, it allows the determination of the allergen primary structure by deducing the amino acid sequence from the gene nucleotide sequence. Second, sequence information can give an insight into allergen biological activity by searching for similar sequences in computer databases. Third, it may allow homology modeling of the allergen three-dimensional structure. Fourth, the cloned allergen gene can be used as a DNA vaccine for immunotherapy. And finally, cloning of allergen genes gives the opportunity to express these genes *in vitro* to produce recombinant allergens.

Three of the major peanut allergens, Ara h 1, Ara h 2, and Ara h 3, are abundant proteins that belong to the vicillin, conglutin, and glycinin families of seed storage proteins, respectively[2,4,6,16]. These allergens have now been relatively well characterized, cloned, and sequenced, and their IgE-binding epitopes and T-cell epitopes are partially identified[17]. Ara h 1 is a protein with a mean molecular weight of 63.5 kDa and a mean isoelectric point of 4.55. This protein is recognized by greater than 90% of peanut-sensitive patients, thus establishing it as an important allergen[6]. The majority of serum IgE recognition of the Ara h 1 allergen appears to be due to epitopes within this protein that are linear amino acid sequences that do not contain significant amounts of carbohydrate[6,7]. Twenty three different linear IgE-binding epitopes were identified, located throughout the length of the Ara h 1 protein[17]. An epitope can be considered immunodominant if it is recognized by

serum IgE from the majority of patients with peanut hypersensitivity or if the serum IgE that recognizes a peptide represents the majority of Ara h 1-specific IgE found in a patient[7]. Four of the Ara h 1 IgE binding epitopes (1, 3, 4, and 17) appeared to be immunodominant in that they were recognized by > 80% of the patients and bound more IgE than any of the other Ara h 1 epitopes[7,17].

Current diagnosis of food hypersensitivity relies on a significant clinical history plus evidence of specific IgE to the food allergen in question. The absence of specific IgE to a food means there is a > 95% probability that the ingestion of the food will not lead to clinical symptoms. However, the presence of specific IgE to a particular food has only at best a 50% positive predictive value when correlated with a positive food challenge[18]. One explanation of this low predictive value is the current use of crude allergen mixtures. The use of recombinant allergens should make it possible to obtain diagnostics with optimal concentrations of each allergen or to develop panels of mixtures of large numbers of recombinant allergens and then test for individual components. Another possible use for recombinant peanut allergens is in immunotherapy. Immunotherapy with specific recombinant allergen epitopes rather than the crude allergen mixture could prove to be the more effective treatment modality. The use of recombinant allergens in standard allergen immunotherapy would have several advantages over natural allergens, including better control of the batch to batch variability of the specific allergens and the assurance of the representation of minor allergens in standard amounts[4]. Like this, a recombinant allergen using Ara h 1 known as the major peanut allergen may be useful in the diagnosis and the treatment of peanut hypersensitivity.

So far, the full-length recombinant Ara h 1 has been expressed by using *E. coli*, yeast or an insect cell culture. But unfortunately, it is insoluble possibly due to incorrect folding of the monomer leading to extensive polymerization. In order to use in the skin pinprick test and immunotherapy, this recombinant allergen needs to be expressed in a form that is soluble. We decided to clone the full- length allergen as three separate fragments which we hoped would be unable to be polymerized and therefore remain soluble. In order to obtain fragments that would be expressed in a similar fashion, the full length Ara h 1 clone was divided into three fragments of approximately equal size. Any patient with peanut hypersensitivity generally could be detected with fragment 1-9 and 16-23 because they contain all four peptides (1, 3, 4, and 17). We have developed three recombinant Ara h 1 fragments that is 1-9, 10-15 and 16-23. The fragments were also chosen so that each contained roughly the same proportion of hydrophilic residues. We subcloned the Ara h 1 fragment into pET expression vectors and transformed appropriate *E. coli* host strains. The pET vectors contained an in frame 6X HIS tag at the 3' end of the insert. These constructs allow for easy isolation of full-length recombinant protein by affinity chromatography on nickel columns.

After performing an immunoblot using the serum IgE of the allergic patient to peanuts to check out the cloned recombinant Ara h 1, the estimated size of the recombinant peptides came to 20-23 kDa, which corresponds to the predicted molecular mass encoded by the clones. These fragments are of a similar size, and contain hydrophilic residues so that they aren't thought to form an organic epitope. This study is supposed to observe if the expressed Ara h 1 fragment is combined with the serum of the allergic patient to peanuts through cloning and specifying fragments of the recombinant Ara h 1.

In conclusion, we produced the full-length Ara h 1 in a soluble fashion by dividing it in an insoluble fashion into three fragments, and then by recombining them using *E. coli*. The result of this study is expected to clarify the three fragments of the Ara h 1 and contribute to the desensitization of peanut allergy as well as the diagnosis of it.

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