

Namiko Iwamoto^a
Chiharu Nishiyama^a
Takaomi Yasuhara^a
Ayako Saito^a
Toshihumi Yuuki^a
Yasushi Okumura^a
Hirokazu Okudaira^b

^a Central Research Laboratory, Asahi
Breweries and

^b Department of Medicine and Physical
Therapy, Faculty of Medicine,
University of Tokyo, Japan

Direct Expression of Der f 2, a Major House Dust Mite Allergen, in *Escherichia coli*

Abstract

Der f 2 protein in a highly antigenic form was directly expressed in bacteria. Plasmid pFLU11 derived from pKK233–2 was designed to express methionyl-Der f 2 under the control of the trc promoter and it has the replication origin of pUC118 instead of its original to increase the copy number. This expression plasmid directed the synthesis of recombinant Der f 2 (rDer f 2) protein in an insoluble form of inclusion bodies in *Escherichia coli* cells. The high copy number plasmid pFLU11 conferred the efficient production of the Der f 2 protein in *E. coli*, when compared to a nonchanged origin material. rDer f 2 inclusion bodies were easily solubilized in urea and renatured by dialysis to assume the active form. The rDer f 2 protein was purified by means of anion exchange and gel filtration chromatography. This expression system yielded about 10 mg of purified rDer f 2 protein from the 1L culture. Purified rDer f 2 protein reacted with IgE from patient sera almost identically to the native Der f 2 in the RAST enzyme immunoassay and skin prick test.

Key Words

Der f 2
Allergen
Escherichia coli

Introduction

The house dust mite [1] is one of the major causes of atopic diseases such as bronchial asthma and atopic dermatitis [2–4]. The specific binding of Der f 2 [5–10] to IgE antibodies leads to atopic diseases. However, no information on the structure responsible for the allergic response is available. To clarify the structure-allergenicity relationship of Der f 2 protein, a system needs to be established with which the allergen can be prepared. Although the Der f 2 protein can be prepared from mite bodies [5, 7], it is still difficult to obtain a large amount of the allergen. To overcome this, we cloned the cDNA for Der f 2, which is one of the major allergens produced by the house dust mite *Dermatophagoides farinae* (Der f) [2–6] in *Escherichia coli*. We then established an expression system, in which the Der f 2 protein was produced as a fused protein with the N-terminal portion of

β -galactosidase of *E. coli*. The fused protein reacted with rabbit anti-Der f 2 antisera [10]. To analyze the antigenicity of Der f 2 in detail, however, nonfused Der f 2 protein is necessary. We thus constructed the plasmid that produced methionyl-Der f 2 in *E. coli*.

This paper describes the direct expression of the Der f 2 protein in *E. coli* and the ability of the recombinant Der f 2 (rDer f 2) to bind human IgE antibodies.

Materials and Methods

Strains and Plasmid

The *E. coli* strains used were: DH1 (F[–], recA1, supE44, gerA96, thi-1, hsdR17, relA1, λ), HB101 (F[–], hsdS20, recA13, ara-14, proA2, lacY1, galK2, psL20, xyl-5, mtl-1, supE44, l[–], mcrA⁺, mcrB[–]), JM105 [thi, rpsL, endA, sbcB15, hsdR4, Δ (lac-proAB), (F⁺, proAB, lacIQ, lacZ Δ M15, traD36)], JM109 [recA1, endA1, thi, hsdR17, supE44,

KARGER

E-Mail karger@karger.ch
Fax +41 61 306 12 34

© 1996 S. Karger AG, Basel
1018–2438/96/1094–0356 \$10.00/0

Correspondence to: Dr. Namiko Iwamoto
Central Research Laboratory
Asahi Breweries, Ltd.
2-13-1, Ohmori-kita, Ohta-ku
Tokyo 142 (Japan)

Received:
February 14, 1994
Accepted after revision:
October 23, 1995

relA1, λP^- , $\Delta(\text{lac-proAB})$, (F' , proAB, lacIQ Δ M15, traD36)], and MV1184 [D(srl-recA) 306::Tn10D (lac-pro)alaP $^-$, thi $^-$, rpsL $^-$, Φ 80dlacZ Δ M15 (F' , proAB, lacIQ, lacZ Δ M15, traD36)].

The plasmid pKK233-2 [11], which has a single *NcoI* restriction site (CCATGG) containing an ATG codon for translational initiation, preceded by a possible SD sequence for ribosome binding with a 7-bp space, the trc promoter and the rrnB transcriptional terminator upstream and downstream, respectively, were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). The plasmid pUC118 [12] was from Takara Shuzo (Kyoto, Japan).

Chemicals

All enzymes were purchased from Takara Shuzo. Sodium dodecylsulfate (SDS)-polyacrylamide gels were obtained from the TEF Corporation.

Antisera and Antibodies

Seven human sera with a radioallergosorbent score (RAST SCORE) of over 3 were obtained from mite-allergic patients. The sera were used for RAST enzyme immuno assay (EIA) [13, 14] and skin prick test [15], and pooled for Western blotting [16].

Construction of Expression Plasmid

The expression plasmid pFLU11 was constructed as follows. Plasmid pFL11 [10] was cleaved with *KpnI* to isolate a DNA fragment of a 0.5 kbp containing Der f2 cDNA. The resulting 0.5-kb *KpnI* fragment recovered by agarose gel electrophoresis was digested with *Sau3AI*, blunt-ended using the Klenow fragment, then digested with *NcoI*. The *Sau3AI* (filled)-*NcoI* fragment of 63 bp was prepared by polyacrylamide electrophoresis. The 0.5-kb *KpnI* fragment prepared in the same manner was digested with *HinfI*, blunted with the Klenow fragment, then digested with *NcoI*. The *NcoI*-*HinfI* (filled) fragment of 390bp which was recovered by agarose gel electrophoresis was ligated with the above 63-bp DNA fragment. The resultant plasmid named pFLK11 was further digested with *PvuI* plus *PvuII* and the larger portion of 3,376 bp was ligated with the *PvuI*-*PvuII* fragment of 1,438 bp of pUC118 containing the replication origin. The resulting designated plasmid pFLU11 has the replication origin of pUC118 instead of that of pKK233-2, which may efficiently express Der f2 protein due to its high copy number in *E. coli* cells. The nucleotide sequences of the portion encoding Der f2 cDNA in this plasmid were verified by dideoxynucleotide termination [17] with a DNA sequencer (Model 370A, Perkin-Elmer, USA) according to the manufacturer's instructions.

Purification of Recombinant Der f2

E. coli JM109 harboring pFLU11 was grown overnight in LB broth (10 g bactotrypton, 5 g bacto-yeast extract, 5 g NaCl/l, pH 7.4) supplemented with 50 μ g/ml ampicillin at 30°C. One milliliter of the culture was transferred into 100 ml of the same fresh LB medium and incubated under the same conditions. When the turbidity of the culture medium at 600 nm reached 0.5, isopropyl- β -D-thio-galactopyranoside was added to a final concentration of 0.1 mM and incubated for another 6 h. The cells harvested by centrifugation were washed with 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and phenylmethylsulfonyldifluoride (PMSF), and resuspended in 2 ml of the same buffer. DNaseI and sodium deoxycholate were added to a final concentration of 0.16 mg/ml and 20 mM, respectively; the mixture was incubated at 4°C for 15 min. The mixture was then subjected to freeze-thaw treatment, followed by sonication (30 s, 25 times) with an Olympus Bio-ruptor HP-200 (200 W, 200 kHz), and then the precipitates containing

inclusion bodies were collected by centrifugation at 7,000 g for 15 min at 4°C. The inclusion bodies were solubilized with 100 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF and 8 M urea at 10°C overnight, then dialyzed against 20 mM Tris-HCl (pH 8.5) for refolding. The dialysate was centrifuged at 70,000 g for 30 min at 4°C to remove insoluble materials.

The supernatant was applied onto an anion exchange column on an FPLC system (Hiload 26/10 Q-Sepharose, Pharmacia LKB Biotechnology) equilibrated with 20 mM Tris-HCl (pH 8.5) containing 0.01% NaN₃. Correctly refolded rDer f2 was eluted with 20 mM Tris-HCl (pH 8.5) containing 80 mM NaCl. The eluate was concentrated to 0.25 ml using a Centricon-10 (Amicon, Verly, USA), then applied to a gel filtration column (HR 10/30 Superdex 75, Pharmacia LKB Biotechnology) on an FPLC system, equilibrated with phosphate-buffered saline (pH 7.4) containing 0.01% NaN₃. A major peak was collected. The rDer f2 protein migrated as a homogeneous protein on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [18]. The N-terminal amino acids were determined with a gas phase protein sequencer (Model 473A, Perkin-Elmer, USA).

Immunoblotting

Der f2 samples were resolved by SDS-PAGE [17] and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, USA) for Western blotting [17] against antisera from mite-allergic patients as described above.

RAST-EIA

Direct RAST-EIA was performed using CNBr-activated filter paper disks (3 mm) and the Pharmacia RAST-EIA kit (Kabi Pharmacia Diagnostics, Uppsala, Sweden). The antigens were diluted with 0.1 M borate to 0.07, 0.7, 7.0, 70, or 700 ng/ml for rDer f2, and 0.15, 1.5, 15, 150, or 1,500 ng/ml for nDer f2. The disks were soaked in these solutions overnight at room temperature. The antigen-coupled disk was washed with 0.5 ml of 0.1 M NaHCO₃, and blocked with 0.2 ml of 0.1 M monoethanolamine (pH 9.0) for 3 h at room temperature. The disk was then washed once with 0.5 ml of 0.1 M NaHCO₃ and 3 times with 0.5 ml of 0.1 M acetate buffer (pH 4.0). Serum (50 μ l) containing 15–340 RPU/ml anti-Der f IgE antibodies from 7 patients was added to the disks and incubated for 4 h at 37°C. The amount of IgE antibodies binding to the allergen was determined with a RAST-EIA kit according to the manufacturer's instructions. Standard curves for the interaction between native Der f2 (nDer f2) and IgE antibodies were obtained as described [13].

Results

Production and Purification of rDer f2

To produce a methionyl-Der f2 protein, we constructed the pFLU11 (fig. 1). This plasmid harbored Der f2 cDNA controlled by the trc promoter and a pUC118 replication origin. Agarose gel electrophoresis after alkaline plasmid extraction [19] revealed that the copy number of this plasmid in one *E. coli* (JM105) cell was higher than that of pFLK11 having pKK233-2 replication origin [data not shown]. We also examined rDer f2 production in various *E. coli* strains (DH1, HB101, JM105, JM109, and MV1184). Since the

Fig. 1. Construction of pFLU11 to produce Der f2 protein. Der f2 cDNA encodes 129 residues of mature Der f2 with a hydrophobic leader sequence, which was removed by cloning as described in Materials and Methods. This product started with an Asp residue at nucleotide 40. Sites for *Kpn*I, *Nco*I at the 5' end and *Kpn*I at the 3' end were derived from linkers [see 10]. An ATG codon was located within a unique *Nco*I site (CCATGG) at the 5' end. Ptrc = trc promoter, identical to tac 12 promoter [11] with the exception of a 1-bp change; 5S = 5S ribosomal RNA gene; T1T2 = transcription terminator; Apr = ampicillin resistance gene.

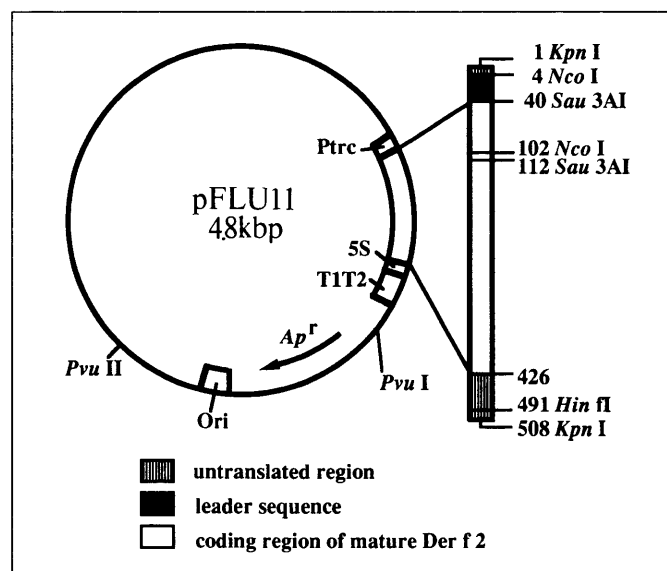
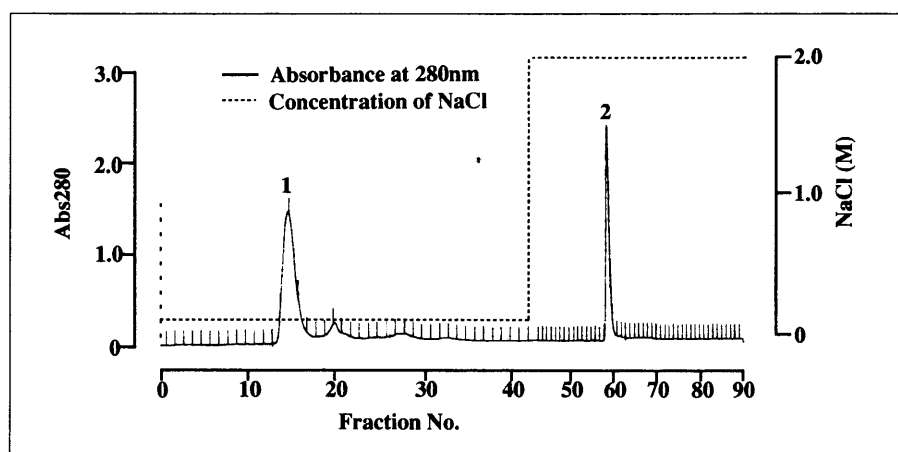


Fig. 2. Elution profile of rDer f2 from Q-Sepharose. Peak 1 contained rDer f2.



JM109 transformant produced the most rDer f2, we selected this strain as the host [data not shown].

The rDer f2 produced in JM109 was an insoluble material probably forming inclusion bodies. To obtain the correctly refolded molecule, denaturation and renaturation were required. Like the Der f2 proteins fused with β -galactosidase, a large portion of insoluble rDer f2 protein was refolded by exposure to 8 M urea and the successive dialysis to remove the denaturant. After refolding, rDer f2 was purified by anion exchange chromatography (fig. 2) followed by gel filtration chromatography (fig. 3). As shown in table 1, about 10 mg of rDer f2 protein were purified from 1 liter of culture with a yield of 35%. The purified rDer f2 migrated as a single band with a molecular weight 14 kDa on SDS-PAGE (fig. 4B), which coincided well with the molecular mass deduced from the nucleotide sequence. And this mate-

rial also had the same degree of reactivity to nDer f2 on Western blotting using IgE from patient sera (fig. 4A). We determined the N-terminal sequence of 25 amino acid residues and found that it was identical to that deduced from the nucleotide sequence of Der f2 cDNA, except that a methionine residue remained uncleaved at the N-terminus [20].

IgE-Binding Ability of rDer f2

The IgE-binding ability of rDer f2 was analyzed by direct RAST-EIA (fig. 5). When IgE-binding ability of rDer f2 was compared with that of nDer f2, there were no differences throughout the range of Der f2 concentrations studied. Figure 6 shows the RAST-EIA with 50 ng/ml of rDer f2 and nDer f2 against 7 patient sera. The IgE-binding abilities of rDer f2 and nDer f2 were similar against all patient sera.

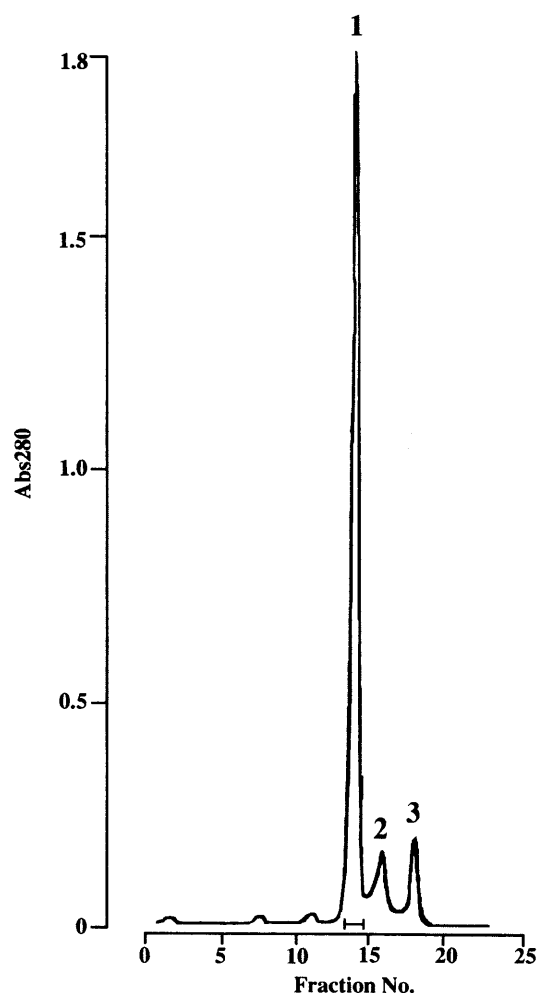


Fig. 3. Elution profile of rDer f2 from Superdex 75. Peak 1 was purified rDer f2.

Table 1. Summary of rDer f2 purifications

Purification step	Total protein ¹ mg	rDer f2		Yield ⁴ %
		mg ²	purity ³	
Whole cell	356	31	8.7	100
Sonication	311	27	8.9	87
×12,000 g	51	20	39	65
×70,000 g	40	18	45	58
Q-Sepharose	12	11	95	35
Superdex 75	11	11	~100	35

¹ Estimated by the Lowry method with BSA as a standard.

² Calculated by the densitometry of Coomassie brilliant blue staining of separated Der f2 protein on SDS-PAGE.

³ Der f2(mg)/total protein (mg) × 100.

⁴ Recovery from total production of Der f2 protein.

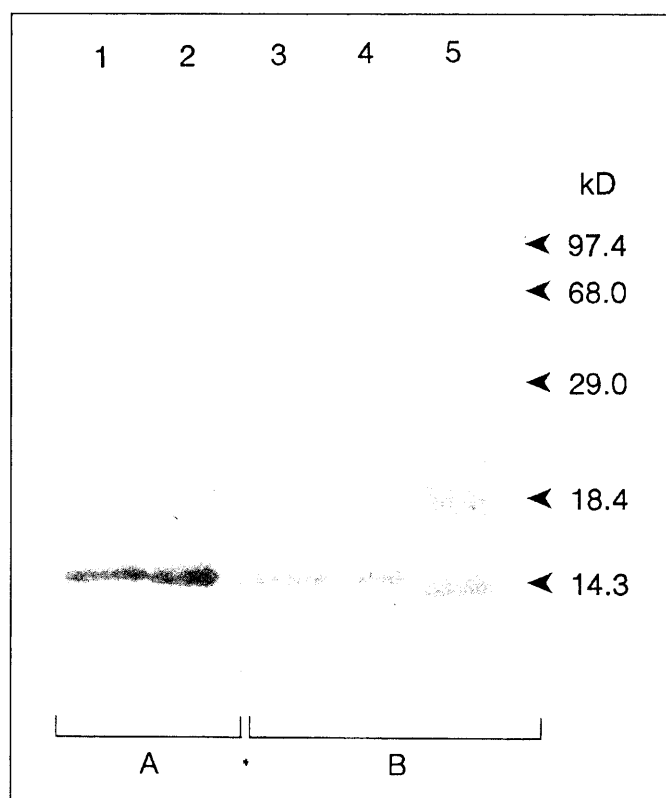


Fig. 4. SDS-PAGE of rDer f2 and nDer f2. Immunoblotting with IgE in patient sera (**A**) and Coomassie brilliant blue staining (**B**) of the purified fractions. Lanes 1 and 3 represent nDer f2, lanes 2 and 4 rDer f2 and lane 5 molecular weight marker.

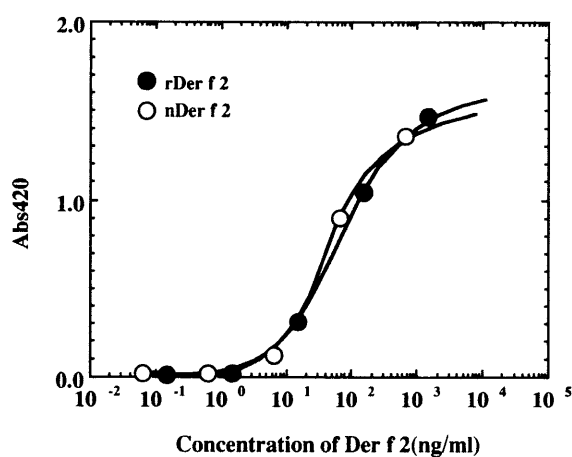
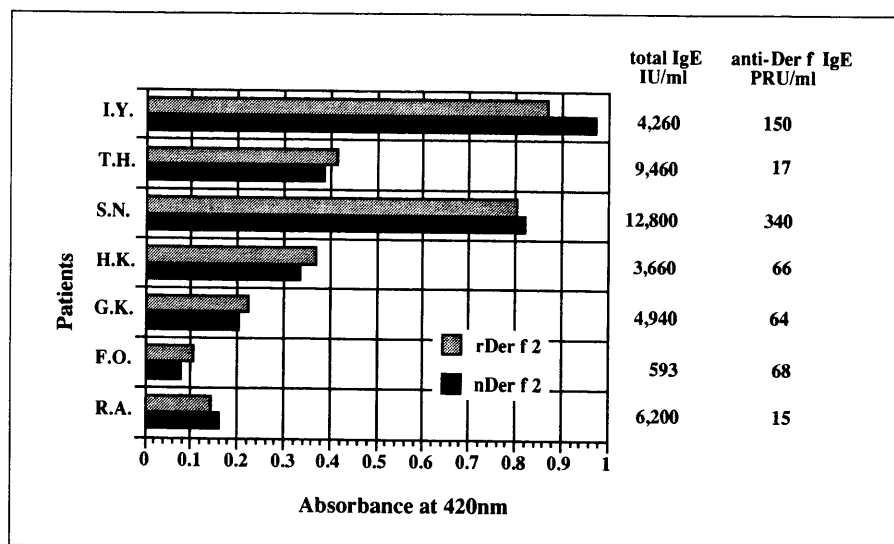


Fig. 5. A comparison of the allergenicity of rDer f2 with nDer f2. The allergenicity of both was measured with RAST-EIA using 4-fold dilutions of serum from an atopic patient (anti-Der f1 IgE, 340 PRU/ml, one of the patients shown in fig. 6) and coupled disks with various antigen concentrations (nDer f2, 0.07, 0.7, 7, 70, 700 ng/ml. rDer f2, 0.15, 1.5, 15, 150, 1,500 ng/ml).

Fig. 6. Comparative reactivity of IgE in serum of allergenic patients with rDer f 2 and nDer f 2 by RAST-EIA. Results are shown at a Der f 2 concentration of 50 ng/ml.



We examined the allergenic activity of nDer f 2 and rDer f 2 by skin prick test. Both antigens caused positive reactions in the 7 patients at concentrations of less than 3 $\mu\text{g/ml}$, but not in the healthy volunteers even at concentrations of 10 $\mu\text{g/ml}$. These results indicated that rDer f 2 has a conformation identical to that of nDer f 2.

Discussion

A high level (10 mg of 1L culture) of nonfused-type rDer f 2 protein was produced using an expression system with a trc promoter. We found that nDer f 2 has three disulfide bonds at Cys8-Cys119, Cys21-Cys27 and Cys73-Cys78, and when nDer f 2 is reduced, it loses the ability to bind IgE antibodies, and becomes unstable [13]. These observations

suggest that the disulfide bonds stabilize the structure of the Der f 2 molecule, which is responsible for the allergenicity of this protein. The methionyl-rDer f 2 prepared here had an antigenicity that was almost identical to that of nDer f 2, suggesting that the rDer f 2 possessed the same conformation as the nDer f 2 protein.

The efficient product system for rDer f 2 described here may be useful not only to elucidate the structure-antigenicity relationship of Der f 2, but also for the practical use of this recombinant protein as a therapeutic reagent for diseases mediated by Der f 2, because hyposensitization with the mite allergens is effective against these diseases.

Determination of epitopes of Der f 2 for IgE as well as its three-dimensional structure is now in progress. This could elucidate the mechanism of atopic diseases via Der f 2.

References

- Hart BJ, Fain A: Morphological and biological studies of medically important house dust mite. *Acarologia* 1988;3:285-295.
- Voorhorst R, Spijkstra-Boezeman MIA, Spijkstra FTM: Is a mite (*Dermatophagoides* sp.) the producer of the house dust allergen? *Allergen Asthma* 1964;10:329.
- Platts-Mills TAE, de Weck AL: Dust mite allergens and asthma - A worldwide problem. *J Allergy Clin Immunol* 1989;83:416-427.
- Beck H-I, Korsgaard J: Atopic dermatitis and house dust mites. *Br J Dermatol*. 1989;120:245-251.
- Yasueda H, Mita H, Yui Y, Shida T: Isolation and characterization of two allergens from *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* 1986;81:214-223.
- Miyamoto T, Oshima S, Ishizaki T, Sato S: Allergenic identity between the common floor mite (*Dermatophagoides farinae* Hughes, 1961) and house dust as a causative antigen in bronchial asthma. *J Allergy* 1968;42:14-28.
- Platts-Mills TAE, Chapman MD: Dust mite: Immunology, allergic diseases, and environmental control. *J Allergy Clin Immunol* 1987; 80:755-775.
- Trudinger M, Chua KY, Thomas WR: cDNA encoding the major mite allergen Der f 2 in *Escherichia coli*. *Clin Exp Allergy* 1991;21:33-37.
- Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TAE: Antigenic and structural analysis of group 2 allergens (Der f 2 and Der p 2) from house dust mites (*Dermatophagoides* spp.). *J Allergy Clin Immunol* 1989;83:1055-1067.

- 10 Yuuki T, Okumura Y, Ando T, Yamakawa H, Suko M, Haida M, Okudaira H: Cloning and expression of cDNA coding for the major house dust mite allergen Der f2 in *Escherichia coli*. *Agric Biol Chem* 1991;55:1233-1238.
- 11 Amman E, Brosius J, Ptashne M: Vector bearing a hybride trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene* 1983;25:167-178.
- 12 Vieira J, Messing J: Production of single-stranded DNA; in Wu R (ed): *Method in Enzymology*. New York, Academic Press, 1987, vol 153, pp 3-11.
- 13 Nishiyama C, Yuuki T, Takai T, Okumura Y, Okudaira H: Determination of three disulfide bonds in a major house dust mite allergen, Der f2. *Int Arch Allergy Appl Immunol* 1993;101:159-166.
- 14 Aalberse RC, Koshte V, Clemmens JGJ: Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol* 1981;68:356-364.
- 15 Van der Zee JS, Van der Swieten P, Jensen HM, Aalberse RC: Skin tests and histamine release with P1-deleted *Dermatophagoides pteronyssinus* body extracts and purified P1. *J Allergy Clin Immunol* 1988;81:884-896.
- 16 Towbin H, Staehelin T, Gordon V: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350-4354.
- 17 Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977;74:5463-5467.
- 18 Laemmle UK: Cleavage of structural proteins during assembly of the head of T4 bacteriophage. *Nature* 1970;227:680-681.
- 19 Maniatis T, Fritsch EF, Sambrook J: *Molecular Cloning. A Laboratory Manual*. New York, Cold Spring Harbor Laboratory, 1982.
- 20 Hiral PH, Schumitter JM, Dessen P, Fayat G, Blanquet S: Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci U S A* 1989;86:8247-8251.