

Production of Recombinant Mite Allergen Der fI in Insect Cells and Characterization of Products——Removal of Pro-sequence Is Essential to IgE-Binding Activity——

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Der fI is a major mite proteinaceous allergen found in house dust. We produced recombinant Der fI (reDer fI) in insect cells using a baculovirus expression system. Based on the molecular mass and N-terminal amino acid (aa) sequence analysis, reDer fI was found to be comprised of a mixture of two proteins, each of which includes a pro-sequence of different length. The reDer fI had IgE-binding activity at only 20% of that of native Der fI. The removal of the pro-sequence in an acidic solution drastically increased IgE-binding activity to almost the same as that of native Der fI, showing that the presence of the pro-sequence is inhibitory to the IgE-binding activity of Der fI.

Key words: IgE-binding protein; baculovirus expression system; secretion; precursor; processing

The binding of IgE and allergens induces allergic diseases such as atopic dermatitis and atopic asthma, and patients who suffer from allergic disease are often treated by immunotherapy using the allergen. However, a sufficient amount of the native allergen may not be available for the treatment. Therefore, we need artificial production of allergens that have IgE-binding activity.

The house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* produce potent proteinaceous allergens, Der f and Der p, respectively, which include the group I allergens (Der fI and Der pI) and the group II allergens (Der fII and Der pII). These allergens have been proved to react with the majority of patients allergic to mites.¹⁾ Native Der fI has cysteine protease activity.²⁾ cDNAs coding for these mite allergens have already been cloned.^{3–6)} The genes encoding Der f were found to be highly homologous to the corresponding Der p-encoding genes.^{3,7)} Group II allergens expressed in *E. coli* had the same IgE-binding activity as native proteins,^{6,8)} but group I allergens expressed in *E. coli* had little IgE-binding activity.⁹⁾ The recombinant Der pI (reDer pI) expressed in yeast was found to bind to IgE,¹⁰⁾ although quantitative data on its binding activity to IgE has not been reported. Production of reDer pI in yeast seems less satisfactory, because the product was mostly insoluble and required laborious purification processes including solubilization, dialysis, and affinity chromatography with an immobilized monoclonal antibody. In addition, the final yield in yeast culture was only 1 mg/liter.

Mammalian cells have superior ability in producing some type of recombinant proteins that have the same function as the native proteins. However, the production by mammalian cells is costly and limited in amount. In contrast to mammalian systems, the baculovirus expression system

has become increasingly popular in the production of recombinant proteins. A variety of the recombinant proteins expressed in insect cells have the same functions as the native proteins, and the amount of products are usually greater than those by mammalian cells.

We report the production of reDer fI in insect cells with a baculovirus expression system. We found the IgE-binding activity of reDer fI required activation by removal of the pro-sequence from reDer fI.

Materials and Methods

Construction of recombinant virus. The cDNA coding for entire Der fI (Der fI, 1.1-kb *Bam*HI fragment) was cloned by the method of described previously,⁵⁾ using rabbit anti-Der fI anti-serum as the primary antibody. The *Bam*HI fragment was ligated into the *Bam*HI site of the transfer vector pVL941 (pVL941-Der fI). pVL941-Der fI and *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (Invitrogen) were cotransfected into *Spodoptera frugiperda* Sf9 cells (Invitrogen) using Lipofectin (Gibco-BRL) according to the manufacturer's instruction. The recombinant virus was screened through the visibility of occlusion-deficient plaques followed by three rounds of individual plaque purification.¹¹⁾ The production of reDer fI, in the culture medium and cells, was monitored by Western blot analysis.

Production of reDer fI in insect cells. Sf9 cells were cultured at 27 °C in TC-100 (Gibco-BRL) with 10% (v/v) fetal bovine serum (FBS) in monolayer or suspension culture. Cells were routinely passed every 3 days with gentamicin (50 µg/ml). Exponentially growing Sf9 cells were infected with the recombinant virus at a multiplicity of 5 plaque-forming units/cell. The virus was allowed to adsorb for 1 h at room temperature with gentle rocking. The inoculum was removed and replaced with TC-100 with 10% FBS and incubated at 27 °C. After 24 h, the culture medium was changed to TC-100 without FBS so as to prevent potential contamination with high molecular mass proteins from FBS, and incubated at 27 °C.

Sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis (PAGE) and Western blot analysis. The culture medium was centrifuged at 1000 × g at 4 °C for 10 min. The resultant precipitates that were resuspended in

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Abbreviations: A, absorbance (1 cm); aa, amino acid(s); AcNPV, *Autographa californica* nuclear polyhedrosis virus; FBS, fetal bovine serum; kDa, kilodalton; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; re-, recombinant; SDS, sodium dodecyl sulfate.

phosphate-buffered saline (PBS), and the supernatant were separated by SDS PAGE (gel concentration was 12.5%). The gel was stained with Page-Blue 83 (Fluka). Western blot analysis was done as described elsewhere using rabbit anti-Der fI anti-serum as the primary antibody and peroxidase-conjugated donkey anti-rabbit $F(ab')^2$ (Amersham) as the secondary antibody.

Purification of native Der fI and reDer fI. Native Der fI was purified from lyophilized mite *Dermatophagoides farinae* by the method of Yasueda *et al.*¹²⁾ reDer fI was purified from the culture medium at 72 h after infection. The culture medium was centrifuged at $1000 \times g$ at 4 °C for 10 min. The supernatant was passed through a 0.45- μ m ultrafilter and gel-filtered using a Sephadex G-50 (Pharmacia) column equilibrated with 20 mM Tris-HCl (pH 8.0). The passed through fraction was put onto a DEAE-Sephacel (Pharmacia) column that was equilibrated with the same buffer. reDer fI was eluted with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. The reDer fI-containing fractions were collected and were gel-filtered using a Sephadex G-50 column to change buffer to that 10 mM phosphate buffer (pH 7.2) and passed through a hydroxyapatite (Bio-rad) column that was equilibrated with the same phosphate buffer (pH 7.2). Purity was monitored by SDS PAGE.

N-Terminal amino acid sequence analysis. reDer fI was separated with SDS PAGE, transferred to a polyvinylidene difluoride (PVDF)-coated membrane, and stained with Page-Blue 83. The stained band was cut off, and its N-terminal amino acid sequence was analyzed using an automated Edman degradation protein sequencer Model 470A (Applied Biosystems). Protein was measured by the protein assay reagent (Pierce) using bovine serum albumin as a standard.

Measurement of IgE-binding activity. Each reDer fI and native Der fI was coupled to cyanogen bromide activated paper discs. Each disc was washed by a 0.1 M NaHCO_3 solution and incubated for 3 h at room temperature in a 1 M 2-ethanolamine solution (pH 9.0). The discs were washed successively by 0.1 M NaHCO_3 , 0.1 M acetate (pH 4.0), and PBS containing 0.1% Tween-20. The washed discs were shaken for 3 h at 37 °C with human anti-mite IgE serum, washed by PBS containing 0.1% Tween-20, and incubated for 12 h at room temperature with β -galactosidase-conjugated anti-human IgE antibody (Pharmacia). The discs were washed by PBS containing 0.1% Tween-20, and reacted with 2-nitrophenyl- β -D-galactopyranoside. One-tenth M NaHCO_3 was added to the reaction mixture, and absorbance (A) at 420 nm was measured.

Acid treatment of reDer fI. One ml reDer fI (0.2 mg/ml) was gel-filtered using a Sephadex G-50 column equilibrated with 0.2 M acetate at appropriate pH (4.0–4.5) and incubated at 4 °C for 2 days. After incubation, the solution of reDer fI was gel-filtered using a Sephadex G-50 column by PBS to neutralize it.

Results

Production of reDer fI

Sf9 cells were infected with the recombinant baculovirus containing cDNA coding for entire Der fI. Culture media (lanes 1–4) and cell lysates (lanes 5–8) were analyzed for production of reDer fI by Western blot analysis using anti-Der fI rabbit anti-serum (Fig. 1). Proteins reactive to the specific antiserum appeared in both Sf9 cells and culture medium in a time-dependent manner, indicating the production of reDer fI by Sf9 cells. A protein of 33 kDa gave clear bands from the first of the production but another protein of about 35 kDa, forming faint bands, accompanied it. At 48 h, reDer fI (33-kDa protein and 35-kDa protein) began to be detected (lanes 2 and 6). At 72 h, about a half of reDer fI was present in the culture medium (lane 3), and the other half of reDer fI was associated with Sf9 cells (lane 7). Decomposition products were already evident at 72 h, of which the smallest detectable size was 28 kDa. Because we could not purify only the 28-kDa protein, we did not refer the characterization of the protein in this report. At 96 h, a significant amount of the

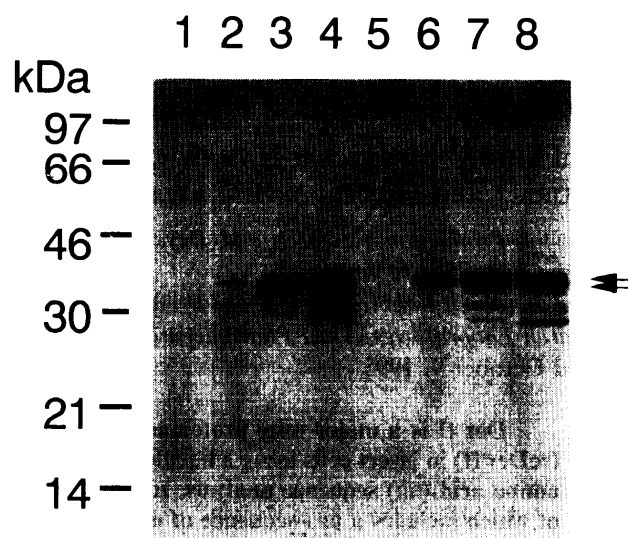


Fig. 1. Production of reDer fI in Culture Media and Cell Lysates.

The culture media and the cell lysates at 24 h (lanes 1 and 5), 48 h (lanes 2 and 6), 72 h (lanes 3 and 7), and 96 h (lanes 4 and 8) after infection were analyzed by Western blot analysis. Lanes: 1–4, culture media of the infected cells; 5–8, infected cell lysate. Arrows show 33 kDa and 35 kDa. The numbers on the left mark the positions of the reference proteins (kDa).

immunoreactive product had decomposed to form smaller proteins (lanes 4 and 8).

Purification of reDer fI

We attempted to purify reDer fI from the culture medium at 72 h after infection. There were some proteins in the culture medium. But main ones were reDer fI and the high molecular weight proteins that might be derived from FBS. The culture medium was cleared by centrifugation and put through two steps of column chromatography, DEAE-Sephacel and hydroxyapatite. After DEAE-Sephacel treatment, there were still obstructive proteins in the fractions containing reDer fI. Fortunately these proteins were adsorbed to the hydroxyapatite but reDer fI wasn't. The purified reDer fI gave two bands of 33 kDa and 35 kDa by SDS-PAGE (Fig. 2, lane 2), and was almost free from other contaminating proteins, except for the one of 28 kDa which gave a subtle band. Overall yield of the purified reDer fI was 50 mg from 1 liter of culture medium.

Characterization of purified reDer fI

The purified reDer fI was comprised of 33-kDa and 35-kDa proteins (Fig. 2, lane 2). The molecular mass of 35-kDa protein was similar to that of a polypeptide deduced from Der fI cDNA, which would be 35 kDa after the removal of an N-terminal signal sequence for secretion. Both reDer fI proteins are larger in molecular mass than native Der fI, which was 28 kDa (Fig. 2, lane 1).

N-terminal amino acid sequence analysis showed that the purified reDer fI was comprised of two pro-proteins that differed in the length of the N-terminal pro-sequence (Fig. 3). One, which constituted about 20% of the mixture, had a pro-sequence of 80 amino acids starting from Arg-[–80]. The other, which constituted about 80%, had a pro-sequence of 59 amino acids starting from Val-[–59]. These two types of proteins seems to correspond to 33-kDa and 35-kDa proteins appearing in SDS-PAGE analysis (Fig. 2, lane 2).

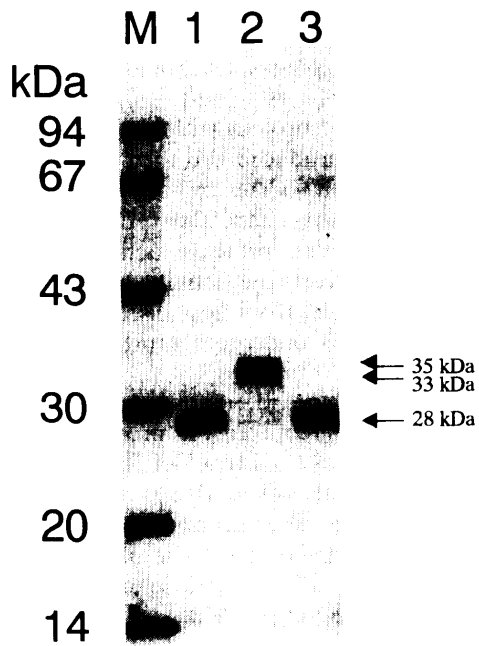


Fig. 2. Purified Der fI Proteins and Their Acid-treated Derivative.

Native Der fI, the purified reDer fI and the purified acid treated reDer fI were analyzed by SDS PAGE, respectively, and stained with Page-Blue 83. Lanes: M, molecular mass standards; 1, native Der fI; 2, purified reDer fI; 3, acid treated reDer fI. The upper two arrows show 33-kDa and 35-kDa protein for the non-processed reDer fI, and a lower arrow shows 28-kDa processed reDer fI and native Der fI. The numbers on the left mark the positions of the reference proteins (kDa).

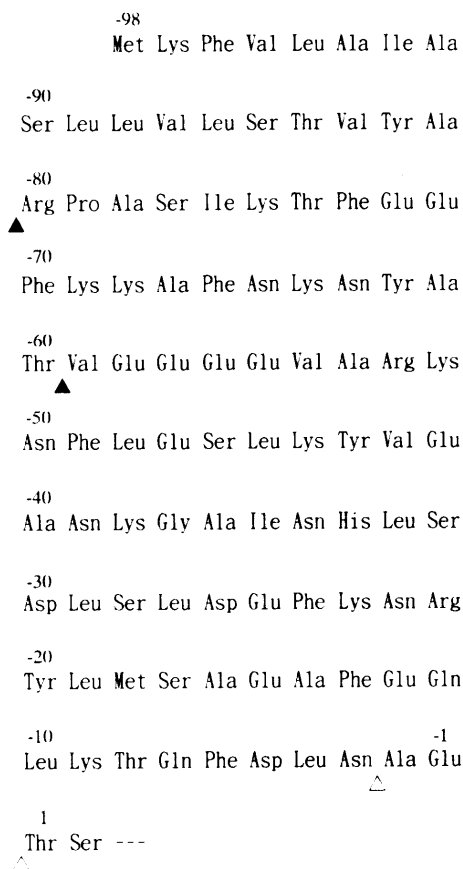


Fig. 3. N-Terminal Amino Acid Sequence of reDer fI.

N-Terminal amino acid sequences of the purified reDer fI and the acid treated reDer fI were analyzed using an automatic protein sequencer (see Materials and Methods). Amino acids are negatively numbered from the N-terminal of native Der fI. (▲) shows the cleavage sites of the purified reDer fI. (△) shows the cleavage sites by the acid treatment.

The IgE-binding activity of reDer fI, which was a mixture of the two types of pro-proteins, was measured using human anti-mite IgE serum (Fig. 4). It turned out that the purified reDer fI had binding activity, although it was only 20% of native Der fI. The baculovirus expression system advantageously gave soluble reDer fI, which facilitated the subsequent purification. We did experiments to characterize the reDer fI, regarding the relation between the pro-sequence and low IgE-binding activity of reDer fI.

Effects of acid treatment of reDer fI

Treatment of reDer fI with an acidic buffer gave smaller degradation products. reDer fI was incubated in an acidic

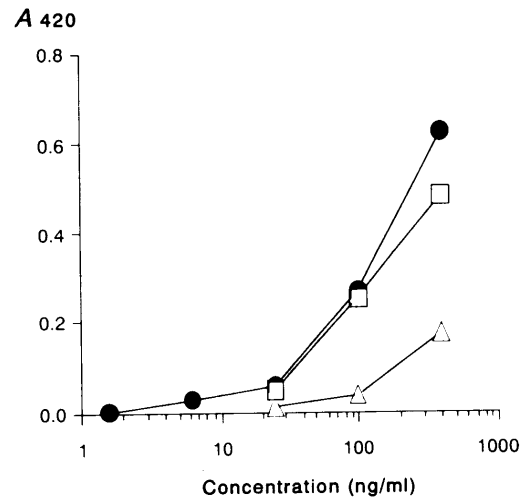


Fig. 4. IgE-binding Activity of Native Der fI and reDer fI.

IgE-binding activity of each Der fI was analyzed (see Materials and Methods). (●) shows IgE-binding activity of native Der fI. (△) shows IgE-binding activity of the purified reDer fI. (□) shows IgE-binding activity of purified 28-kDa protein(s).

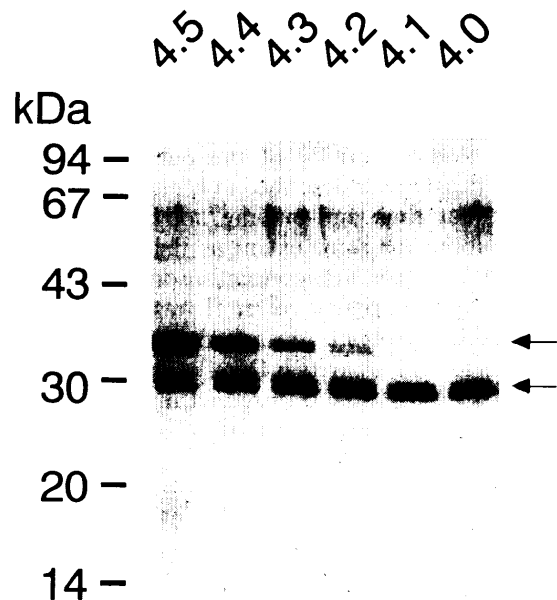


Fig. 5. Effects of Acid Treatment on reDer fI.

Purified reDer fI was treated with 0.2M acetate buffer varying pH 4.5 4.0 and incubated at 4 °C for 2 days. Proteins in each solution are separated by SDS PAGE and stained with Page-Blue 83. Lanes: 4.5 4.0, pH values in acidic conditions. The upper arrow shows 33-kDa and 35-kDa protein for the non-processed reDer fI, and a lower arrow shows 28-kDa processed reDer fI. The numbers on the left mark the positions of the reference proteins (kDa).

solution of pH 4.0 to 4.5 for 2 days at 4°C, and then neutralized as described in Materials and Methods. The resultant reDer fI was analyzed by SDS-PAGE. Figure 5 shows that the sample treated at pH 4.5 gave three major bands of 28 kDa, 33 kDa, and 35 kDa. In contrast, the samples treated at pH 4.1 and 4.0 gave two bands of 28 kDa and 30 kDa. These results suggest that the proteins of 33 kDa and 35 kDa were degraded to the proteins of 28 kDa and 30 kDa as the pH decreased from 4.5 to 4.0. Although the ratio of 28-kDa and 30-kDa proteins were variable, depending on experiments, the 28-kDa proteins was always predominant. By incubating reDer fI with a solution of pH 4.5 for 30 days at 4°C, SDS-PAGE gave only one band of 28 kDa (data not shown). When treated with pH lower than 3, reDer fI proteins and 28-kDa protein were not detected by SDS-PAGE. The temperature was kept constant at 4°C over 2 days since the IgE-binding activity of native Der fI was sensitive to heat.

Characteristics of the 28-kDa degradation product

To examine the properties of the 28-kDa degraded product by the acid treatment as pH 4.0 for 2 days at 4°C, we further purified it by gel filtration as described in Materials and Methods. The final, overall yield was 20 mg from 1 liter of culture medium.

Figure 2 shows the results of SDS-PAGE analysis of the purified 28-kDa protein (lane 3). A somewhat broad, but only one band of 28-kDa was seen, and its mobility was almost equal to that of native Der fI (Fig. 2, lane 1). N-Terminal amino acid sequence analysis showed that the purified 28-kDa protein was actually a mixture of two proteins of about an equal amount (Fig. 3). They were different in N-terminal amino acids; one started at Ala-[2], and the other started at Thr-[1]. The N-terminal of the latter protein was identical to that of native Der fI. From these results, the purified 28-kDa protein(s) were expected to be almost identical in structure to the native Der fI, and native Der fI itself should be a processing product in which the N-terminal pro-sequence is proteolytically removed.

The IgE-binding activity of the purified 28-kDa protein(s) was measured as described in Material and Methods. We found that their IgE-binding activity drastically increased to about the same extent as that of native Der fI (Fig. 4). For example, at a concentration of 100 ng/ml, the absorbance by the original reDer fI was 0.04, but that by purified 28-kDa protein(s) was 0.25 (6-folds), which was comparable to 0.27 by native Der fI. This result indicates that the 28-kDa degradation product matches structurally and functionally the native Der fI.

Discussion

We succeeded in producing reDer fI in large quantity by using a baculovirus expression system, and found conditions to process the products to provide an active reDer fI preparation.

The purified reDer fI was comprised of two types of pro-proteins: one has 59 additional amino acids and the other has 80 additional amino acids, which correspond to 33-kDa and 35-kDa proteins in reDer fI preparation, respectively. The structure of 35-kDa protein is consistent with the pro-protein proposed by Dilworth *et al.*³⁾ Based

on the analysis of that of the cDNA sequence, they supposed that the N-terminal 18 amino acids (Met-[-98]-Ala-[-81]) constitute a pre-sequence and the next 80 amino acids sequence (Arg-[-80]-Glu-[-1]) is a pro-sequence. We propose that the protein with a pro-sequence of full length was initially produced and then it was converted to the other form with a shorter pro-sequence by proteolytic cleavage. In this conversion, the reDer fI itself, which reportedly has cysteine protease activity,²⁾ might have played a role. The cleavage activity of the pro-sequence might be also provided from host insect cells, wherein the recombinant proteins undergoes proteolysis by proteases released by cell lysis.¹³⁾

We found that the acid treatment of the reDer fI at pH 4.0 substantially removed the pro-sequence to give a protein quasi-identical to native Der fI and a protein with two additional amino acids. It has been reported that pro-sequences are cut off under acidic conditions,^{14,15)} and that the mutation of the active site of the protease itself prevents processing of the pro-sequence under acidic conditions.¹⁶⁾ These suggest that reDer fI itself on its degraded products acts as a processing enzyme under acidic conditions to remove the pro-sequence. However, it is also conceivable that contaminating proteases removed the pro-sequence of reDer fI. The formation of the protein with the two additional amino acids, which has not been observed *in vivo*, suggests a different mechanism in pro-sequence removal between *in vitro* and the as yet unknown maturation conditions *in vivo*.

Our data indicated that the pro-sequence of reDer fI was inhibitory in the access of IgE to reDer fI. There could be two different mechanisms in this inhibitory effect. In one mechanism, the pro-sequence structurally blocks IgE to bind the antigenic determinant of reDer fI. In the other mechanism, the pro-sequence induces changes in three-dimensional conformation of reDer fI to decrease the affinity of IgE to reDer fI. This type of conformational change was reported in porcine pepsinogen, of which the pro-sequence induces the changes of three-dimensional configuration.¹⁷⁾ The binding of the allergen to IgE probably needs not only the primary sequence of the antigenic determinant but also the correct three-dimensional conformation.

The IgE-binding activity of 28-kDa proteins are about the same as that of native Der fI, suggesting that the two proteins different in two amino acids at N-terminal have similar IgE-binding activity. The processed reDer fI thus can be widely applied in the medical field. In combination with the development of reDer fII with equal IgE-binding activity to native Der fII,⁸⁾ a sufficient amount of major mite allergens for medical treatment and diagnosis of allergic patients is available.

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