

Production of Enzymatically and Immunologically Active Der f 1 in *Escherichia coli*

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Key Words

Der f 1 · Recombinant Der f 1 · Allergen · Protease ·
Escherichia coli

Abstract

Der f 1 is a major house dust mite allergen belonging to the cysteine protease family. Because of the great demand for clinical and research use of this allergen, much effort to establish an efficient method of preparing purified Der f 1 has been made. We constructed an isopropyl- β -D-thiogalactopyranoside-inducible expression plasmid to produce the pro-form of Der f 1 in *Escherichia coli*. The recombinant product was accumulated as insoluble inclusion bodies in cells. The solubilized inclusion bodies were found to be successfully renatured by two-step gel filtration chromatography. About 70 mg of pro Der f 1 were properly refolded by this method from 1 liter of culture. Acid treatment of the renatured pro Der f 1 resulted in the autocatalytic removal of the pro-sequence. The obtained mature form of Der f 1 bound IgE in patient sera and induced the release of histamine from peripheral blood leukocytes equally to native Der f 1. Furthermore, mature Der f 1 obtained by this method had identical protease activity with native Der f 1. We also discussed the contribution of the pro-sequence and the sugar chain of Der f 1 to its antigenic

and enzymatic activity. This is the first report to produce an active mature form of recombinant Der f 1 in *E. coli*.

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Introduction

The house dust mites, *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* (Der p), produce proteins inducing allergy. Group 1 (Der f 1 from *D. farinae* and Der p 1 from *D. pteronyssinus*) and group 2 (Der f 2 and Der p 2) allergens from these mites are reported to react with the majority of patients suffering from atopic dermatitis and atopic asthma [1].

Group 1 allergens are mainly contained in the feces of mites. They belong to the cysteine protease family [2] and are thought to function as a digestive enzyme in the intestine of mites. cDNA clones encoding Der f 1 and Der p 1 have been already isolated [3, 4] and were found to be highly conserved. The cDNA sequence for Der f 1 consists of three regions encoding pre (a signal sequence of 18 amino acids), pro (80 amino acids) and mature (223 amino acids) peptides. Native group 1 allergen in mite feces is a mature form.

The schematic protein structure of the Der f 1 molecule is given in figure 1. Seven cysteine residues are

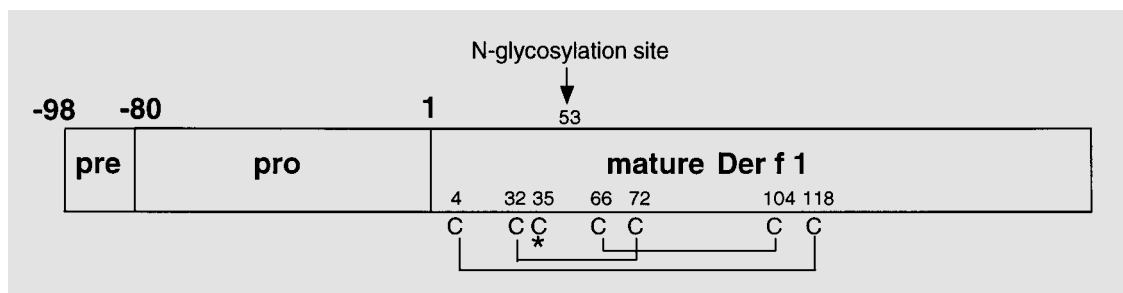


Fig. 1. Schematic protein structure of the Der f 1 molecule. The Der f 1 molecule is firstly synthesized as prepro-form in vivo. The numbers are those of amino acid residues counting from the first residue of the mature protein.

present in a functional enzyme of the mature form. Cys³⁵ (indicated by an asterisk in fig. 1) is the active site of the enzyme, and the other residues are predicted to form three disulfide bonds [5, 6]. The mature form of Der f 1 has a potential N-glycosylation site at Asn⁵³.

There is a great demand of group 1 allergens for research use. In addition, a large amount of highly purified allergens are needed for allergen-specific immunotherapy whose effect has been proved for this decade [7]. Much effort has been made to replace extracts by purified allergens for diagnosis and treatment. A procedure for purifying group 1 allergen from mite feces has been established [8]. However, it is too complex and inefficient for industrial application. Although affinity chromatography using monoclonal antibodies can save several purification steps, it still requires preparation of the extract from mite feces. To overcome the poor availability of purified allergen, the production of recombinant group 1 allergen has been attempted. Recombinant Der f 1 (rDer f 1) was successfully produced in a Baculovirus expression system [9, 10]. However, in addition to the high costs of the medium, the product had a sugar chain different from the native one. It may acquire antigenicity due to this different sugar chain. Recently, Yasuhara et al. succeeded in a more efficient expression of rDer f 1 in methylotrophic yeast, *Pichia pastoris* [pers. commun]. The product displayed much higher molecular weight than the native Der f 1 (nDer f 1) and was shown to be highly glycosylated. The disruption of the N-glycosylation site enabled it to be generated as a protein without a sugar chain, but resulted in a change of an amino acid residue. *Escherichia coli*, which has no ability to add sugar chains, grows fast in inexpensive medium and is easily handled. It is therefore suitable as a host to produce rDer f 1 industrially. However, there is no example to express the active mature form of rDer f 1. Here we established a method to produce

the active form of rDer f 1 in *E. coli*. The product had the same amino acid sequence as nDer f 1 and no sugar chain. rDer f 1 prepared by this method had enzymatic and antigenic properties identical to nDer f 1.

Materials and Methods

Native Der f 1

nDer f 1 purified from mite feces was kindly provided by Torii Pharmaceuticals (Japan).

Construction of Expression Plasmid

The cDNA encoding Der f 1 was isolated and inserted at the *Bam*H I site of pUC118 as described previously [9] (pUC118:prepro Der f 1). pGEMEX-1 (Promega, Madison, Wisc., USA) was digested with *Xba*I and self-ligated. The resulting plasmid was digested with *Nde*I and self-ligated after filling-in reaction to disrupt the *Nde*I site close to fl ori (pGEMEX-1A). The fragment obtained by *Xba*I-digestion of pGEMEX-1 was inserted into the *Xba*I site of pGEMEX-1A to produce a plasmid with a unique *Nde*I site downstream of the T7 promoter (pGEMEX-1B). Synthetic oligonucleotides 5'-TATGACAAGCGCTATCGATG-3' and 5'-GATCCATCGATAGCGCTTGTCA-3' were annealed and inserted at the *Nde*I/*Bam*H I site of pGEMEX-1B (pGEMEX-1C). The fragment encoding Der f 1 obtained by *Aor*51H I/*Bam*H I digestion of pUC118:prepro Der f 1 was inserted at the *Aor*51H I/*Bam*H I site of pGEMEX-1C (pGEMEX-1:mature Der f 1). The DNA fragment amplified by PCR using pUC118:prepro Der f 1 as a template and oligonucleotides 5'-CCC-ATATGCGTCCAGCTTCAATCAAAC-3' and 5'-AATACTCGCAAGAGTAGTTG-3' as primers was inserted into the *Nde*I/*Aor*51H I site of pGEMEX-1:mature Der f 1 (pGEMEX:pro Der f 1).

Expression and Inclusion Body Preparation

E. coli BL21 (DE3) was transformed with pGEMEX:pro Der f 1. One percent of the overnight culture of the transformant was inoculated into fresh medium and cultured at 30°C until OD₆₀₀ = 0.4–0.5. After the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.1 mM, cells were cultured for an additional 3–6 h. After centrifugation, the cell pellet was washed with TEP (100 mM Tris·Cl (pH 8.5), 10 mM EDTA, 1 mM PMSF) and frozen at –80°C.

Cells were disrupted by sonication and centrifuged at 15,000 *g* for 15 min. The pellet was washed with TEP and used as inclusion bodies.

Solubilization, Renaturation, and Maturation
After the addition of TEP containing 8 *M* urea and 1% dithiothreitol, the inclusion bodies were stirred gently at room temperature for 1–2 h. The solubilized sample was applied to a Sephacryl S-200 16/60 gel filtration column (Amersham Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris·Cl buffer (pH 8.6). The fraction containing monomeric pro Der f 1 was dialyzed with PBS (8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄·12H₂O/l, pH 7.4) and subjected to a second gel filtration using the same column equilibrated with PBS. The preferred fraction was dialyzed with 100 mM acetate buffer (pH 4.0) at 4 °C overnight to remove pro-sequence.

Sodium Dodecyl Sulfate Polyacrylamide-Gel Electrophoresis
Proteins were separated by SDS-PAGE using 16% polyacrylamide gel according to the method of Laemmli [11] and stained with Coomassie brilliant blue.

Analysis of N-Terminal Amino Acid Sequence
Amino-terminal sequences were determined by 473A protein sequencer (Applied Biosystems, Foster City, Calif., USA).

Protease Activity
Pro rDer f 1, mature rDer f 1 and nDer f 1 (1.6 µg of each) were incubated in 10 mM potassium phosphate buffer (pH 6.0) containing 0.01 *M* cysteine at 37 °C for 10 min, respectively. Synthetic substrates, N-suc-Leu-Leu-Val-Tyr-MCA (Sigma, St. Louis, Mo., USA), Boc-Val-Leu-Lys-MCA (Peptide Institute, Osaka, Japan) and Suc-Ala-Pro-Ala-MCA (Peptide Institute) were added at a final concentration of 0.1 mM, and the mixtures were incubated at 37 °C for 30 min. Ten percent acetic acid were added to stop the reaction, and the fluorescence (excitation wavelength 380 nm, emission wavelength 460 nm) of released aminomethylcoumarin (MCA) was measured by a F-2000 fluorometer (Hitachi, Tokyo, Japan).

Reactivity to IgE in Allergic Patient Sera
The reactivity of pro rDer f 1, mature rDer f 1 and nDer f 1 to IgE in the sera of 14 allergic patients was determined by RAST-EIA as described previously [12] using the RAST-FEIA kit (Amersham Pharmacia). Briefly, each allergic patient's serum (diluted 1/4) was incubated with paper discs coupled with nDer f 1 or rDer f 1. The IgE bound to the disc was detected with β-galactosidase-conjugated anti-human IgE antibody by measuring the fluorescence at 355 nm for excitation and 460 nm for emission. The patients' sera were kindly provided by Dr. Okudaira (Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, Japan).

Histamine Release Assay
Leukocytes were obtained from the peripheral blood of an allergic volunteer by separating heparinized blood by dextran sedimentation. After being washed with Tyrode buffer three times, the leukocytes were incubated with serially diluted pro rDer f 1, mature rDer f 1 and nDer f 1 at 37 °C for 40 min. The histamine released from the stimulated leukocytes in the cell-free supernatant was quantitated by the Histamine-ELISA Kit (ICN Biomedical Research Product, Costa Mesa, Calif., USA).

Results

Refolding of rDer f 1
The inducible T7-based expression vector of pro Der f 1 was introduced into *E. coli* BL21. On induction with isopropyl-β-D-thiogalactopyranoside, pro Der f 1 accumulated as insoluble inclusion bodies in the cells. The cells were disrupted, and the collected inclusion bodies were solubilized with 8 *M* urea containing 1% dithiothreitol. The denatured pro Der f 1 was refolded by two-step gel filtration chromatography. The elution pattern profiles of the first and second gel filtrations are shown in figure 2. The first gel filtration was carried out in the presence of 20 mM Tris·Cl (pH 8.6) without NaCl. Although the solubilized pro Der f 1 was separated into fractions A (frA) and B (frB) under this condition (fig. 2A), only frB was able to be renatured. frA was thought to be aggregated protein. The ratio of frA to frB was determined by the ionic intensity of the equilibration buffer. For example, no fraction corresponding to frB was eluted in the presence of 100 mM NaCl (data not shown).

frB was dialyzed with PBS and loaded onto the same column equilibrated with PBS (pH 7.4; fig. 2B). Both fraction C (frC) and fraction D (frD) contained pro Der f 1, but only frD was able to be processed to the mature form. The amount of each fraction depended on the pH of the equilibration buffer. An additional peak containing aggregated pro Der f 1 was eluted at lower pH and the amount of frC, which was monomeric but could not be activated, was increased at higher pH (data not shown). The fractions of each step were analyzed by SDS-PAGE (fig. 3A, lanes 2–4). A band with an apparent molecular weight of 35 kD corresponded to pro Der f 1. A protein with a slightly lower molecular weight appeared after the dialysis with PBS. It was revealed to be a protein lacking the N-terminus of the pro-region including 21-amino acid residues (table 1A). The latter of the two peaks contained in frD probably corresponded to this protein. It was thought

Table 1. Amino-terminal sequences of rDer f 1 in the process of refolding and processing

A	-61 V EEEEVARKNPL	
B		1 TSA (C) RINSVNV
C		-2 AETSA (C) RINSV

A = Lower band in lane 4 of fig. 3A; B/C = band in lane 5 of fig. 3A. The numbers are those of amino acids counting from the first residue of the mature form.

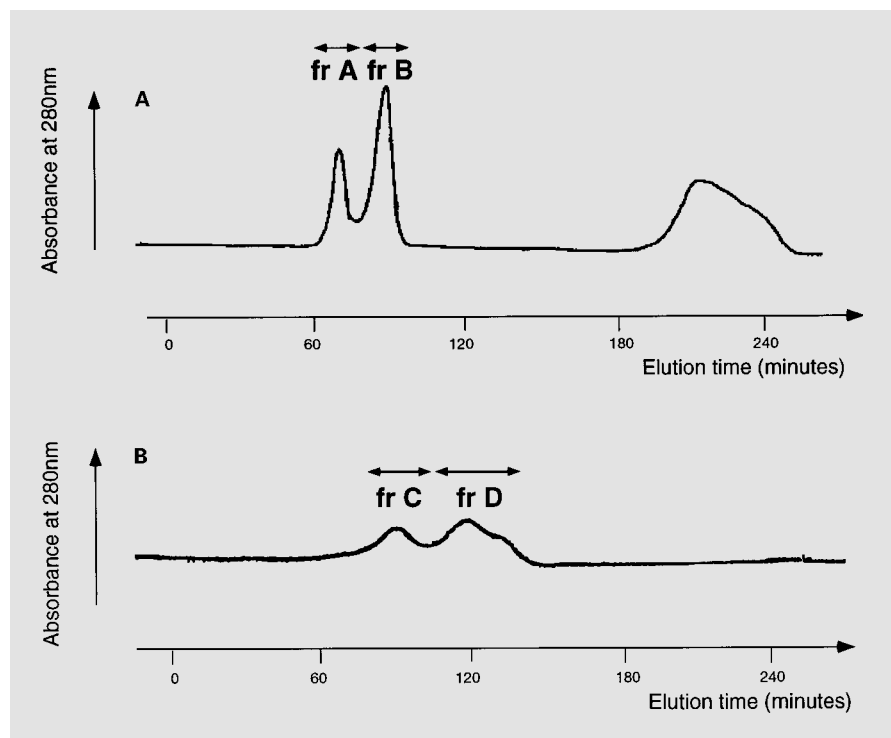


Fig. 2. A Elution pattern of the first gel filtration chromatography. The first gel filtration chromatography was performed in the presence of 20 mM Tris·Cl (pH 8.6) without NaCl using a Sephacryl S-200 column (Amersham Pharmacia). Bed volume of the column: 120 ml, flow rate: 0.5 ml/min. The absorbance was monitored at 280 nm. **B** Elution pattern of the second gel filtration chromatography. The second gel filtration chromatography was performed in the presence of PBS (pH 7.4). The other conditions were the same as for the first gel filtration.

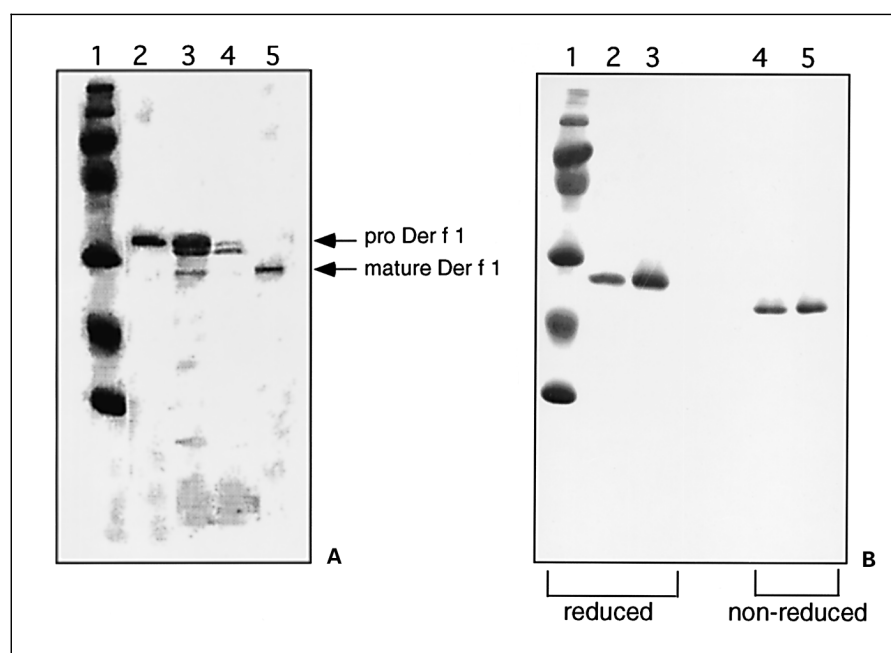


Fig. 3. A SDS-PAGE analysis of rDer f 1 in the process of refolding and processing. Lane 1 = Molecular weight markers (15, 19, 28, 46, 68, 97 and 200 kD), lane 2 = frB in the first gel filtration; lane 3 = after the dialysis with PBS; lane 4 = frD in the second gel filtration; lane 5 = after the dialysis with acetate buffer. **B** SDS-PAGE analysis of mature rDer f 1 and nDer f 1. The obtained mature rDer f 1 and nDer f 1 were analyzed by SDS-PAGE under reduced (lanes 2, 3) and non-reduced (lanes 4, 5) conditions. The gel was stained with Coomassie brilliant blue. Lane 1 = Molecular weight markers (15, 19, 28, 46, 68, 97 and 200 kD); lanes 2, 4 = mature rDer f 1; lanes 3, 5 = native Der f 1.

that the change in pH and ionic intensity caused a structural change in pro Der f 1 in frB and resulted in the autocatalytic cleavage of the Thr⁶⁰-Val⁵⁹ bond. Approximately 70 mg of pro Der f 1 were properly renatured by this two-step gel filtration method from 1 liter of culture.

Processing of Pro Der f 1

frD from the second gel filtration was dialyzed with acetate buffer (pH 4.0) for the autocatalytic removal of pro-sequence. The SDS-PAGE pattern of rDer f 1 before and after the acid treatment are shown in fig. 3A (lanes 4,

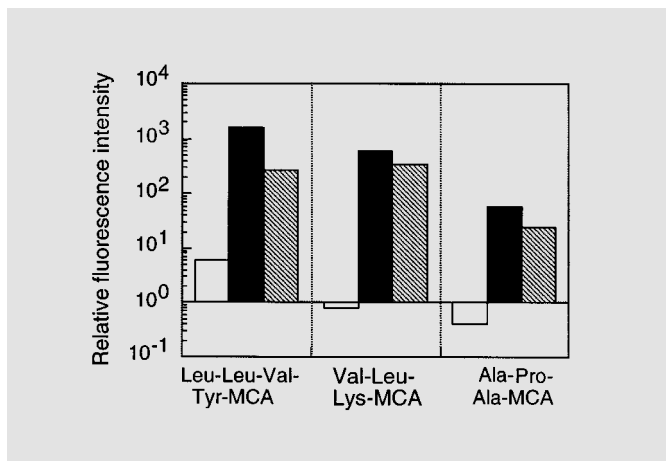


Fig. 4. Protease activities of pro rDer f 1, mature rDer f 1 and nDer f 1. Digestion of synthetic peptide substrates was detected by the fluorescence of released MCA. Fluorescence intensity in the absence of Der f 1 was subtracted from the values in the presence of each Der f 1. White bar = Pro rDer f 1; black bar = mature rDer f 1; hatched bar = nDer f 1.

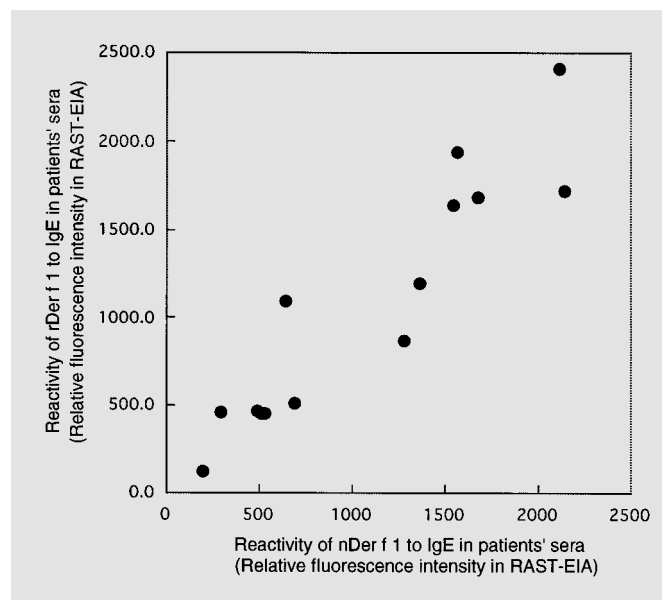


Fig. 5. Reactivity of rDer f 1 and nDer f 1 to IgE in the sera of mite-allergic patients. The reactivity of mature rDer f 1 and nDer f 1 to IgE in each patient's serum was determined by RAST-EIA. The binding activities are represented as relative values of fluorescence intensity yielded from the β -galactosidase-digested substrate.

5). Both of the double bands in lane 4 were shifted to a lower band of about 28 kD in lane 5. The analysis of the N-terminal amino acid sequence of this 28-kD protein revealed the presence of two polypeptides. As shown in table 1 (B, C), one started from the first amino acid of nDer f 1 and the other had two additional amino acid residues of pro-sequence. The proportion of the latter was about 10%. The obtained mature rDer f 1 was dialyzed with PBS and subjected to further analysis. The final product was analyzed by SDS-PAGE under reduced and non-reduced conditions (fig. 3B). Because it stained as a single band, the proteins from *E. coli* were fully removed. Furthermore, it showed the same mobility as nDer f 1 under both reduced and non-reduced conditions, which suggested that the three disulfide bonds predicted to exist in the nDer f 1 molecule (fig. 1) [5, 6] were correctly formed in mature rDer f 1.

Protease Activity

The protease activities of pro rDer f 1, mature rDer f 1 and nDer f 1 were determined using synthetic peptide substrates. As shown in figure 4, mature rDer f 1 had equal protease activity with nDer f 1 and the removal of pro-sequence was necessary for the cysteine protease ac-

tivity. Both mature rDer f 1 and nDer f 1 digested N-Suc-Leu-Leu-Val-Tyr-MCA and Boc-Val-Leu-Lys-MCA more efficiently than Suc-Ala-Pro-Ala-MCA. This result showed that they had similar substrate specificity.

IgE Binding Activity

The reactivity of nDer f 1 and rDer f 1 to IgE in the sera of 14 patients was compared (fig. 5). The reactivity of the two proteins to each serum was highly correlated ($r = 0.93$). From this result, mature rDer f 1 was revealed to have the same IgE binding activity as nDer f 1.

Histamine-Releasing Activity

The capacity of pro rDer f 1, mature rDer f 1 and nDer f 1 to release histamine from an allergic patient's basophils was next determined. As shown in figure 6, mature rDer f 1 and nDer f 1 induced histamine release at concentrations higher than 10^{-5} mg/ml. On the other hand, pro rDer f 1 induced it only at 100-fold higher concentration. The capacity of mature rDer f 1 and nDer f 1 to release histamine was almost equal, and pro Der f 1 acquired the antigenicity by cleaving the pro-sequence.

From these results, mature rDer f 1 was shown to have the same ability to bind IgE as nDer f 1.

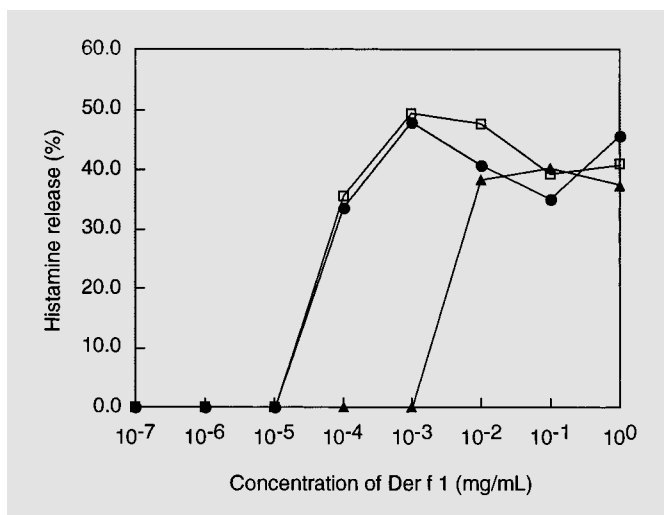


Fig. 6. Histamine release capacity of pro rDer f 1, mature rDer f 1 and nDer f 1. The histamine released from an allergic patient's peripheral blood leukocytes stimulated with each Der f 1 was quantitated by ELISA. The total amount of histamine in the patient's leukocytes was quantitated by disrupting the cells with Triton X-100. The percentage of released histamine to total histamine is shown. Black triangle = Pro rDer f 1; black circle = mature rDer f 1; white square = nDer f 1.

Discussion

The refolding efficiency of denatured protein varies widely with the kind of protein, and there is no generally applicable rule for renaturation. It is required to find the optimal conditions for renaturation in each case. Group 1 allergens (Der f 1 and Der p 1) belong to the cysteine protease family which includes papain, cathepsin B, H, L, and S, and actinidin. They have conserved cysteine residues at enzymatically active sites and those forming disulfide bonds. There are only a few examples of this class of protease being expressed even at low levels in *E. coli* because of their difficulty in refolding [13–18]. The most common method to remove the denaturing solvent is dialysis or dilution. However, neither of these methods allowed for Der f 1 to be refolded efficiently. Techniques for renaturation using size exclusion and gel filtration chromatography were recently reported [19–21]. They have the advantage of preventing the progression of aggregation, which is the major cause of unsuccessful refolding, by suppressing the interactions of partially folded proteins and separating the aggregated refolding intermediates from monomeric ones. In addition, it is also possible to separate the product from the proteins from *E. coli*. The

pro-form of rDer f 1 was efficiently refolded and purified by two-step gel filtration chromatography.

Der f 1 is firstly synthesized as a prepro-form in *D. farinariae*. The pre-sequence directs proteins to the endoplasmic reticulum. When the prepro-form of protein was expressed in *E. coli*, the pre-sequence often showed a cytotoxic effect to suppress the cell growth [14] and impair the protein synthesis [22]. In contrast, the pro-peptide, which regulates the enzymatic activity of the protein [23–25], is usually essential for proper folding [26, 27]. The pro-sequence was necessary for the renaturation of rDer f 1. When rDer f 1 was expressed as mature form, the inclusion bodies could not be renatured after solubilization (data not shown). The time course observation revealed that the protein lacking 21 amino acids of the pro-sequence generated by dialysis with PBS was processed more efficiently than the protein with the entire pro-sequence. However, when this deletion mutant was expressed in *E. coli*, it was unable to be renatured from inclusion bodies (data not shown). This indicates that the complete pro-peptide is required for correct folding although it moderately impairs autocatalytic processing.

nDer f 1 has a potential N-glycosylation sequence. We showed that nDer f 1 was actually glycosylated by detecting a sugar chain with the NaIO₄ oxidation method [unpubl. data]. From the comparison of the reactivity of nDer f 1 and rDer f 1 to IgE in patients' sera (fig. 5), the sugar chain of nDer f 1 did not seem to be antigenic in the majority of patients. In addition, it was not responsible for the protease activity, because mature rDer f 1 and nDer f 1 had almost identical protease activity and substrate specificity (fig. 4). Thus, the sugar chain of nDer f 1 was thought to be neither involved in the allergenic nor in the enzymatic activity of this molecule. Similarly, the unglycosylated form of cathepsin B was shown to be functionally identical to its glycosylated liver counterpart [28].

Here we established a method to produce enzymatically and immunologically active Der f 1. In addition to contributing to the field of recombinant allergens for clinical use, this method can provide sufficient material for the analysis of both the enzymatic and allergenic properties of the Der f 1 molecule. Several studies have focused on the allergenicity of group 1 allergens from house dust mites related to its protease activity [29–32]. Recently, Der p 1 from *D. pteronyssinus* was reported to selectively cleave the low affinity IgE receptor (CD23) [33]. The authors speculated that this loss of cell surface CD23 from IgE-secreting B cells promoted an IgE-mediated immune response by ablating a feedback mechanism which limited the IgE synthesis. Because the obtained mature rDer f 1

had the same protease and IgE-binding activity as nDer f 1, it will be a powerful tool for the study of this relationship. Mature rDer f 1 is thought to have a very similar structure to nDer f 1 judging from its enzymatic and allergenic properties. The determination of the three-dimensional structure of rDer f 1 and the analysis of various rDer f 1 mutants will not only reveal electrostatic interaction of the enzyme but also B and T cell epitopes of this allergen.

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Acknowledgment

We thank Dr. Hirokazu Okudaira, University of Tokyo, for providing patients' sera, and Torii Pharmaceuticals for providing purified nDer f 1.

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