

Dilution Method to Refold Bacterially Expressed Recombinant Der f 2 and Der p 2 to Exhibit the Secondary Structure and Histamine-Releasing Activity of Natural Allergens

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Key Words

Circular dichroism · Der f 2 · Der p 2 · Dilution ·
Histamine release · House dust mites ·
Inclusion bodies · Recombinant allergens · Refolding

Abstract

Background: Structurally refolded recombinant forms of major house dust mite group 2 allergens, Der f 2 and Der p 2, expressed in *Escherichia coli*, were prepared by solubilizing the insoluble products with urea and subsequently dialyzing against buffer. In this study, we determined conditions for refolding the urea-denatured recombinant Der f 2 and Der p 2 by one-step dilution as an alternative to dialysis, which requires several steps of handling and much time and cost. **Methods:** The insoluble bacterial product containing recombinant Der f 2 was solubilized with a buffer containing 8 M urea, and the solution was diluted to various urea concentrations. The refolding efficiency in each dilution was estimated from the height of the peak corresponding to the folded recombinant Der f 2 and that containing the aggregated form on anion exchange chromatography. The structure and allergenicity of the purified recombinant Der f 2 and Der p 2 refolded using the dilution method were analyzed based on circular dichroism and a basophil histamine-

releasing assay, respectively. **Results:** Although the refolding efficiency decreased as the urea concentration in the dilution increased, experimental conditions whereby the protein and urea concentrations in the dilution were less than 0.5 mg/ml and 0.8 M, respectively, achieved maximum refolding efficiency. The recombinant allergens prepared by the dilution method exhibited the secondary structure and histamine-releasing activity of natural allergens purified from mite culture. **Conclusions:** The dilution method established in this study is more convenient in terms of handling, time, and cost than the dialysis method and will be useful for large-scale production and for the preparation of numbers of mutants to analyze IgE epitopes.

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Introduction

House dust mites of the *Dermatophagoides* species (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) are a major source of indoor allergens associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis. Group 1 (Der f 1 and Der p 1) and group 2 (Der f 2 and Der p 2) allergens derived from house dust mites are considered major aller-

gens based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract [1].

Der f 2 from *D. farinae* [2–4] and Der p 2 from *D. pteronyssinus* [5–7] are single-domain proteins with an immunoglobulin fold composed of 129-amino-acid residues. Natural and recombinant Der f 2 have three disulfide bonds [8–11]. Their IgE epitopes are dependent on the integrity of the conformational structure rather than the contiguous sequence of amino acids [10–16]. Their amino acid sequences are homologous to other group 2 allergens from different mite species, esr16 found in moth trachea, and Niemann-Pick type C2 disease protein (NPC2/HE1) [17–22]. Recently, a structural relationship between the Der f 2 homologues and MD-2, which associates with Toll-like receptor 4, was indicated [23].

Recombinant Der f 2 and Der p 2 can be bacterially expressed and have been used by various researchers. Structurally refolded recombinant Der f 2 and Der p 2 have been prepared by solubilizing the insoluble product with urea [9, 24] or guanidine [25], and subsequently dialyzing against buffer without the denaturants. However, the dialysis involves several steps and consumes much time and cost particularly for large-scale production. Here, we try to improve the refolding process by replacing the dialysis with dilution and determine efficient conditions under which to refold the urea-denatured recombinant Der f 2 and Der p 2 by one-step dilution.

Materials and Methods

Cloning of cDNAs Coding for Der f 2 and Der p 2

Total RNA was prepared from cultured mites, and the cDNA fragments of Der f 2 and Der p 2 were synthesized, amplified by RT-PCR, subcloned into vectors and sequenced as described previously [26]. The PCR primers used to amplify Der f 2 cDNA were designed to correspond to the region upstream of the initiation codon and that downstream of the termination codon, while those used to amplify Der p 2 cDNA were designed to correspond to a region containing the initiation codon and a region containing the termination codon.

Expression of Recombinant Der f 2 and Der p 2

The cDNAs encoding Der f 2 and Der p 2 were subcloned into an expression vector, a modified pGEMEX-1 (Promega, Madison, Wisc., USA). The vectors were designed to express mature amino acid sequences of Der f 2 and Der p 2 with an additional methionine at the N-termini.

The recombinant Der f 2 and Der p 2 were expressed in *Escherichia coli* BL21 (DE3) transformed with the vectors, and the insoluble inclusion bodies accumulated in the cells were recovered as described previously [9, 24].

Refolding and Purification of Recombinant Der f 2 and Der p 2

Inclusion bodies recovered from 1-liter cultures of *E. coli* cells using a method described [9, 24] contained approximately 100 mg of protein. The inclusion bodies were solubilized to a protein concentration of 5–10 mg/ml with a buffer (20 mM Tris-HCl, pH 8.5, 0.01% NaN₃) containing 8 M urea with gentle shaking overnight at 4°C. The protein concentration of the solution was estimated by the Bradford procedure using a protein assay kit (Bio-Rad, Richmond, Calif., USA). The solution was diluted to various urea concentrations by rapid addition of a buffer (20 mM Tris-HCl, pH 8.5, and 0.01% NaN₃) containing various concentrations of urea at room temperature. After a 1-hour incubation, the solution was subjected to anion exchange column chromatography (HiTrapQ, 5 ml; Amersham Pharmacia, Uppsala, Sweden). The column was washed with the buffer without urea, and the peak fractions eluted with the buffer containing 90 mM NaCl (stepwise) corresponding to the folded monomeric recombinant Der f 2 and Der p 2 were recovered. Concentrations of the purified proteins were estimated by means of the Bradford procedure and calculations based on the absorbance at 280 nm and the number of tyrosine and tryptophan residues in recombinant Der f 2 and Der p 2.

Natural Der f 2 and Der p 2

Natural Der f 2 and Der p 2 were purified from whole culture extracts of house dust mites, *D. farinae* and *D. pteronyssinus*, respectively, as described by Yasueda et al. [27].

Circular Dichroism

Circular dichroism (CD) spectra were measured on a JASCO J-820 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 0.1-cm cell at 25°C over 190–260 nm. Samples at 0.05 mg/ml were dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The experimental parameters were: bandwidth 1.0 nm, sensitivity 5 millidegrees, step resolution 0.5 nm/datum, scan speed 50 nm/min, and scans 20.

Histamine Release

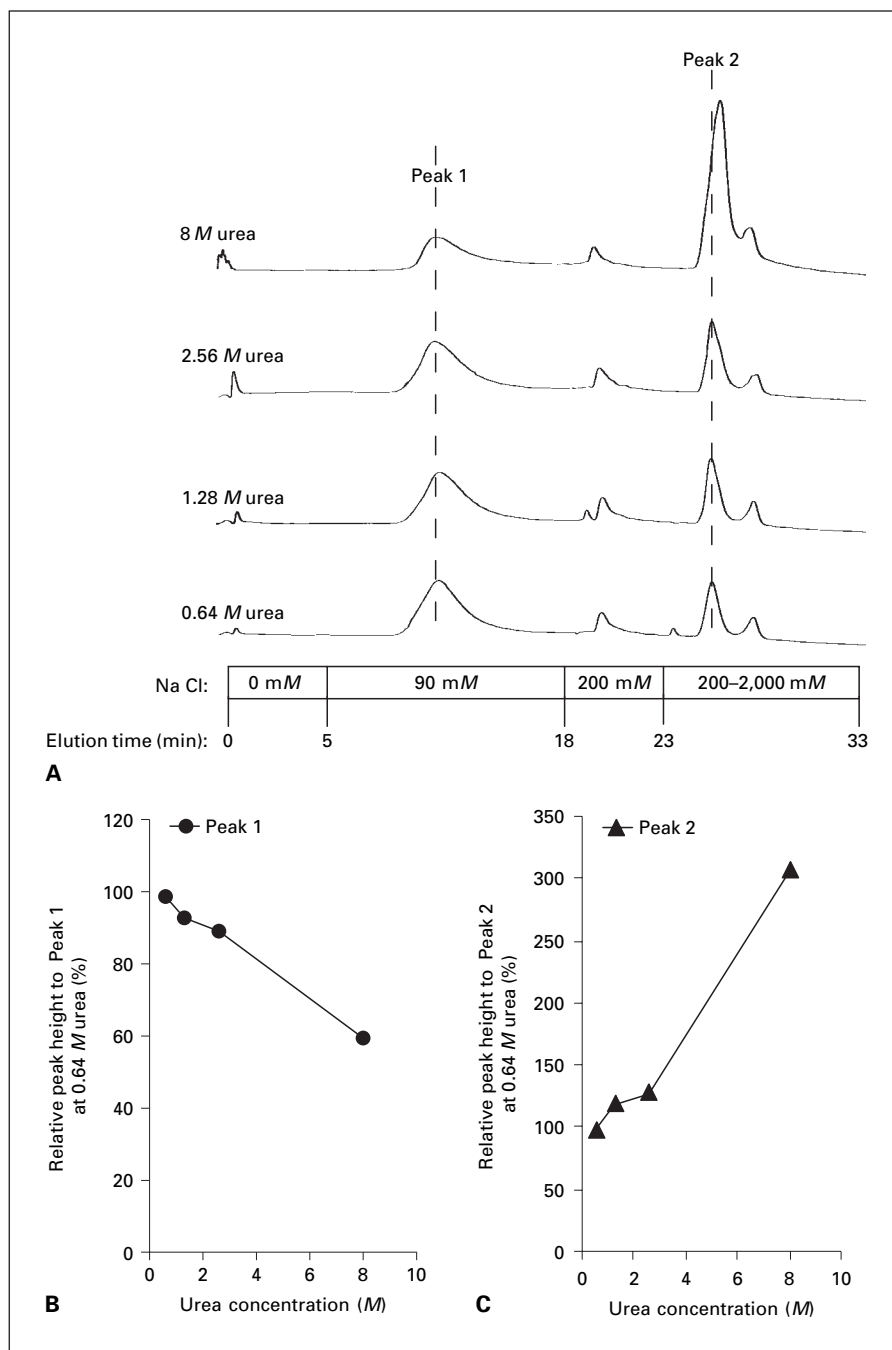
The method employed was as described [28] using HRT-Shionogi (Shionogi, Osaka, Japan) with some modifications [29]. The percentage of histamine released was calculated as the relative amount of histamine in the supernatant to the total amount of histamine. The amount released after incubation with 0.2 mg/ml digitonin was used as the total histamine content for the calculation.

Results

Cloning of cDNAs Coding for Der f 2 and Der p 2

Six cDNA clones for Der f 2 were sequenced. Four of the clones were identical in the deduced amino acid sequence to clone 11/Der f 2.0103, one was identical to clone 1/Der f 2.0101, and one was identical to clone 2/Der f 2.0102 originally reported by Yuuki et al. [2, 3]. Six cDNA clones for Der p 2 were sequenced. Two of the clones were identical in the deduced amino acid sequence to Der p 2.0101 except for residue –1 in the signal sequence, one was identical to Der p 2.0104, one was iden-

Fig. 1. Refolding of recombinant Der f 2 by the dilution method. Insoluble inclusion bodies were solubilized with a buffer containing 8 M urea, and the urea-denatured recombinant Der f 2 was refolded by dilution in buffers with various urea concentrations and subjected to anion exchange column chromatography. **A** Elution profiles of samples diluted in buffers containing various urea concentrations (8, 2.56, 1.28, and 0.64 M). After the loading of each sample, buffers (20 mM Tris-HCl, pH 8.5, 0.01% NaN₃) without NaCl (5 min at a flow rate of 5 ml/min), with 90 mM NaCl (13 min at 1 ml/min), with 200 mM NaCl (5 min at 5 ml/min) and with a gradient of 200 mM to 2 M NaCl (10 min at 5 ml/min) were sequentially loaded onto the column. Absorbance at 280 nm was monitored. Peak 1 = Peak corresponding to folded recombinant Der f 2; peak 2 = peak containing aggregated recombinant Der f 2. **B** Relation between refolding and urea concentration of the dilution. **C** Relation between aggregation and urea concentration of the dilution. Relative peak height: percentage relative to the peak height at the urea concentration of 0.64 M. The clone used was clone 1/Der f 2.0101 [2, 3].



tical to Der p 2.0105 first reported by Chua et al. [5, 30] and Smith et al. [31], and two were identical to the '2nd clone' originally reported by Park et al. [32]. The amino acid residue at -1 was alanine in all clones isolated in the present study, whereas it is arginine in Der p 2.0101 in a study by Chua et al. [5].

Optimization of the Urea Concentration in Dilution to Refold Recombinant Der f 2

The clone 1/Der f 2.0101, which has been used for analysis of the NMR structure of Der f 2 [4], was used. Inclusion bodies from *E. coli* expressing recombinant Der f 2 were solubilized with a buffer containing 8 M urea. The solution was diluted to a protein concentration of

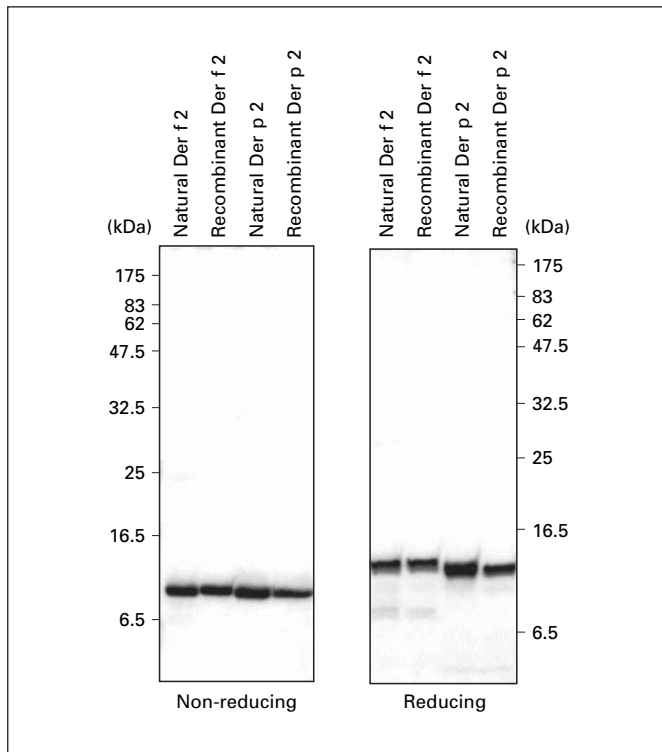


Fig. 2. SDS-PAGE of purified recombinant Der f 2 and Der p 2 refolded by the dilution method and natural Der f 2 and Der p 2. The samples boiled in sample buffers without (non-reducing) and with 2-mercaptoethanol (reducing) were subjected to SDS-PAGE (15–25%). The gels were stained with Coomassie brilliant blue. The clones used were clone 11/Der f 2.0103 [2, 3] and Der p 2.0101 [5].

0.2 mg/ml and various urea concentrations by adding cocktails of a buffer without urea and that with 8 M urea at various ratios, and subjected to anion exchange column chromatography (fig. 1A). The NaCl concentration for the stepwise elution was optimized to elute only the folded monomeric recombinant Der f 2 without contamination by aggregated forms and dimers, and determined as 90 mM. Peak 1 in figure 1A corresponded to a folded monomeric recombinant Der f 2, and peak 2, aggregated recombinant Der f 2. The lower the urea concentration of the dilution was, the higher was the height of peak 1 (fig. 1B) and the lower was the height of peak 2 (fig. 1C). Thus, the refolding efficiency increased as the urea concentration of the dilution decreased. The refolding efficiency at a urea concentration of 0.64 M in the dilution was the highest among the concentrations tested in figure 1. Ten to 20 mg of refolded and purified recombinant Der f 2 was obtained from a 1-liter culture.

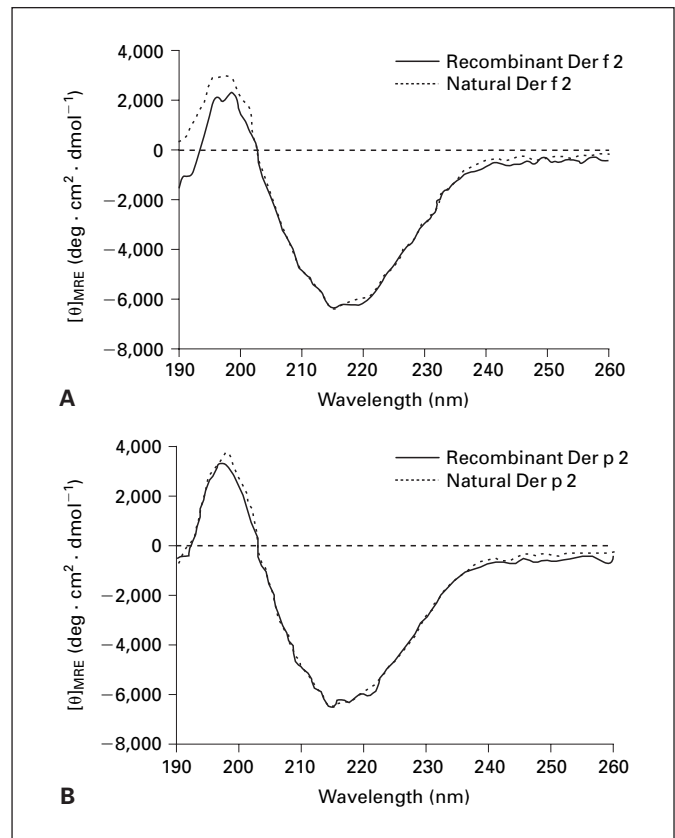


Fig. 3. Comparison of CD spectra of purified recombinant Der f 2 and Der p 2 refolded by the dilution method and natural Der f 2 and Der p 2. The clones used were clone 11/Der f 2.0103 and Der p 2.0101. MRE = Mean residual ellipticity.

This efficiency was almost equivalent to efficiencies under two other sets of conditions; a protein concentration of 0.5 mg/ml and urea concentration of 0.8 M and a protein concentration of 0.05 mg/ml and urea concentration of 0.08 M, and equivalent to or higher than that obtained using the dialysis method (data not shown). No apparent insoluble precipitate was observed using the dilution or dialysis method, although small insoluble fractions were removed by centrifugation and filtration before loading onto the column.

Purification of Recombinant Der f 2 and Der p 2 Refolded by the Dilution Method

Recombinant Der f 2 and Der p 2 with amino acid sequences identical to clone 11/Der f 2.0103, which has been used for IgE epitope analysis [10, 11, 33, 34], and Der p 2.0101, respectively, were expressed, refolded by the dilution method, and purified. Refolding efficiencies

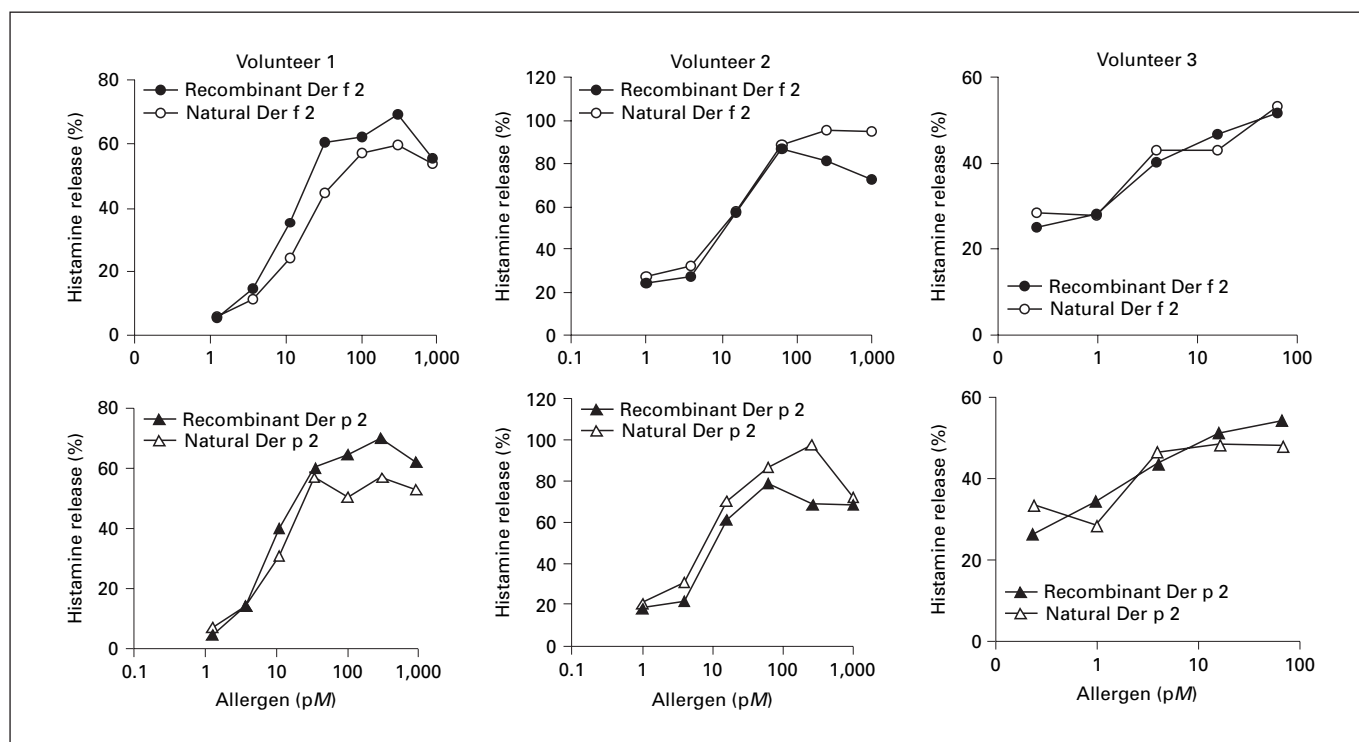


Fig. 4. Basophil histamine-releasing activity of purified recombinant Der f 2 and Der p 2 refolded by the dilution method and natural Der f 2 and Der p 2. Peripheral blood was obtained from 3 mite-allergic volunteers. Spontaneous histamine release in the 3 volunteers was 4, 19 and 30%, respectively. The clones used were clone 11/Der f 2.0103 and Der p 2.0101.

of these clones were similar to that for clone 1/Der f 2.0101 described above. Each of the purified recombinant and natural allergens was detected as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (fig. 2). Mobilities of the recombinant Der f 2 and Der p 2 were equivalent to those of natural Der f 2 and Der p 2, respectively. The allergens were less mobile under reducing conditions than under non-reducing conditions. Once the recombinant allergens were refolded and purified, they were stable and soluble during storage at 4° or -80°C at the tested concentrations of 1–4 mg/ml.

Secondary Structure and Allergenicity of Recombinant Der f 2 and Der p 2

The purified recombinant Der f 2 (clone 11/Der f 2.0103) and Der p 2 (Der p 2.0101) were used for a comparison of secondary structure and allergenicity with the natural Der f 2 and Der p 2. CD spectra of the purified recombinant Der f 2 and Der p 2 were identical to those of the natural types (fig. 3). The biological activities of the

recombinant Der f 2 and Der p 2 to stimulate peripheral blood basophils to release histamine were almost equivalent to those of the natural types in three mite-allergic volunteers (fig. 4).

Discussion

The production of recombinant allergens by microorganisms has major advantages in handling, time, and cost compared to the purification of natural allergens from natural extracts of mites, pollens, food, and so on. Recombinant allergens, which can be prepared in large amounts and with high purity and homogeneity, will be useful for the standardization of allergens for diagnosis and therapy, and research uses, such as analysis of IgE epitopes and engineering allergens for future allergen-specific immunotherapy [35, 36]. Major house dust mite group 2 allergens, Der f 2 and Der p 2, are the allergens that have been the most extensively analyzed by the use of recombinant proteins [1]. *E. coli* and yeast expression systems to pro-

duce recombinant Der f 2 and/or Der p 2 have been reported previously [9, 24, 25, 37]. In the *E. coli* expression system, the recombinant Der f 2 and Der p 2 were accumulated as insoluble inclusion bodies within the cells, solubilized with a buffer with a high concentration of denaturant, such as urea, and refolded to retain the correctly folded structure and allergenicity by subsequent dialysis against the buffer without the denaturant [9, 24, 25]. In the yeast *Saccharomyces cerevisiae* expression system [37], recombinant Der p 2 was detected as a soluble folded protein in the culture supernatant, however, the productivity in the yeast system seemed significantly changed by mutations in the recombinant Der p 2 introduced for analysis of IgE epitopes [38]. While the advantages of the *E. coli* system are that *E. coli* grows more rapidly than yeast and that the productivity was not drastically different among mutants [10, 11, 33, 34, 39], a disadvantage is that steps of denaturing and dialysis are required. Particularly in large-scale production, the dialysis involves several steps and consumes both time and money.

In the present study, we improved the refolding process by replacing the dialysis with dilution and determined efficient conditions to refold the urea-denatured recombinant Der f 2 and Der p 2 by one-step dilution. Although the recombinant Der f 2 denatured with 8 M urea loaded onto the anion exchange column could be refolded, the refolding efficiency was relatively low when diluted in a buffer with 8 M urea (fig. 1A, 8 M urea, peak 1; fig. 1B), indicating that a decrease of the urea concentration before loading onto the column is necessary for effective refolding. The refolding efficiency at a urea concentration of 0.64 M in the dilution was the highest among the concentrations tested in figure 1. Thus, the refolding efficiency with the dilution method is as good as that using the dialysis method to refold the urea-denatured recombinant Der f 2 if the protein concentration and urea concentration are low enough. A protein concentration and urea concentration lower than 0.5 mg/ml and 0.8 M, respectively, were enough to achieve maximum refolding efficiency under the conditions tested.

Less mobility on SDS-PAGE under reducing conditions (fig. 2) is considered a result of elimination of the disulfide bonds, and suggests the formation of the correct disulfide bonds in the recombinant proteins [11]. Although CD spectra for natural [40] and recombinant Der f 2 [11, 33, 34, 41] have been reported, neither spectra for the natural nor the recombinant Der p 2 have been reported so far. Here, we presented CD spectra of recombinant Der f 2 and Der p 2 refolded by the dilution method and natural Der f 2 and Der p 2 (fig. 3). CD spectra of

the recombinant Der f 2 and Der p 2 were identical to those of the natural types, showing that secondary structures were the same.

The biological activities of the recombinant Der f 2 and Der p 2 to stimulate basophils from mite-allergic volunteers to release histamine were almost equivalent to those of the natural types (fig. 4). However, interestingly, Park et al. [32] reported that two recombinant histidine-tagged isoforms of Der p 2 showed a difference in IgE binding capacity in some patient sera although they could not find differences in biological activity estimated from the weal size in an intradermal test. Indeed, we also observed that recombinant Der p 2.0101 prepared in the present study showed less direct IgE binding in a serum than natural Der p 2 (data not shown). Therefore, the analysis of the IgE binding capacity by means of assays for direct IgE binding and/or IgE binding inhibition using numbers of sera should be addressed in future studies, although the present study indicated that the recombinant allergens retained the structure and biological activity of the natural allergens.

Although the recombinant Der f 2 and Der p 2 could be successfully refolded using both the dialysis and dilution methods, this might not always be the case for various recombinant allergens other than Der f 2 and Der p 2. For example, pro- and mature forms of the house dust mite major group 1 allergen Der f 1 expressed as inclusion bodies in *E. coli* were not refolded by either the simple dialysis or dilution method [42, 43]. Pro-Der f 1 was refolded to the monomer and converted to the prosequence-removed mature form by the use of size exclusion chromatography to remove urea [42]. Furthermore, recombinant mite group 1 allergens Der f 1 and Der p 1 seem to need assistance from their propeptides, which function as intramolecular chaperones, to be correctly folded and matured to be enzymatically functional [26, 29, 42–44]. Therefore, expression and/or refolding systems should be optimized allergen by allergen. However, if the dilution method is effective for the refolding of a recombinant allergen, the process for large-scale production and preparation of numbers of mutants for molecular analysis will be simplified and accelerated. The dilution method is valuable in the case where the recombinant allergen is expressed as an insoluble product in spite of the fact that it might not always be successful.

In summary, a dilution method to refold recombinant house dust mite major group 2 allergens, Der f 2 and Der p 2, to retain the structure and basophil histamine-releasing activity of the natural Der f 2 and Der p 2, was established in this study. In addition, this paper includes the

first representation of CD spectra of natural and recombinant Der p 2. The dilution method is more convenient in terms of handling, time, and cost than the dialysis method and will be useful for large-scale production of the recombinant house dust mite major group 2 allergens and for the preparation of numbers of mutants to analyze IgE epitopes.

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