

Recombinant Fusion Proteins Assembling Der p 1 and Der p 2 Allergens from *Dermatophagoides pteronyssinus*

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Key Words

Allergy vaccine • Der p 1 • Der p 2 • Fusion protein • House dust mite • Immunotherapy • Recombinant allergen

Abstract

Background: Fusion proteins assembling multiple allergens can be engineered by recombinant DNA technologies in order to produce tools for diagnostic and immunotherapeutic purposes. Herein, we developed and characterized chimeras assembling Der p 1 and Der p 2 allergens as potential candidate vaccines against house dust mite allergy. **Methods:** Fusion proteins encompassing Der p 2 with either mature or proDer p 1 were expressed in *Escherichia coli* or *Pichia pastoris*. Forms with mutation in Der p 1 catalytic site were also engineered. Purified chimeras were characterized by immunoblotting, circular dichroism, disulfide bond mapping, basophil and T lymphocyte stimulation assays. **Results:** Four fusion proteins were expressed in *E. coli* as inclusion bodies, whereas only chimeras comprising proDer p 1 were obtained in yeast. All such hybrids formed polymers and aggregates, and yeast-expressed chimeras were unstable. Circular dichroism analysis performed after refolding of bacteria ex-

pressed chimeras encompassing mature Der p 1 confirmed partial folding, consistent with the occurrence of both correct and inappropriate intramolecular disulfide bonds. All fusion molecules were recognized by Der p 1- and Der p 2-specific human IgEs, monoclonal and polyclonal antibodies. Fusion proteins activate basophils from mite-allergic patients and trigger the proliferation of specific CD4⁺ T cells, albeit to a lower level when compared to individual allergens. **Conclusions:** Production of multiple Der p 1-Der p 2 fusion proteins exhibiting partial folding and proper antigenic properties has been achieved. Nonetheless, significant solubility and stability issues currently limit the application of such chimeras for immunotherapy or diagnostic.

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Introduction

House dust mites (HDM) represent a common source of allergens worldwide. Prevalent mite species in Europe and North America include *Dermatophagoides pteronyssinus* and *D. farinae* which share antigenic specificities. Current therapeutic vaccines against HDM allergy are

based on complex biological extracts made from either one or both species [1]. Given significant amino acid sequence homologies between *D. pteronyssinus* and *D. farinae* allergens and resulting IgE cross-reactivities [2, 3], a vaccine based solely on *D. pteronyssinus* allergens could likely be useful to treat allergies to either one of those two mite species.

In order to develop such a vaccine, recombinant allergens represent an attractive alternative to allergenic extracts, since they can be produced in large quantities as well-defined and standardised molecules. A valid candidate vaccine against HDM allergy should likely combine at least group I and II allergens, for example Der p 1 and Der p 2 major allergens from *D. pteronyssinus*. The latter are highly clinically relevant, with a majority of mite-allergic patients exhibiting high seric IgE titres against those molecules [4–6]. One option for immunotherapy, most particularly via the sublingual route, is to rely upon allergens in a natural conformation with a preserved immunogenicity in terms of IgE, IgG and T lymphocyte recognition when compared to natural allergens [7]. To this aim, group I and II allergens can be expressed either independently or as a fusion protein in GMP-compliant prokaryotic or eukaryotic systems. The latter approach has already been applied for instance to pollen [8, 9] and more recently mite allergens [10].

Der p 1 and Der p 2 are 25.1- and 14.1-kDa proteins, respectively, each comprising 3 disulfide bonds. Der p 2 has been produced successfully in various cell expression systems, including *Escherichia coli*, the yeast *Saccharomyces cerevisiae* and tobacco cells [11–13]. Der p 1 is a more complex protein expressed as a preproenzyme. As such, attempts to express the mature protein in *E. coli* led to poor yields and altered conformation of the molecule [14]. Furthermore, only proDer p 1 forms have been successfully expressed in various eukaryotic systems such as plants, insect and mammalian cells or the methanotrophic yeast *Pichia pastoris* [15–20], implying that the propeptide region is absolutely required for Der p 1 expression in eukaryotic systems. Such recombinant proforms of Der p 1 exhibit IgE reactivity and can be processed into active mature forms [18, 21–23].

Herein, we report the expression of recombinant group I and II mite allergens as various fusion proteins in either *E. coli* or *P. pastoris*. Whereas all chimeras are expressed in bacteria, only fusion proteins comprising proDer p 1 are produced in yeast. Extensive characterization of such chimeras indicates that the two mite allergens are recognized by specific antibodies and T lymphocytes, despite being only partially folded.

Material and Methods

Construction of Mite Fusion Chimeras

Four fusion proteins assembling Der p 1 and Der p 2, termed F1 to F4 were constructed. Detailed cloning methods and used primers are described in the online repository available at the journal site (see online supplementary tables 1 and 2, www.karger.com/doi/10.1159/000312631). DNA matrices pPICZ α -Der p 1 and pPICZ α -Der p 2 (gift from Dr. Ronald van Ree, Sanquin, Amsterdam, Netherlands) were used to generate Der p 1/Der p 2 fusions by an overlap-PCR strategy. Alternatively, codon-optimised sequences were synthesised by Genecust (Evry, France) or Genearth (Regensburg, Germany) with a mutation of the initial amino acid of the first moiety to a serine residue, in order to facilitate cleavage of the amino-terminal methionine. To abrogate cysteine protease activity (Enz- forms), the cysteine in the active site of Der p 1 was substituted by a valine residue (C132V).

Fusion-coding ORFs were subcloned into pIX2.0 according to the manufacturer's instructions (Qiagen, Les Ulis, France) to generate His-tagged fusions by in vitro or in vivo expression. They were also subcloned into pET24 a (+) (Novagen, Nottingham, UK) at *NdeI* and *NotI* sites and in frame with the α -factor sequence signal into pPICZ α (Invitrogen, Cergy Pontoise, France) at the *XhoI* and *NotI* sites.

Protein Expression in *E. coli*

Fusion proteins were expressed in BL21 (DE3) and Origami2 (DE3) *E. coli* strains (Novagen, Nottingham, UK) with or without pLysS plasmid. Cells were transformed with either one of the various plasmids by the heat shock method, according to the manufacturer's instructions. Transformed clones were selected on LB plates containing appropriate antibiotics (50 μ g/ml carbenicilline or 30 μ g/ml kanamycine with or without 34 μ g/ml chloramphenicol; all from Sigma-Aldrich, Lyon, France). Construct integrity was systematically confirmed by PCR. For recombinant protein expression, single colonies were inoculated and grown overnight at 37°C in LB medium plus antibiotics. Cultures were then diluted 1:100 and grown up to 0.4–0.8 OD_{600nm}. Target protein expression was induced with 1 mM IPTG. Cell pellets were collected by centrifugation and stored at –20°C for further analysis. For lysis, cell pellets were frozen in liquid nitrogen and thawed at 37°C five times, then resuspended in lysis buffer (50 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA) and incubated on a rotator for 30 min at 4°C. Samples were then sonicated (10 times for 10 s, 50 W, on ice) and clarified by centrifugation. Both lysis supernatants and pellets were subjected to Western blot analysis. Inclusion bodies were solubilised in 8 M urea buffer. His-tagged proteins were purified by nickel affinity chromatography on a His-Trap column (GE Healthcare, Orsay, France) under denaturing conditions using 20 mM sodium phosphate pH 7, 0.5 M NaCl, 0.05% Tween 20, 8 M urea, 1 mM DTT, 0.03 M imidazole as binding buffer. Proteins were eluted in 20 mM sodium phosphate pH 7.8, 0.05% Tween 20, 8 M urea, 1 mM DTT, 1 M imidazole, and then stored at –20°C after purification in elution buffer. Endotoxin levels in 10 μ g/ml fusion protein solutions measured by a Limulus Amoebocyte Lysate assay range from 15 to 377 EU/ml.

Refolding of Fusion Proteins out of Inclusion Bodies

Cell pellets obtained from *E. coli* cultures were resuspended in 1/25 culture volume with lysis buffer (50 mM Tris-HCl pH 8.0, 50

mM NaCl, 1 mM EDTA), frozen/thawed 5 times and sonicated 3 times for 7 min on ice (UP 200S, cycle 50% active, amplitude 80%; Hielscher, Teltow, Germany). Soluble and insoluble fractions were separated by centrifugation (30 min at 4°C at 46,000 g). The insoluble fraction was resuspended v/v in 2 M NaCl, incubated for 1 h at 4°C under shaking and spun down for 30 min at 4°C and at 46,000 g. The same steps were repeated successively in the presence of 2% X100 Triton and 1 M urea, respectively. Inclusion bodies were then solubilised in 8 M urea (1/100 of the initial culture volume). After an overnight incubation at 8°C under shaking, samples were centrifuged for 30 min at 4°C at 46,000 g. Urea-solubilised proteins were diluted 1:20 in refolding buffer and incubated overnight at 4°C and centrifuged afterwards at 46,000 g and 4°C for 30 min. After visual check for the presence of a precipitate, supernatants were further analyzed by both SDS-PAGE under reducing or non-reducing conditions followed by Coomassie Blue staining, as well as Western blot and size exclusion chromatography. Online supplementary table 2 gives an overview of selected refolding conditions yielding soluble fusion proteins.

Protein Expression in *P. pastoris*

Fusion proteins were expressed in methanotrophic *P. pastoris* GS115 or X33 yeast strains, according to the manufacturer's instructions (Invitrogen). pPICZα plasmids containing the different constructs were first digested with *SacI* and used to transform yeast cells by electroporation. Positive clones were selected on YPD zeocine (100 µg/ml) plates and construct integration was confirmed by PCR. Selected clones were inoculated in buffered glycerol complex medium (BMGY pH 6) and grown overnight at 30°C up to 2–6 OD_{600nm}. Cells were then diluted in buffered methanol complex medium (BMMY pH 6) to an initial OD_{600nm} of 1 and further grown for 24 h at 30°C. Culture supernatants were harvested after centrifugation and stored at –20°C until use. The F2 (Der p 2-proDer p 1) fusion protein expressed in yeast was purified by weak anionic exchange chromatography onto a DEAE column (GE Healthcare, Saclay, France). Briefly, supernatants (20 ml) were thawed and diluted 10-fold in binding buffer (50 mM Tris HCl, pH 9) and applied onto a 20-ml CV DEAE column. After washing with binding buffer, columns were submitted to a linear gradient of 50 mM Tris HCl, 1 M NaCl pH 7.5 buffer with fusion proteins being eluted at 150 mM NaCl. Collected fractions were pooled and concentrated onto an Amicon Cell, using a 10-kDa cut-off membrane (Millipore, St Quentin en Yvelines, France) to at least 100 µg/ml as measured by Bradford assay. Concentrated proteins were aliquoted and stored at –20°C until characterization.

SDS-PAGE and Western Blot Analysis

Proteins were separated by SDS-PAGE using Nupage 4–12% Bis-Tris acrylamide gels with MES buffer under non-reducing conditions and then transferred onto nitrocellulose membranes, as per the manufacturer's instructions (Invitrogen). Apparent molecular masses were estimated using the SeeBlue Plus2 ladder (Invitrogen). Membranes were treated with Qentix Western blot signal enhancer (Pierce, Rockford, Ill., USA) prior to blocking non-specific sites with TBS buffer containing 1% non-fat milk (BioRad, Marne La Coquette, France). Specific antibodies were added overnight in TBS buffer supplemented with 0.2% non-fat milk and 0.1% Tween 20. Antibodies used included the anti-Dpx antibody (0.2 µg/ml; Indoor Biotechnologies Limited, Warmin-

ster, UK) as well as Kori 221B and Kori 75B mouse monoclonal antibodies (0.2 µg/ml; Stallergènes, Antony, France) directed to Der p 2 or Der p 1, respectively, the P1B anti-Der p 1 sheep polyclonal antibody (1:1,000; a gift from Dr. R. van Ree) or a serum pool from HDM-allergic patients (1:30). Secondary antibodies included, as appropriate, either an HRP-conjugated sheep anti-mouse IgG (1:1,000; Sigma-Aldrich), a mouse anti goat/sheep IgG (1:5,000; Sigma-Aldrich) or a rabbit anti-human IgE (1:1,000; Dako, Trappes, France) followed by an HRP-conjugated goat anti-rabbit IgG (1:10,000; Calbiochem, Nottingham, UK). Detection was performed using either the SuperSignal West Pico or West Femto chemiluminescent substrates (Pierce) and a Chemismart 2000 CCD camera (Vilbert Lourmat, Marne la Vallée, France).

Natural and Recombinant Der p 1 and Der p 2 Major Allergens

Natural Der p 1 and Der p 2 allergens were purified from a *D. pteronyssinus* extract [1] by two chromatographic steps. The two allergens were first separated onto a phenyl sepharose column (GE Healthcare) using an ammonium sulfate gradient. Der p 1 was further purified by chromatofocusing onto a PBE 94 column (GE Healthcare) using a gradient starting from pH 7.5 to 4. As for Der p 2, it was submitted to ion exchange chromatography onto a SP XL column (GE Healthcare Saclay, France). Recombinant proDer p 1 expression and purification from tobacco plants were described elsewhere [16]. Recombinant Der p 2 was expressed in *P. pastoris* and purified by ion exchange chromatography. After loading onto an SP XL column (GE Healthcare), it was eluted with 10 mM Na acetate 150 mM NaCl pH 5. Fractions containing rDer p 2 allergens were pooled, concentrated by ultrafiltration using an Amicon PM-3 membrane (Millipore) and dialysed against PBS.

Deglycosylation with Endoglycosidase H

Proteins were placed in a 50 mM sodium acetate pH 5.2 buffer by buffer exchange with Vivaspin 500 filters (PES MWCO 10 kDa; Sartorius, Palaiseau, France). Subsequently, 0.04% (w/v) SDS was added and 50-µl samples were then incubated with or without 5 mU endoglycosidase H (Roche Diagnostics, Meylan, France) at 37°C for 4 h prior to SDS-PAGE analysis.

Determination of Cysteine Protease Activity

Cysteine protease activity was measured as described by Schulz et al. [24] using a fluorescent peptidic substrate coupled to AMC (Z-FR-AMC; Interchim, Monluçon, France) in 96-well plates. A *D. pteronyssinus/D. farinae* freeze-dried extract was used as a positive control (0.0125 µg/ml). Proteins were first diluted at 0.12 µg/ml in incubation buffer (50 mM sodium phosphate pH 7, 1 mM EDTA, 1 mM DTT). Sample volumes were adjusted to 62.5 µl with incubation buffer and 5 µl of 1 mM DTT was added for 5 min under shaking at 37°C. Subsequently, 62.5 µl of either DMSO and 125 µl of incubation buffer were added. After adding 25 µl of Z-FR-AMC substrate (40 µM in DMSO) or DMSO for 30 min under shaking at 37°C, fluorescence intensity was measured with λ_{ex} = 385 nm and λ_{em} = 465 nm.

Structural Analyses

Circular dichroism structural analyses were performed as follows to evaluate the secondary structure content of proteins. Material refolded in buffer B19 (online suppl. table 2) was filtered (0.45 µm) and loaded at 23 cm/H on a Superdex S200 Hi Load size

exclusion chromatography column (GE Healthcare). Two-milliliter fractions were collected with PBS as elution buffer. Scans were acquired in the far UV at a 1- μ M concentration on a Jasco J-810 spectropolarimeter (Jasco, Bouguenais, France) with a scan speed of 50 nm $\cdot$ min⁻¹ at 20°C. Spectra were recorded as the average of 10 scans between 195 and 250 nm using 0.1-cm path length cells with the baseline established with buffer spectra. Similar experiments were performed with nDer p 1 and nDer p 2, both purchased from Indoor Biotechnologies Limited (Warminster, UK), and an equimolar mix of those molecules.

For disulfide bridges mapping, protein samples were treated with urea, alkylated with iodoacetamide and digested with trypsin (Promega, Madison, Wisc., USA). Digests were applied on a LTQ mass spectrometer (Thermo, Waltham, Mass., USA) using an Agilent 1100 nano pump (Santa Clara, Calif., USA), 100 μ m $\times$ 10 cm, 5 μ m C18 analytical and 200 μ m $\times$ 1 cm, 5 μ m C18 trapping columns. Peptides were eluted with a linear gradient ranging from 5 to 45% acetonitrile developed over 120 min at room temperature, at a flow rate of 500 nl/min, and effluents were electrosprayed into the LTQ mass spectrometer. The source was operated in a positive ion mode with 3.0 kV electrospray potential, a sheath gas flow of 15 arbitrary units, and a capillary temperature of 200°C. Data were collected in the 'Triple-Play' mode (MS scan, Zoom scan and MS/MS scan) using Xcalibur 2.0 software (Thermo).

Basophil Activation Test

Basophil activation was measured by flow cytometry (FC500; Beckman Coulter, Villepinte, France) using the Allergenkit (Beckman Coulter) as described elsewhere [16]. Briefly, heparinised whole blood samples were obtained from 3 HDM-allergic patients after informed consent and incubated in absence or presence of various concentrations of fusion proteins or natural Der p 1 and Der p 2 at 37°C for 15 min. Once excluding T lymphocytes (CD3+ cells), activated basophils were detected by dual staining with anti-CRTH2 and anti-CD203c antibodies. Whole blood cells incubated with either PBS or anti-IgE antibodies were used as negative and positive controls, respectively.

T-Cell Proliferation Assay

Der p 1- and Der p 2-specific polyclonal CD4+ T cell lines were generated from PBMCs of 3 HDM-allergic patients as previously described [16]. PBMCs were isolated from heparinised venous blood and separated by gradient centrifugation. Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin (all from Invitrogen) and 10% human AB serum (PAA, Les Mureaux, France) at a density of 5 $\times$ 10⁶ cells/ml and cultured in presence of 10 μ g/ml of either natural purified Der p 1 or Der p 2. Cells were fed every 3–4 days with 20 U/ml IL-2 starting at day 5. Cultures were subsequently restimulated after 2 weeks with natural allergens (10 μ g/ml) and left in medium without IL-2 for at least 3 days prior to performing proliferation assays.

Fusion proteins stored in 8 M urea were dialysed against RPMI medium. Der p 1- or Der p 2-specific T cells were resuspended at 10⁷ cells/ml in serum-free PBS and incubated with 0.8 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oreg., USA) for 10 min at 37°C. CFSE labelling was stopped by adding an equal volume of medium containing

10% human AB serum. After several washes, 0.5 $\times$ 10⁶ cells/well were plated in 48-well plates in RPMI medium containing 10% human AB serum and stimulated with 10 μ g/ml of the various allergens or medium alone as a negative control. After 3 days, IL-2 was added and after 7 days, cells were harvested, washed and incubated with PC5-conjugated anti-CD4 and PC7-conjugated anti-CD3 antibodies (Beckman Coulter) for 15 min at 4°C. Proliferating CD3+CD4+ T cells were identified by cytofluorometry (FC500; Beckman Coulter) as cells with a decreased CFSE fluorescence intensity.

Results

Der p 1/Der p 2 Fusion Proteins Are Expressed in both E. coli and P. pastoris

We engineered 4 categories of fusion proteins, termed F1 to F4, assembling Der p 2 (isoform 2.0101) at the N- or C-terminal end of either mature Der p 1 (isoform 1.0105) or proDer p 1 (fig. 1). Constructs were designed to be expressed in *E. coli* cytoplasm either as N-terminally His-tagged or non-tagged forms. In parallel, the same constructs were expressed as secreted proteins in *P. pastoris* after fusion with the yeast α -mating factor signal peptide. DNA encoding fusion proteins were obtained by an overlap PCR strategy or gene synthesis. Table 1 provides an overview of the various fusion proteins which have been produced in either *E. coli* or *P. pastoris*, termed Fx_{ec} and Fx_{pp}, respectively.

Production in *E. coli* resulted in expression of the four F1 to F4 fusion proteins as inclusion bodies. Interestingly, only the two proDer p 1-containing fusions, F2 and F4, were successfully produced in *P. pastoris* (fig. 2a, b). The apparent molecular masses of proteins produced in *E. coli* matched their theoretical molecular mass (that is 39 kDa and 48 kDa for F1_{ec}/F3_{ec} and F2_{ec}/F4_{ec}, respectively). Noteworthy, for unclear reasons, F3_{ec} and F4_{ec} fusions consistently migrated at slightly lower apparent molecular masses, when compared with F1_{ec} and F2_{ec}, respectively. MALDI-TOF analyses performed on F1_{ec} and F3_{ec} fusions confirmed that the two proteins have very similar molecular masses (data not shown) and that apparent differences observed during SDS-PAGE are neither due to a premature translation stop nor to protein cleavage. Also, F3_{ec} migrated as a more diffuse band when compared with F1_{ec} (fig. 2b). As expected, the two F2_{pp} and F4_{pp} fusion proteins were hyperglycosylated (fig. 2c) in agreement with other studies demonstrating that multiple N-glycosylation sites are used within Der p 1 following expression in yeast [18, 22, 25]. As summarized in table 1, most chimeras, including forms in which the cysteine

Fig. 1. Schematic representation of Der p 1-Der p 2 fusion proteins. HDM allergen fusions encompassing Der p 2 and either mature Der p 1 or proDer p 1 were engineered. Fragments of each allergens included in fusion proteins are numbered relatively to the amino acid sequences of proDer p2 and preproDer p1, respectively. Numbering above each representation refers to boundaries within each fusion protein.

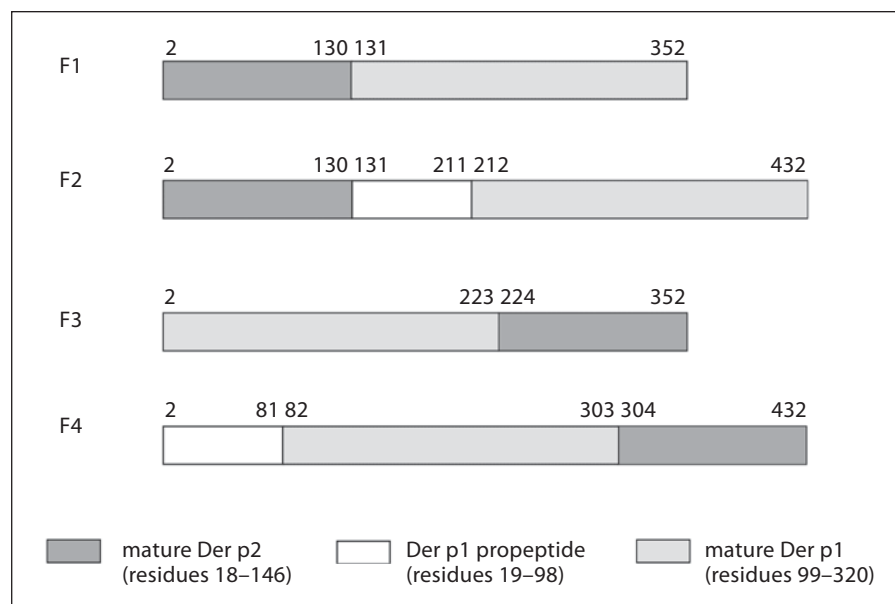


Table 1. Properties of Der p 1/Der p 2 fusion proteins expressed in *E. coli* (ec) and *P. pastoris* (pp)

Fusion proteins	Characteristics	Protein yield after purification ^b	Stability, homogeneity, solubility	Characterization tests performed on purified proteins
F1 _{ec} ^a , F2 _{ec} ^a , F3 _{ec} ^a , F4 _{ec} ^a	Expressed as inclusion bodies in <i>E. coli</i> either His-tagged or not Preserved catalytic site in Der p 1 moiety	30 mg/l for all proteins, except F4 _{ec} (16 mg/l) ^c	Degradation products are observed before purification in cell lysates for all fusion proteins Tendency to form aggregates and degradation following urea removal ^d Presence of multimers after refolding documented for F1 _{ec} and F3 _{ec}	His-tagged proteins were used without refolding in both IgE recognition, basophil activation and T cell proliferation assays Non-tagged proteins were used for refolding experiments and subsequent structural analyses
F1 _{ec} Enz-, F2 _{ec} Enz-, F3 _{ec} Enz-, F4 _{ec} Enz-	Expressed as inclusion bodies in <i>E. coli</i> Mutated enzymatic site (C132V) in Der p 1 moiety	ND	Degradation products are observed in cell lysates for all fusion proteins Presence of multimers after refolding documented for F1 _{ec} Enz- and F3 _{ec} Enz-	The non-His-tagged F1 _{ec} Enz- and F3 _{ec} Enz- proteins were submitted to refolding experiments and subsequent structural analyses
F2 _{pp} , F4 _{pp}	Expressed in <i>P. pastoris</i> as secreted proteins in culture supernatants Native catalytic site in Der p 1 moiety F1 _{pp} and F3 _{pp} could not be expressed	8 mg/l (F2 _{pp})	Soluble, monomeric, hyperglycosylated F4 _{pp} unstable during purification Degradation (i.e. cleavage products) observed with F2 _{pp} after purification	F2 _{pp} was used in basophil activation tests

ND = Not determined.

^a Similar results were obtained with His-tagged and non-tagged chimeras. ^b Expressed as equivalent BSA, estimated by Bradford assay. ^c Yield determined with His-tagged proteins. ^d Aggregates and degradation products have been observed upon extensive dialysis to remove urea deleterious for T cells.

protease activity had been abrogated by point mutation, were partially degraded during expression as evidenced by the presence of low molecular mass degradation products (fig. 2). Also, all fusion proteins expressed in *E. coli* tend to form multimers and aggregates. Neither a decreased IPTG concentration nor a reduced growth tem-

perature during expression and induction phases allowed soluble expression. Expression of fusion proteins in *E. coli* Origami strain, in which disulfide pairing and folding are facilitated, did not improve protein solubility (data not shown). During purification by anion-exchange chromatography, both F2_{pp} and F4_{pp} fusion proteins also

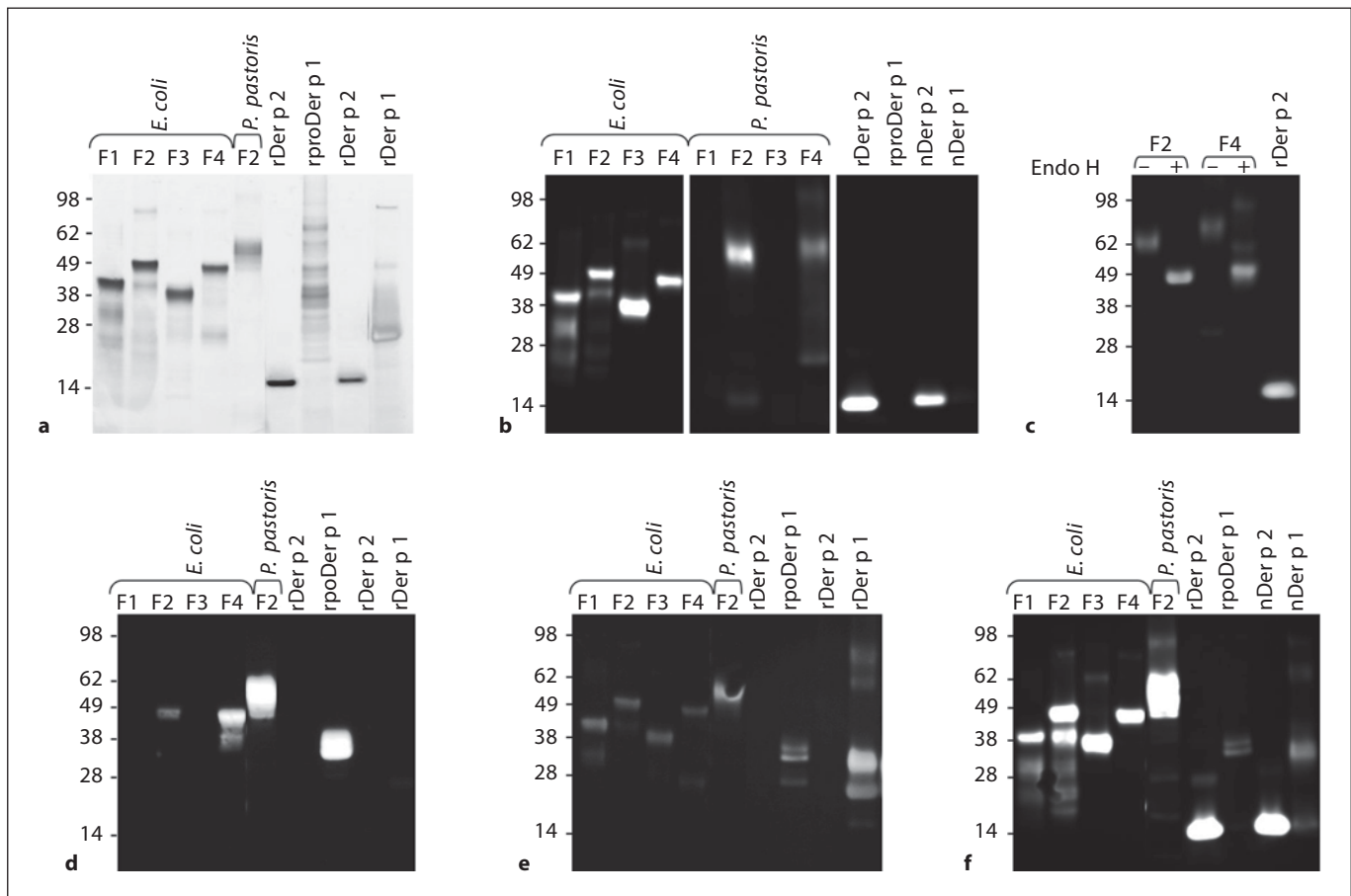


Fig. 2. Immunoblot analysis of Der p 1-Der p 2 fusion proteins produced in *E. coli* and *P. pastoris*. Western blot analysis of fusion proteins (100 ng) expressed in *E. coli* (ec) and *P. pastoris* (pp) was performed under non-reduced conditions with different antibodies. All analyses were done using purified protein except **b** (middle panel) and **c** for which proteins from *P. pastoris* culture supernatants were used. Natural and recombinant Der p 2 (100 ng), re-

combinant proDer p 1 (100 ng) and natural Der p 1 (750 ng) are used as controls. **a** Silver staining of purified fusion proteins. **b** Detection with anti-Dpx (anti-Der p 2). **c** Recognition with anti-Dpx after deglycosylation with EndoH of F2_{pp} and F4_{pp}. **d** and **e** Detection with anti-Der p 1 Kori75B and P1B antibodies, respectively. **f** Detection with a serum pool from HDM-allergic patients.

proved to be very unstable with appearance of multiple degradation products. Nevertheless, we were able to purify F1_{ec}, F2_{ec}, F3_{ec}, F4_{ec}, and in lower amounts F2_{pp}, to perform characterization studies.

Immunoreactivity of Der p 1/Der p 2 Fusion Proteins

Antibody reactivities of fusion proteins were first assessed by immunoblot and recognition by either anti-Der p 2, anti-Der p 1 (monoclonal or polyclonal) antibodies or seric IgE from HDM-allergic patients. All fusion proteins reacted well with anti-Der p 2 antibodies (fig. 2b). The Kori75B murine monoclonal antibody only detected forms containing the Der p 1 propeptide, whereas the P1B polyclonal antibody reacted with the five fusion proteins

(fig. 2d, e). All of them were also recognized by a serum pool from allergic patients (fig. 2f). Experiments conducted after depletion of either Der p 1- or Der p 2-specific IgEs suggested that the two mite allergens are recognized within the fusions by patient IgEs, with possibly a better reactivity with Der p 2 (data not shown). The latter result could be explained by a better folding of Der p 2 versus Der p 1 within the fusions, or by the presence of linear IgE epitopes within Der p 2, as previously described [26, 27]. Alternatively, it could reflect the well-known poor detection of Der p 1 in immunoblots [5]. Collectively, these results establish that all fusion proteins are properly recognized by a set of specific antibodies directed to either Der p 1 or Der p 2 molecules.

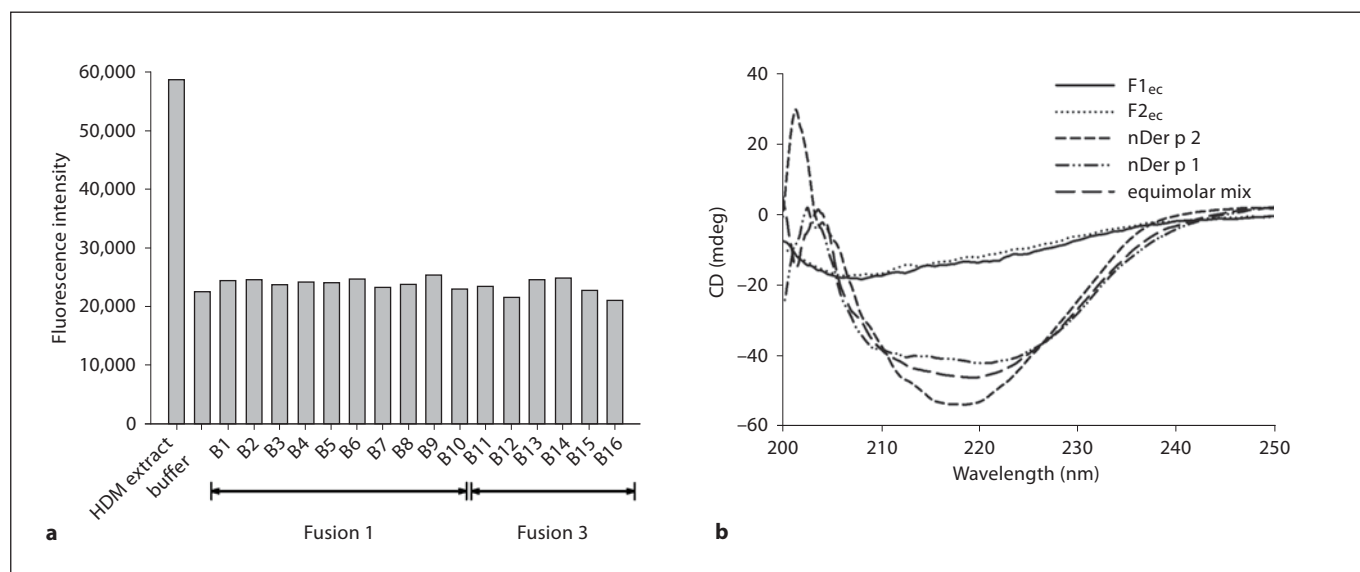


Fig. 3. Structural analysis of Der p 1-Der p 2 fusion proteins and cysteine protease activity. **a** Cysteine protease activity was measured after cleavage of an AMC-coupled fluorescent peptidic substrate as described in Material and Methods. F1_{ec} and F3_{ec} molecules were tested after refolding. Each bar represents a different refolding condition. A *D. pteronyssinus*/*D. farinae* extract is used as a positive control and buffer alone plus Z-FR-AMC substrate is used as negative control. **b** Secondary structure of F1_{ec} and F3_{ec}

were analysed by circular dichroism. Fusion proteins were refolded in B19 buffer (online suppl. table 2) under conditions yielding both soluble and monomeric proteins after purification by SEC chromatography using PBS as an elution buffer. Near-UV spectra were recorded as the average of 10 scans. CD spectra were superimposed with those of nDer p 1, nDer p 2 and an equimolar mix of these two molecules.

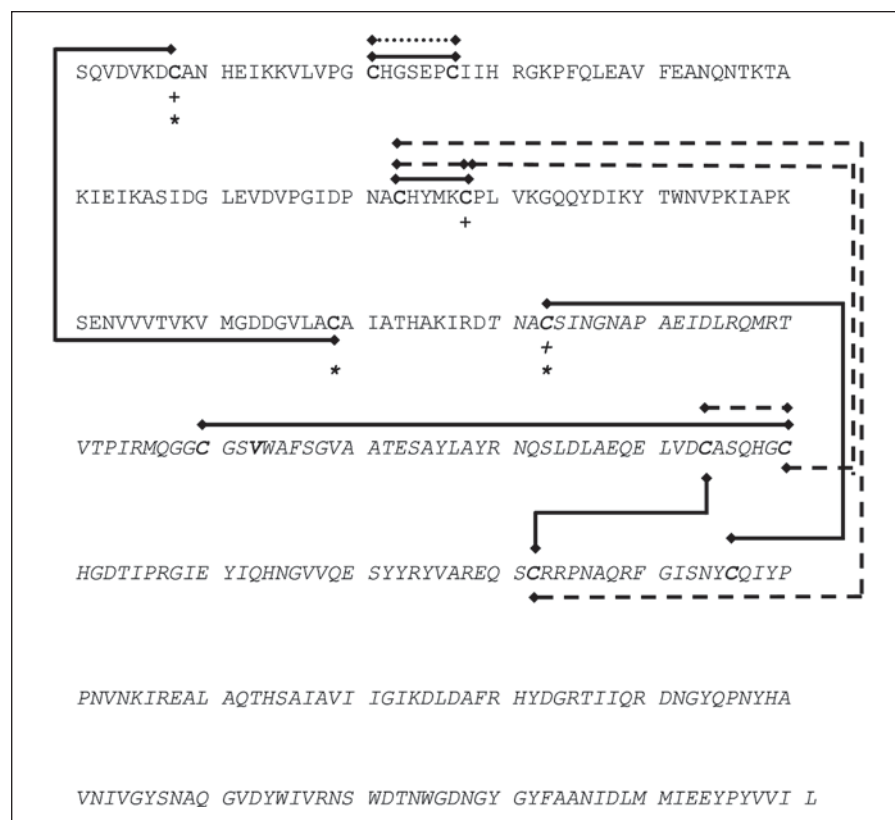
Structural Analyses of Fusion Proteins Produced in *E. coli*

Given their relative stability and thus the possibility to purify sufficient amounts of material, we focused in those analyses on F1_{ec} and F3_{ec} fusion proteins. Purification by anion-exchange chromatography before refolding resulted either in the absence of binding to the chromatographic column or in aggregation in the binding buffer, thus pointing to a need for refolding. In this context, multiple pH, salt, redox potential and detergent conditions were screened as described in Material and Methods (and in the online repository) to allow proper refolding of fusion proteins. Solubility was first assessed by checking visually the presence of precipitates. Samples were then analysed by SDS-PAGE, Western blot and size exclusion chromatography. Of 45 and 74 conditions tested for F1_{ec} and F3_{ec}, respectively, none resulted in a fully satisfactory refolding of the molecules, with multimers still observed under most conditions.

Molecular modelling predicts that F1 (Der p 2-Der p 1) and F3 (Der p 1-Der p 2) adopt slightly different conformations (data not shown), but that the enzymatic active site is likely accessible to the substrate in both fusion

proteins. We further reasoned that proper folding of the Der p 1 molecule could be assessed by measuring the cysteine protease activity. Focusing on refolding conditions yielding monomeric F1 or F3 fusion proteins, we evaluated the capacity of those proteins to cleave a fluorescent AMC-coupled substrate. As shown in figure 3a, no proteolytic activity was detected in any of the fusion proteins when compared to the negative control (buffer alone), irrespective of refolding conditions, whereas an enzymatic activity was clearly found in HDM extract. Thus, we infer from these results that Der p 1 folding is likely incomplete in the fusion proteins, even if we cannot exclude that the proximity of Der p 2 could also preclude access of the substrate to the active site, due to steric hindrance. We subsequently analysed by circular dichroism secondary structures on F1 and F3 fusions obtained under a condition leading to monomeric molecules as judged by SEC chromatography. CD spectra of F1_{ec} and F3_{ec} established the presence of some secondary structures in both fusions. Nonetheless, comparison with CD spectra obtained from purified nDer p 1, nDer p 2 or an equimolar mix of those molecules indicates that fusion proteins are only partially folded (fig. 3b).

Fig. 4. Mapping of disulfide bridges in Der p 2-Der p 1 F1 fusion protein. Disulfide bridges were mapped by mass spectrometry analysis of tryptic digests. Theoretical disulfide bonds (plain line) and those observed for F1_{ec} Enz- under two distinct refolding conditions [B17 (dashed line) or B18 (dotted line)] are positioned with the primary sequence of the fusion protein. Crosses and asterisks indicate free cysteines observed in solution B17 and B18, respectively.

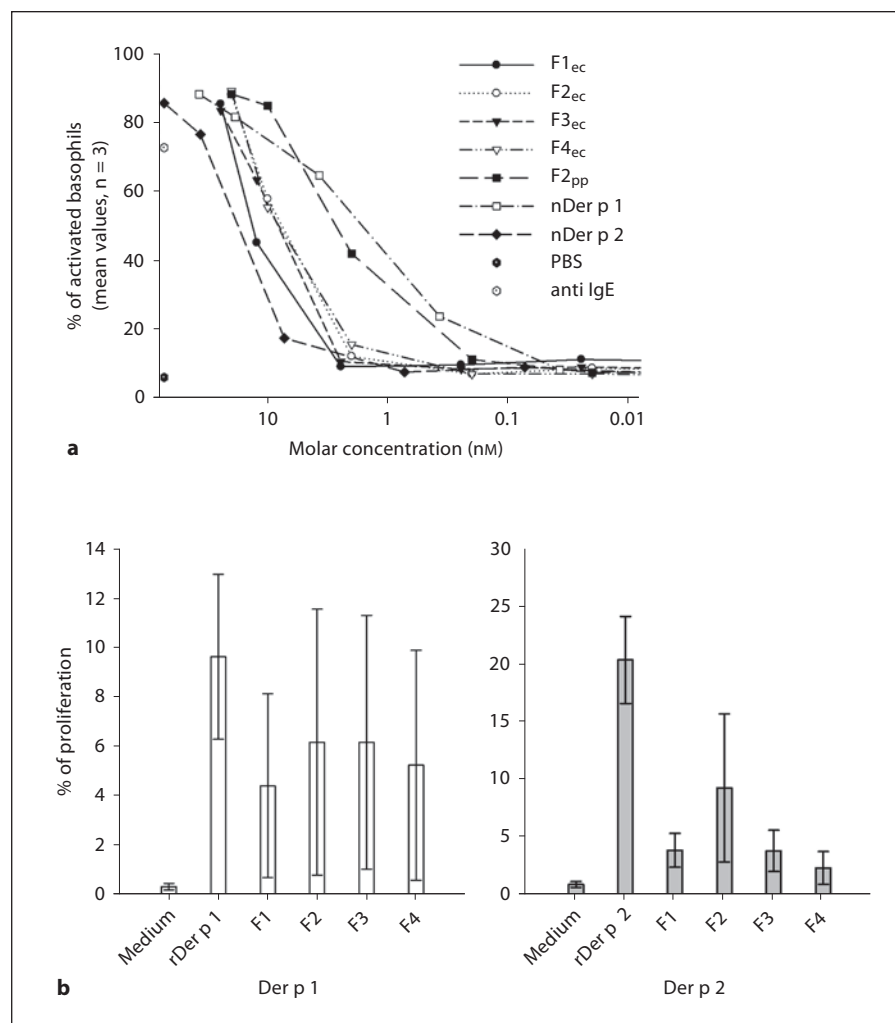


To further explore why Der p 1/Der p 2 fusion molecules form multimers and aggregates, we also investigated whether cysteine residues present in those chimeras are engaged in inappropriate intermolecular bridging, as suggested by differences in behaviour under reduced versus non-reduced SDS-PAGE conditions or in SEC analyses (data not shown). To prevent the possible involvement of the cysteine residue from the Der p 1 active site in such disulfide bridges, we focused on Enz- forms (C132V) in those experiments (table 1). Although F3_{ec} Enz- was highly heterogeneous and multimeric, soluble monomeric F1_{ec} Enz- molecules were obtained after refolding and submitted to peptide-mapping analysis. As shown in figure 4, of 6 expected disulfide bridges (that is 3 in each moiety) [25, 28, 29], only 2 correct proximal bonds were found. Multiple additional irrelevant disulfide bridges were detected, for example between Der p 1 and Der p 2. Other cysteine residues were unengaged or unassigned, demonstrating significant molecular heterogeneity with respect to disulfide bonds. Altogether, these results establish that fusion proteins are only partially folded and that inappropriate cysteine pairing is likely causing intermolecular association and aggregation.

Basophil and T Cell Activation with Fusion Proteins

Fusion proteins were compared to either purified Der p 1 or Der p 2 for their ability to activate basophils from 3 HDM-allergic patients, as detected by the up-regulation of surface expression of the CD203c activation marker in presence of serial allergen concentrations. In agreement with patterns of IgE reactivity previously observed (fig. 2f), all fusion proteins activated basophils in a comparable manner, with the exception of F2_{pp} which was significantly more efficient than the other chimeras (fig. 5a). Furthermore, the capacity of fusion proteins expressed in *E. coli* to stimulate T lymphocytes was assessed using CD4+ T cell lines specific for either Der p 1 or Der p 2 derived from 3 HDM-allergic donors. As shown in figure 5b, all fusion proteins were recognized by CD4+ T cells, albeit to a lower level when compared to single allergens. The reason for such a difference is presently unclear but could be due to aggregates and degradation products observed following extensive dialysis needed to remove urea otherwise toxic to T lymphocytes (data not shown).

Fig. 5. Immunological characterisation of Der p 1-Der p 2 fusion proteins. **a** Basophils from HDM-allergic donors ($n = 3$) were exposed to varying amounts of fusion proteins. The expression of the CD203c activation marker was determined by cytofluorometry among CRTH2 cells. Data are represented as mean percentage values of CD203c^{high} basophils obtained in the 3 independent experiments in presence of varying concentrations of allergens. **b** Three distinct CD4⁺ T cell lines specific for either Der p 1 or Der p 2 were derived from independent HDM allergic patients as described in Material and Methods. Cells were stimulated with 10 $\mu\text{g}/\text{ml}$ of either Der p 1-Der p 2 fusion proteins and recombinant Der p 1 or Der p 2 molecules (left and right panels, respectively). Results are expressed as mean percentages of proliferating T cells ($\pm$ SEM) obtained in the 3 experiments in presence of allergens.



Discussion

Second-generation allergy vaccines will likely rely upon well-defined recombinant allergens. When multiple allergens are involved in patient sensitisation, a valid option is to create hybrid molecules assembling those allergens. This approach, which has previously been applied to grass and *Parietaria* pollens, bee and wasp venom allergens and more recently to mite [8–10, 30–32], leads to the production of a single molecule, thus facilitating manufacturing and development, whilst establishing a fixed molar ratio between allergens. With the aim to develop a recombinant candidate vaccine against respiratory allergies associated with common HDM species, we applied this strategy to two major allergens from the *D. pteronyssinus* species, namely Der p 1 and Der p 2, in the present study.

Four distinct chimeras encompassing Der p 1 and Der p 2 in the absence of any in-between linker sequences have been engineered and expressed in *E. coli*, irrespective of the presence of the Der p 1 propeptide or the relative orientation of the two moieties (that is Der p 1-Der p 2 vs. Der p 2-Der p 1). Der p 1/Der p 2 fusion proteins expressed in *E. coli* are found in inclusion bodies, thus requiring a refolding step to reach natural conformation. Interestingly, in contrast, only fusion proteins containing the Der p 1 propeptide were expressed in *P. pastoris*, in agreement with previous studies indicating that such propeptide is needed for Der p 1 expression in eukaryotic hosts [16–20].

We observed an inherent instability of all fusion proteins, manifested in the form of degradation products or aggregates. Thus, we focused for further characterization on fusion proteins obtained in *E. coli* as well as F2_{pp} for

which sufficient amounts could be obtained. All such hybrid molecules were reactive in Western blot with a set of monoclonal or polyclonal antibodies directed to either one of the two moieties as well as with seric IgEs from HDM-allergic patients. Our attempts to refold F1_{ec} and F3_{ec} led to partially refolded monomeric soluble proteins exhibiting secondary structure. Nonetheless, we failed to detect any cysteine protease activity in those fusion proteins, despite the prediction by computer models that the active enzymatic site is likely accessible to the substrate. That these fusion proteins are partially folded is confirmed by the presence of both proper but also inappropriate cysteine pairing detected by peptide mapping analysis in mass spectrometry. We also infer that engagement of free cysteine residues in intermolecular disulfide bonds is a cause for multimerisation and aggregation.

Interestingly, all HDM fusion proteins activate basophils, consistent with their capacity to react with specific IgE in Western blot. F2_{pp} was slightly more efficient in this regard, possibly as a consequence of a higher affinity for IgEs which could result in a stronger cross-linking of FcεRI receptors. Surprisingly, fusion proteins only elicit a weak, albeit readily detectable stimulation of Der p 1- and Der p 2-specific T cell lines. Although we cannot exclude that some T cell epitopes derived from the 2 allergens are poorly processed or presented by antigen-presenting cells out of fusion proteins, we rather favour that aggregation and degradation of fusion proteins occurring after dialysis in aqueous buffer is an explanation for the observed diminished T cell recognition.

The difficulty to completely refold Der p 1/Der p 2 fusion proteins might be the consequence of different folding characteristics or kinetics for the two moieties. Noteworthy, if Der p 2 alone has been previously successfully refolded from inclusion bodies [13], this has not yet been achieved for Der p 1. Whereas the proregion of cysteine proteases is critical for the folding of such enzymes [33, 34], recent data suggest that recombinant proDer p 1 remains prone to aggregation [35], indicating that the presence of the propeptide per se is not sufficient to ensure natural conformation. Although not shown, our preliminary refolding data on F2 and F4 suggest that such fusion proteins tend to remain insoluble and form aggregates. Beyond evidence of cysteine engagement in intermolecular bridges, head to tail fusion of the two allergens in the absence of a flexible linker might cause stringent structural constraints on the two moieties precluding refolding. The characterization in parallel studies of other recombinant allergen hybrids yielded disparate results with respect to their folding properties secondary structure [8–10, 30, 31].

For example, a *Parietaria judaica* Par j 1-Par j 2 fusion protein exhibited a complete loss of secondary structure [30], whereas a refolded grass pollen-chimeric protein assembling Phl p 1, 2, 5 and 6 had a CD spectrum equivalent to the mixture of corresponding allergens [9]. Noteworthy, no tendency to form aggregates has been reported for either of the hybrids described in those studies, suggesting that our own difficulties to express monomeric soluble Der p 1-Der p 2 chimeras are likely due to specific characteristics of those mite allergens. Consistent with this, Asturias et al. [10] recently described two hypoallergenic fusion proteins containing mature Der p 1 either combined with a truncated form of Der p 2 or flanked by the N and C halves of Der p 2. Although the design of these fusion proteins is not identical to ours, such Der p 1/Der p 2 hybrid proteins also formed aggregates [10].

Allergen multimerization and stability issues are critical for the development of recombinant allergy vaccines. In the present study, we obtained Der p 1-Der p 2 fusion proteins exhibiting significant molecular heterogeneity originating from inappropriate inter- and intra-molecular cysteine pairing, leading to partial folding. Even if soluble fusion proteins are obtained under selected conditions, misfolding and poor stability make them unsuitable for diagnostic or sublingual immunotherapeutic purposes for which 'natural-like' recombinant molecules are preferred [7]. In this context, we recommend that a fusion protein approach should be used for allergens easy to refold, or alternatively, for the development of hypoallergenic vaccines for which natural conformation is not an absolute requirement.

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Supplementary table 1: primers used for the construction of the Der p1/Der p2 chimeras fusion proteins

Dp2FW	GGTTGCTCTTCCAACGATCAAGTCGATGTCAAAGAT
Dp2MDp1FW	CATGCTAAAATCCGCGATACTTAACGCCTGCAGTATC
Dp2MDp1RV	GATACTGCAGGCGTTAGTATCGCGGATTTTAGCATG
Dp1-RV	GCGGCCGCTCAGAGAATGACAACATATGGATA
Dp2_FW_XhoI	GGGCTCGAGAAAAGAGATCAAGTCGATGTCAAAGAT
Dp1_RV_NotI	GGCGGCCGCTCAGAGAATGACAACATATGGATA
Dp2PDp1FW	CATGCTAAAATCCGCGATCGTCCATCATCGATCAAA
Dp2PDp1RV	TTTGATCGATGATGGACGATCGCGGATTTTAGCATG
Dp1_FW_XhoI	GGGCTCGAGAAAAGAACTAACGCCTGCAGTATC
Dp1Dp2FW	CCATATGTTGTCATTCTCGATCAAGTCGATGTCAAA
Dp1Dp2RV	TTTGACATCGACTTGATCGAGAATGACAACATATGG
Dp2_RV_NotI	GGCGGCCGCTCAATCGCGGATTTTAGCATGAGT
PDp1_FW_XhoI	GGGCTCGAGAAAAGACGTCCATCATCGATCAAAACT
Dp1Dp2FW	CCATATGTTGTCATTCTCGATCAAGTCGATGTCAAA
Dp1Dp2RV	TTTGACATCGACTTGATCGAGAATGACAACATATGG
His_Dp2_FW	ATCACCATCACCACGGTATGCAAGATCAAGTCGATGTCAAA
Dp1_RV	CTTGGTTAGTTAGTTATTAGAGAATGACAACATATGGATA
His_Dp1	ATCACCATCACCACGGTATGCAAACCTAACGCCTGCAGTATC
Dp2_RV	CTTGGTTAGTTAGTTATTAATCGCGGATTTTAGCATGAGT
His_PDp1_FW	ATCACCATCACCACGGTATGCAACGTCCATCATCGATCAAA

Supplementary table 2: Composition of buffers used to refold F1_{ec} and F3_{ec}

B1	50 mM Tris-Hcl pH8, 0.1 M NaCl, 2 mM GSSG, 4 mM GSH, 10% glycerol
B2	50 mM Tris-Hcl pH8, 0.1 M NaCl, 5 mM DTT, 0.2 M sucrose
B3	50 mM Tris-Hcl pH8, 0.1 M NaCl, 2 mM GSSG, 4 mM GSH, 0.1% Maltoside
B4	50 mM Glycine pH9, 0.1 M NaCl, 5 mM DTT, 0.1% Maltoside
B5	50 mM Glycine pH9, 0.2 M NaCl, 5 mM TCEP
B6	50 mM Glycine pH9, 4 mM GSSG, 8 mM GSH
B7	50 mM Glycine pH9, 5 mM TCEP, 0.1% NDSB 201
B8	50 mM NaPO ₄ pH7, 0.1 M NaCl, 5 mM DTT, 0.01% Maltoside
B9	50 mM NaPO ₄ pH7, 0.1 M NaCl, 5 mM DTT, 0.1% Maltoside
B10	50 mM NaPO ₄ pH7, 5 mM GSSG, 5 mM GSH, 0.1% Tween80, 0.1% DTAB
B11	50 mM Tris-HCl pH8, 0.1 M NaCl, 1 mM DTT, 0.2 M sucrose
B12	50 mM Tris-HCl pH8, 0.1 M NaCl, 5 mM DTT, 0.1% Maltoside
B13	50 mM Glycine pH9, 0.1 M NaCl, 5 mM DTT
B14	50 mM Glycine pH9, 0.1 M NaCl, 5 mM DTT, 0.2 M sucrose
B15	50 mM Tris-HCl pH8, 0.1 M NaCl, 1 mM DTT, 0.1% Tween80 , 0.1% DTAB
B16	50 mM Tris-HCl pH8, 0.1 M NaCl, 1 mM DTT, 0.1% CHAPS
B17	50 mM Tris-HCl pH8, 0.1 M NaCl, 1 mM CaCl ₂ 1 mM MgCl ₂ , 1 mM DTT, 0,05% SDS
B18	50 mM Tris-HCl pH8, 0.05 M NaCl, 5 mM GSSG, 5 mM GSH, 0,1% CTAB
B19	50 mM Tris-HCl pH8, 0.05 M NaCl, 1 mM EDTA, 5 mM GSSG, 5 mM GSH, 0.1% Tween80, 0.1%CTAB