Efficient soluble production of folded cat allergen Fel d 1 in *Escherichia coli*Chi Zhang, Rosario Recacha, Lloyd W. Ruddock, Antti Moilanen^{*}

Faculty of Biochemistry and Molecular Medicine, University of Oulu, Aapistie 7, 90220, Oulu, Finland

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ABSTRACT

The major cat allergen Fel d 1 is one of the most common and potent causes of animal related allergy. Medical treatment of cat allergy has relied on immunotherapy carried out with cat dander extract. This approach has been problematic, mainly due to inconsistent levels of the major allergen in the produced extracts. Recombinant DNA technology has been proposed as an alternative method to produce more consistent pharmaceuticals for immunotherapy and diagnostics of allergy. Current approaches to produce recombinant Fel d 1 (recFel d 1) in the cytoplasm of *Escherichia coli* have however resulted in protein folding deficiencies and insoluble inclusion body formation, requiring elaborate *in vitro* processing to acquire folded material. In this study, we introduce an efficient method for cytoplasmic production of recFel d 1 that utilizes eukaryotic folding factors to aid recFel d 1 to fold and be produced in the soluble fraction of *E. coli*. The solubly expressed recFel d 1 is shown by biophysical *in vitro* experiments to contain structural disulfides, is extremely stable, and has a sensitivity for methionine sulfoxidation. The latter is discussed in the context of functional relevance.

1. Introduction

Exposure to proteins from other species, either airborne or delivered via food or injection, results in an immune response in humans that may lead to allergic sensitization with symptoms ranging from discomforting rhinitis to life-threatening asthmatic inflammatory disease. The classical method to treat allergy is allergen-specific immunotherapy (SIT). During SIT, the allergen is typically administered via several injections of extracts derived from natural sources, to slowly increase tolerance to the allergen. The major disadvantages of using extracts for SIT is inconsistent levels of major allergens in the extracts, which may lead to ineffective and time-consuming SIT, and potential severe side effects [1]. Recombinant DNA technology has been proposed as an alternative production method, which could allow standardized therapeutic options and diagnostics [2].

Airborne protein allergens from domestic cat (*Felis domesticus*) are among the most common and potent causes of allergy worldwide [2]. The major cat allergen, Fel d 1, is known to cause an immune response for more than 90% of cat allergy patients [3]. Typical cat SIT is carried out with cat dander extract that has the above-mentioned disadvantage

of inconsistency. Two different recombinant Fel d 1 (recFel d 1) constructs have been successfully produced as inclusion bodies in *Escherichia coli* and then refolded to their native state [4,5]. While the natural Fel d 1 (nFel d 1) is a tetramer of two heterodimers, in the recFel d 1 molecules, the chains are typically fused together (Fig. 1A). One recFel d 1 fusion construct, that links the C-terminus of chain 2 to the N-terminus of chain 1 (Fig. 1A), forms a monomer, while the other fusion construct (chain 1 to chain 2) produces as a dimer [5,6]. Regardless of the oligomeric state, the nFel d 1 and the recFel d 1 fusion proteins contain identical disulfide patterns (Fig. 1), and each recFel d 1 construct characterized to date elicit IgE-mediated immunoreactivity equal to nFel d 1 [5–7]. Either recFel d 1 fusion protein could therefore act as an alternative allergen source for SIT.

The major obstacle for efficient production of recFel d 1 and many other recombinant allergens is that they do not fold properly in the cytoplasm of *E. coli* and form inclusion bodies. To obtain soluble protein, the recombinant allergen must first be solubilized from the inclusion bodies and then be refolded *in vitro* [5,6,8,9]. These elaborate methods decrease the efficiency of *E. coli* as a rapid and cost-effective production host. In this study, we introduce a method for soluble cytoplasmic

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; ESI-MS, electrospray ionization mass spectrometry; nanoDSF, nano differential scanning fluorimetry; NEM, N-ethylmaleimide; nFel d 1, natural Fel d 1; PDI, protein disulfide isomerase; PDB, Protein Data Bank; recFel d 1, recombinant Fel d 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIT, specific immunotherapy.

^{*} Corresponding author.

E-mail addresses: Chi.Zhang@oulu.fi (C. Zhang), Rosario.Recacha@oulu.fi (R. Recacha), Lloyd.Ruddock@oulu.fi (L.W. Ruddock), Antti.Moilanen@oulu.fi (A. Moilanen).

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expression of folded recFel d 1. We utilize a folding apparatus, CyDisCo, that co-expresses eukaryotic folding factors to carry out both catalysis of disulfide formation as well as isomerization of non-native disulfides to native states [10,11]. CyDisCo is capable of efficient cytoplasmic production of disulfide-containing proteins with wide range of structural and functional properties, including biologically active antibody fragments and enzymes, protein complexes, and extracellular matrix components [11–15]. It can be efficiently scaled up to bioreactors in chemically defined media [16]. We show that recFel d 1 can be produced solubly in the cytoplasm using CyDisCo with three disulfides and an extremely stable fold. A high sensitivity for methionine sulfoxidation was observed, and potential functional implications of the modification are discussed.

2. Materials and methods

2.1. Construction of expression plasmids

The previously described Fel d 1 fusion construct, which links the C-terminus of chain 2 to the N-terminus of chain 1 and contains a C-terminal His₆ tag, was utilized in this work (Fig. 1) [4]. A codon-optimized gene encoding for the fusion construct (GenScript) was subcloned into a modified pET23-based polycistronic vector that co-expresses the eukaryotic folding catalysts yeast Erv1p and human PDI [16]. A negative control plasmid was constructed in similar pET23 background but lacking the genes for Erv1p and PDI [10]. All plasmids were confirmed by DNA sequencing for correct insertion of genes.

2.2. Preparation of expression strains

Plasmids were transformed into *E. coli* strains K12 BW25113 and BL21(DE3) as described [15]. The transformants were plated on agar plates containing ampicillin (100 mg/L) and cultivated at +37 °C overnight. After two rounds of replating from a single colony, glycerol stocks were prepared from LB culture containing ampicillin (100 mg/L) and 16.7% glycerol. The glycerol stocks were stored at −70 °C.

2.3. Protein expression

Expression strains were streaked from glycerol stocks on agar plates containing ampicillin (100 mg/L) and incubated overnight at +37 °C. Starter cultures in LB, 50 mL primary cultures in chemically defined media, induction of protein expression, and cell harvesting were carried out essentially as described [15]. For the cultivations in rich medium, AIM terrific broth base media with trace elements (Formedium) and 0.8% glycerol was used as instructed by the manufacturer. Briefly, sterile media were inoculated with 1:100 (v/v) ratio of a starter LB culture, the primary culture was incubated at +30 °C/250 rpm for 16 h, protein expression was induced with 0.1 mM IPTG, cells were harvested 24 h later by centrifugation and resuspended to lysis buffer with lysozyme and DNase. After 15 min incubation at RT, the resuspensions were frozen at −20 °C. Cells were lysed by two rounds of freeze-thawing before purification.

2.4. Protein purification

300 mM NaCl was added to thawed lysates, followed by centrifugation (3200×g, 20 min, +4 °C). Cleared lysates were transferred to new tubes, and 30 mM imidazole was added to each cleared lysate. After filtration (0.45 μm), protein were bound to a 5 mL (column volume, CV) HisTrap column (GE Healthcare) pre-charged with nickel and pre-equilibrated with binding buffer (50 mM sodium phosphate, 300 mM NaCl, 30 mM imidazole, pH 7.4). The column was washed with 6 CV of binding buffer, and bound proteins were eluted with 1.5 CV of 50 mM sodium phosphate, 300 mM NaCl, 300 mM imidazole, pH 7.4. Buffer was exchanged to equilibration buffer (20 mM sodium phosphate, pH 7.4) with PD-10 columns (GE Healthcare).

Protein were further purified using a strong anion exchanger (6 mL Resource Q, GE Healthcare) pre-equilibrated with equilibration buffer. Protein were bound to the column at 2 mL/min flowrate, washed with 10 CV of equilibration buffer, and eluted by increasing [NaCl] (0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, and 1000 mM; 1.7 CV of each concentration) in equilibration buffer. Protein content of each

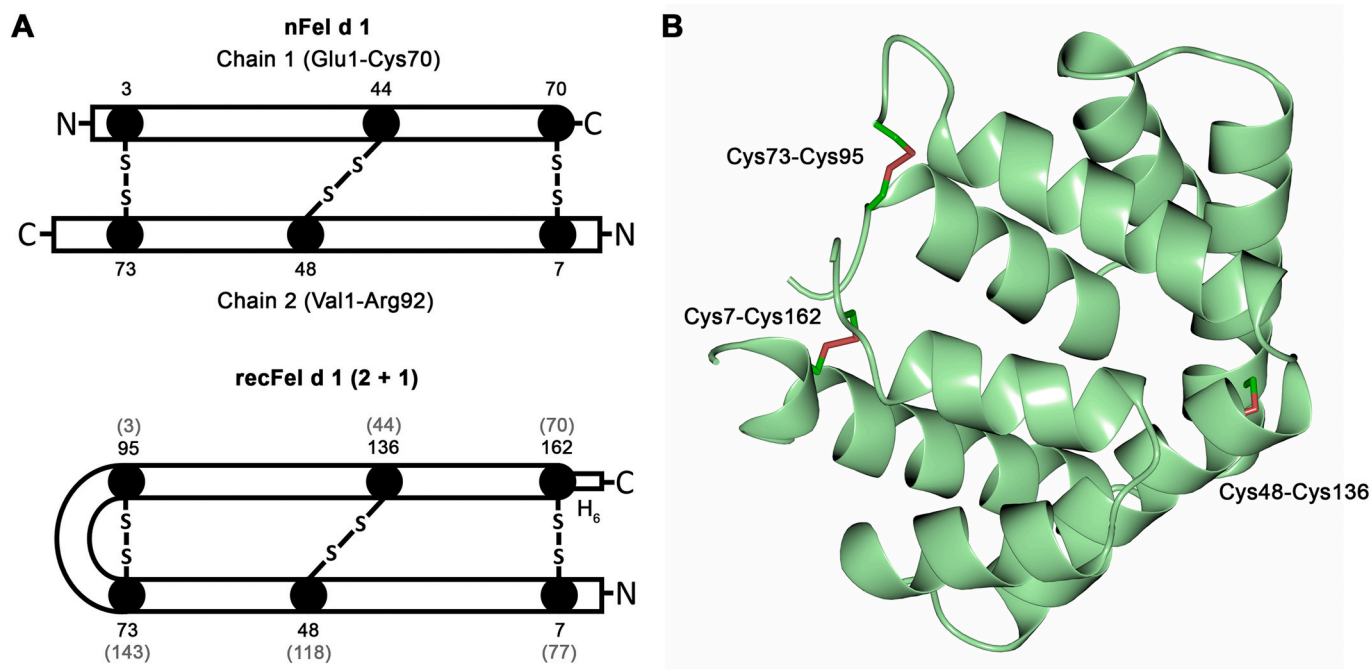


Fig. 1. Structure of Fel d 1. A) Comparison of the polypeptide chains and disulfide patterns of natural Fel d 1 (nFel d 1) and recombinant Fel d 1 (recFel d 1). This work utilizes the 2 + 1 fusion construct, that links the C-terminus of chain 2 to the N-terminus of chain 1 [6]. Another construct (1 + 2, not depicted), connects the C-terminus of chain 1 to the N-terminus of chain 2 [5]. Residue numbers are for mature chains, and they are in black for (2 + 1) and grey for (1 + 2). Black circle, cysteine; -S-S-, disulfide. B) A ribbon structure of recFel d 1 (2 + 1) from 1PUO (PDB code). Labelled disulfides are shown as red sticks.

fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The majority of recFel d 1 was eluted with 150 mM NaCl. The purest fractions were pooled and concentrated using Amicon Ultra centrifugal filters (10k MWCO, Millipore). Concentrated protein was aliquoted, flash frozen in liquid nitrogen, and stored at -70°C .

2.5. Protein concentration

Purified recFel d 1 concentration was determined at 280 nm using calculated average molecular mass (19183 Da) and absorption coefficient ($7825\text{ M}^{-1}\text{cm}^{-1}$).

2.6. Electro-spray ionization mass spectrometry (ESI-MS)

Mass spectrometric analysis was carried out by ESI-MS combined with liquid chromatography using a Q Exactive Plus Biopharma Orbitrap mass spectrometer (Thermo Scientific). Purified recFel d 1 protein samples were acidified with 0.1% trifluoroacetic acid (TFA) before analysis. To alkylate free thiols, protein samples were mixed with 25 mM *N*-ethylmaleimide (NEM) and were incubated 10 min at 25°C before acidification and ESI-MS analysis.

2.7. Far-UV circular dichroism (CD)

Purified and concentrated Fel d 1 was buffer-exchanged to 10 mM sodium phosphate, pH 7.4 using Amicon Ultra centrifugal filters (10k MWCO, Millipore). Spectra were recorded at 0.1 mg/mL protein concentration, at fixed 22°C as described [11], and the thermal unfolding was carried out in similar buffer with a temperature range of 22 – 94°C , taking a scan every 2°C . Each experiment was carried out with two different batches of protein. Analysis and modelling the transitions were done in Chirascan software.

2.8. Nano differential scanning fluorimetry (nanoDSF)

Protein thermal stability was analyzed with a Prometheus NT.48 instrument (NanoTemper Technologies). Capillaries were filled with 10 μL of recFel d 1 protein in 20 mM sodium phosphate with 150 mM sodium chloride (pH 7.4) at a concentration of 2 mg/mL. The temperature was increased from 20 to 95°C at a ramp rate of $1^{\circ}\text{C}/\text{min}$. The excitation wavelength was 280 nm, and the ratio of emission intensities ($\text{Em}_{350\text{nm}}/\text{Em}_{330\text{nm}}$) was recorded. The fluorescence intensity ratio and its first derivative were calculated with the PR.ThermControl software (NanoTemper Technologies).

2.9. Dynamic light scattering (DLS)

The size distribution of purified recFel d 1 was determined using DLS (Wyatt Dyna Pro Reader II, Dynamics 7.17 software). The measurements were carried out at 22°C with 10 acquisitions per measurement. Protein concentration was adjusted to 0.5 mg/mL in 20 mM sodium phosphate with 150 mM sodium chloride, pH 7.4. Samples were centrifuged at $13,000\times g$ for 10 min before loading 30 μL of sample onto an Assay 384 well plate (Corning). Each sample was measured in triplicates.

2.10. Structural analysis

Protein coordinates and the corresponding structure factors for Protein Data Bank (PDB) files 1PUO, 2EJN [5,6], and 1ZKR were downloaded, and the electron density maps $2\text{Fo} - \text{Fc}$ and $\text{Fo} - \text{Fc}$ [17] were generated and analyzed with the graphical program COOT [18]. Methionine residues that showed positive $\text{Fo} - \text{Fc}$ maps were mutated *in silico* to contain methionine sulfoxide (SME) using COOT tools, and the updated models were re-refined with Phenix suite [19] using the experimental data (structure factors) deposited.

3. Results and discussion

3.1. Production of soluble recFel d 1 in the cytoplasm of *E. coli*

Production of correctly folded disulfide-containing allergens in *E. coli* is challenging, as they often form insoluble inclusion bodies [5,6,8,9]. Purification from inclusion bodies typically requires solubilization with chaotropic agents, *in vitro* refolding, and elaborate purification procedures to obtain homogeneously folded soluble allergen. This laborious approach limits the applicability of *E. coli* as an efficient expression host for large scale protein production.

A major issue that leads to inclusion body formation in *E. coli* is that the cytoplasm lacks an oxidative system for disulfide formation. In our previous works, we have shown that CyDisCo system that co-expresses eukaryotic folding catalysts allow soluble production of biologically active disulfide-containing proteins in *E. coli* [10–12,15,20]. We hypothesized that this method could also be used to improve medical and diagnostic tools connected to allergy. As a proof of concept of soluble cytoplasmic production of disulfide-containing allergens, we chose recFel d 1 as the model allergen, which contains three disulfides in the native state (Fig. 1B). To the best of our knowledge, there are no reports of efficient soluble cytoplasmic production of recFel d 1.

We initially screened recFel d 1 expression in *E. coli* strains BL vs K12 and in rich vs chemically defined cultivation media. All combinations with CyDisCo present (co-expressing yeast Erv1p and human PDI as the folding catalysts) resulted in soluble cytoplasmic expression of recFel d 1. This is not surprising as CyDisCo has been reported to work in wide range of expression hosts and media [10,11,16]. We chose *E. coli* strain K12 and chemically defined medium for this proof-of-concept work, as K12-derived strains and defined media are often the preferred choices in bioreactor and industrial protein production.

No soluble recFel d1 was formed without CyDisCo. Co-expression of Erv1p and PDI allowed recFel d 1 to fold to a soluble state in the cytoplasm and to be easily purified (Fig. 2). Purified recFel d 1 migrated faster on a non-reducing gel as a single redox state (compare lanes 4 and 6 in Fig. 2), suggesting that at least one structural disulfide had formed. The purified yields were 36 mg/L or 2.7 mg/L/OD₆₀₀ from shake flask

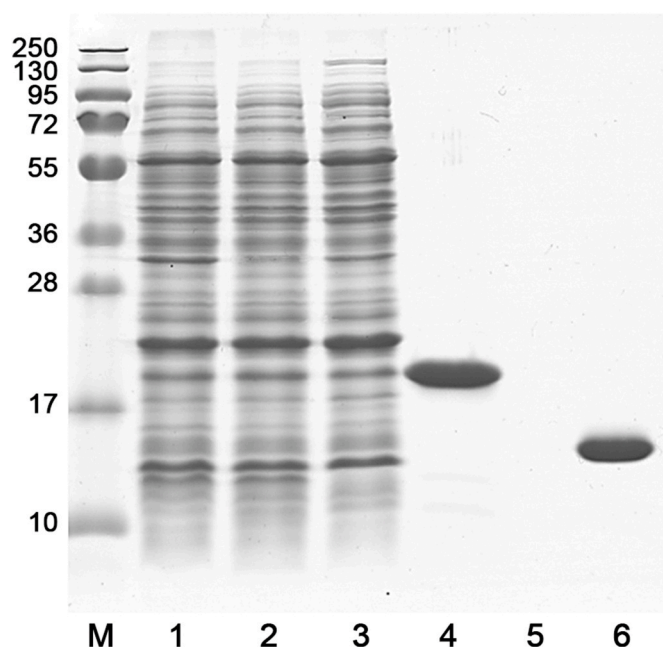


Fig. 2. SDS-PAGE analysis of production of recFel d 1. Lanes: M, molecular marker; 1, total lysate; 2, soluble lysate; 3, flowthrough of immobilized metal affinity chromatography; 4, purified reduced recFel d 1; 5, empty; 6, purified non-reduced recFel d 1.

cultures.

3.2. Biophysical characterization

Soluble expression does not necessarily correlate with native folding and formation of the three disulfides. Therefore, to assess its folded state, we subjected purified recFel d 1 to various biophysical tests. ESI-MS analysis showed consistently a base peak of 19065.5 Da (monoisotopic mass) that accounted for circa 60% of the total intensity (Table 1). Pre-treatment with an alkylating agent NEM, that adds 250 Da per a missing disulfide (125 Da per free alkylated Cys) resulted in similar set of major peaks, including a base peak of similar intensity, and only one additional peak (19190.6 Da, 9% of total intensity). The extra peak likely corresponded to non-specific alkylation (+125 Da) of a non-Cys residue [21]. These results indicate that the recFel d 1 produced had no free cysteines that could be alkylated, and hence three disulfides had formed. The major peak was therefore likely a product of recFel d 1 without a translation initiation methionine (Met0), containing three disulfides, plus 32 Da (Table 1). The identity of the +32 Da mass shift is discussed later in more detail.

Folded recFel d 1 contains mainly α -helices [5,6]. Consistent with this, circular dichroism (CD) spectra of our recFel d 1 showed the typical pattern of an α -helix-rich protein with minima at 208 and 222 nm (Fig. 3A). As this type of analysis probes only the secondary structure and does not necessarily correlate with the tertiary structure or the native fold, we subjected recFel d 1 next to thermal unfolding experiments. It is expected that airborne allergens that contain structure-stabilizing disulfides in the native state would be resistant to various environmental stress factors, including heat [22]. Thermal unfolding observed by CD resulted in one major structural change with a high melting temperature (T_m) of 87.6 °C, supported by a single defined isodichroic point that all the spectra passed through (Fig. 3B). Thermal unfolding carried out by nanoDSF that reads tyrosine fluorescence (Fel d 1 contains no tryptophans) resulted in similar T_m of 89 °C. Dynamic light scattering (DLS) showed our recFel d 1 to be a monomer in solution with an average molecular weight of 20.3 ± 2.9 kDa ($n = 3$, mean $\pm$ STD). Together, these results demonstrate that the solubly produced recFel d 1 had three disulfides and was folded as a stable monomer.

3.3. recFel d 1 is sensitive to methionine oxidation

We observed a consistent +32 Da shift in the ESI-MS major peak of our recFel d 1 (Table 1), regardless of *E. coli* strain (BL vs K12) or expression media (rich vs chemically defined) used during production. DNA sequencing ruled out mutations as the cause of the shift. The most likely explanation that causes a +32 Da mass shift is oxidation (+16 Da) of two amino acid residues. While we were unable to identify which amino acid side chains had been oxidized in our protein, the concept is supported by several reports of allergens that had been modified at methionine residues, specifically, sulfoxidation of the methionine sulfur

Table 1

Five major products of mass spectrometric analyses ($n = 4$) of purified recFel d 1.

Theoretical mass ^a (Da)	Experimental mass (Da)	Relative intensity (% mean $\pm$ STD)	Δ mass (Da)	Explanation ^b
19170.6	19065.5	59.8 ± 6.3	-105.1	-Met0, 3 -SS-, +32 Da
	19033.5	13.8 ± 7.5	-137.1	-Met0, 3 -SS-
	19164.6	8.2 ± 1.9	-6	3 -SS-
	19080.5	4.0 ± 0.4	-90.1	-Met0, 3 -SS-, +48 Da
	19049.5	3.1 ± 1.3	-120.1	-Met0, 3 -SS-, +16 Da

^a Of reduced recFel d 1 with Met0 from Compute pI/MW tool; monoisotopic mass.

^b Abbreviations: Met0, translation initiation methionine; -SS-, disulfide.

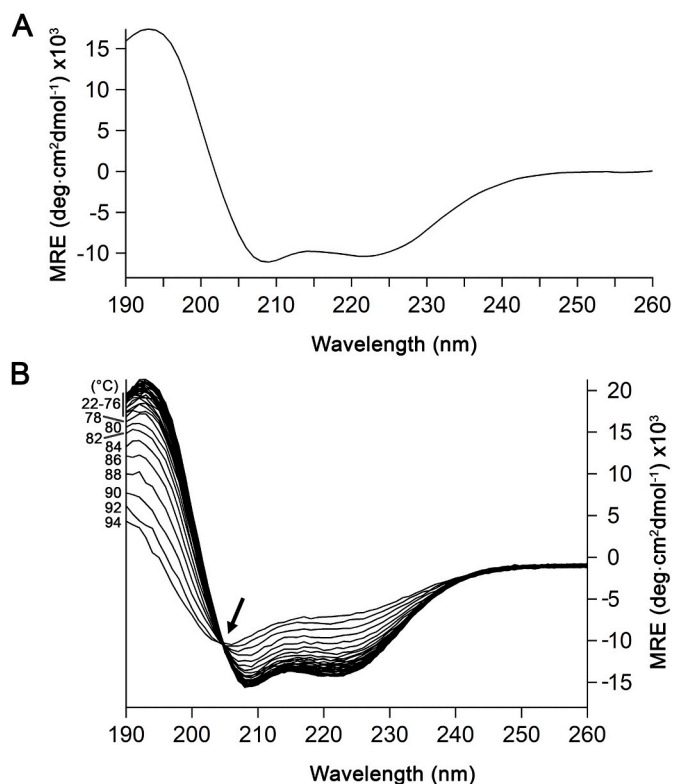


Fig. 3. Biophysical characterization of purified recFel d 1. A) Circular dichroism (CD) spectrum collected at 22 °C. B) Thermal unfolding of recFel d 1 in the temperature range of 22–94 °C observed by CD. The temperature of each trace and the isodichroic point (arrow) are indicated.

atom [7,23–25].

For direct evidence of Met sulfoxidation and potential sites in Fel d 1, we analyzed two published high-resolution crystal structures (PDB codes 1PUO, 1.85 Å and 2EJN, 1.64 Å) [5,6] and an unpublished structure (PDB code 1ZKR, 1.64 Å). None of the structures had assigned sulfoxidation to any of the five Met residues in the protein or to the initiating Met0. Closer inspection of the electron density maps suggested that Met74 in 1PUO and Met49 and Met144 in 2EJN and 1ZKR (that correspond to Met141 and Met74 in 1PUO, respectively) showed positive $F_o - F_c$ maps (Fig. 4A), i.e., they do not fit properly to the experimental data [17]. This suggested that those residues may potentially have contained sulfoxide modification, but they were not interpreted as such. To test this hypothesis, we mutated *in silico* Met49 and Met144 of the high-quality structure 2EJN to contain sulfoxide and re-refined the structure (Fig. 4B). The obtained new R and R_{free} values of 17.8% and 19.7%, respectively, that assess modeling quality [26], showed significant improvement over the original structure (19.0% and 22.0%, respectively). Collectively, the previous reports of sulfoxidation in allergens, as well as the structural analysis conducted here suggest that the consistent +32 Da mass shift in the ESI-MS base peak was caused by sulfoxidation of two Met residues.

The high relative amount of sulfoxidation observed in our ESI-MS analysis (Table 1) is perplexing. We have yet to observe such consistently high Met sulfoxidation levels for any other protein produced in our labs. Sulfoxidation of Met by small molecular weight oxidants such as reactive oxygen species (ROS) is kinetically a slow process and cytoplasmic methionine sulfoxide reductases (MSRs) typically keep sulfoxidation levels low [27]. Long-term storage is one potential cause of oxidation, but this was ruled out, as two out of the four mass analyses were conducted with fresh material stored frozen overnight only. These points and previously reported Met sulfoxidation [7] suggest that Fel d 1 may be intrinsically sensitive to sulfoxidation, and the observed high

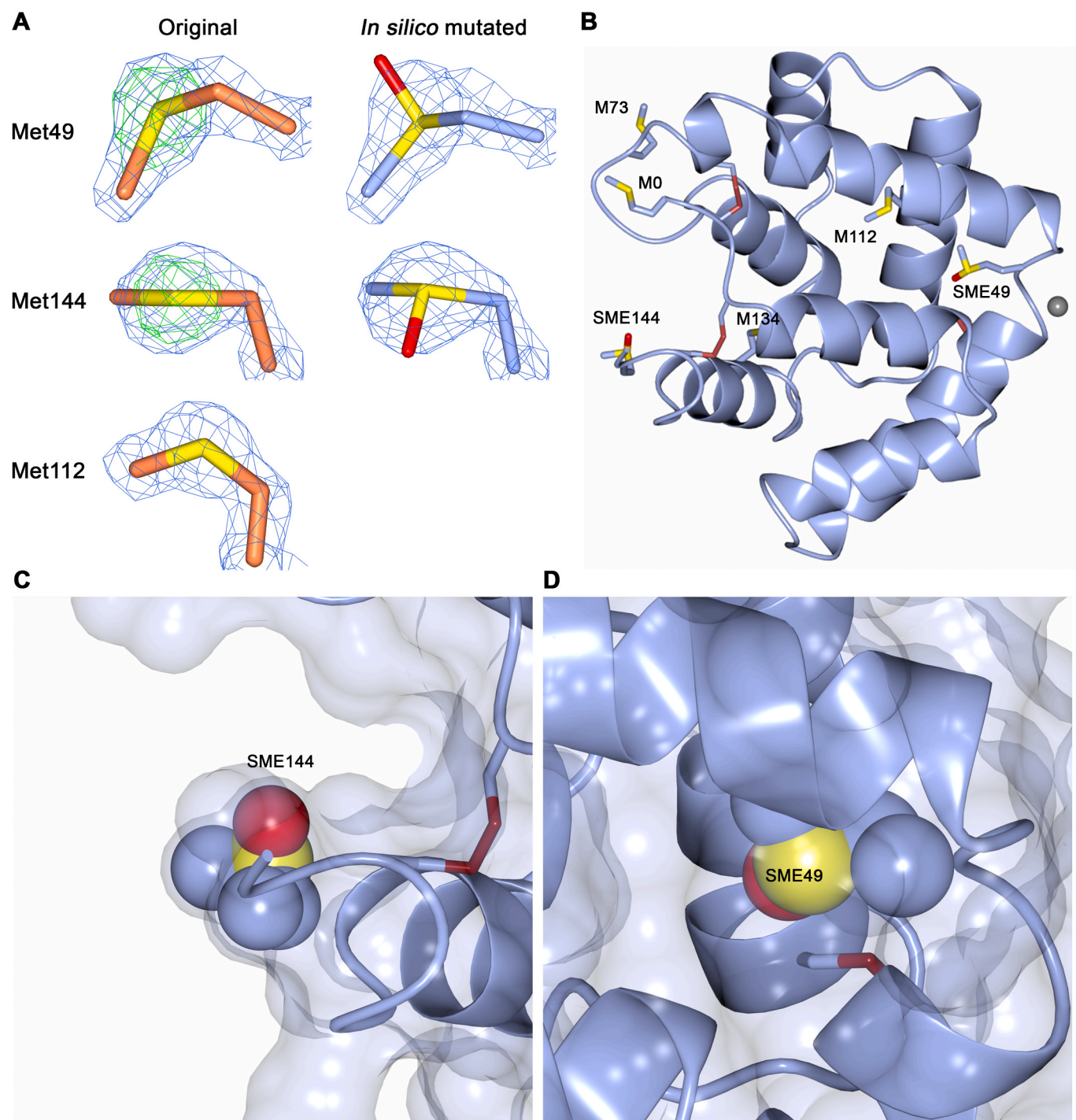


Fig. 4. Structure of *in silico* mutated recFel d 1. **A)** The original (from PDB structure 2EJN) side chains of indicated Met residues (coral color) and their 2Fo-Fc (blue color) and positive Fo-Fc (green color) maps compared to respective *in silico* mutated side chains (ice blue color) containing methionine sulfoxide modifications (SME, red color). Sulfur atom is in yellow. Met112 is depicted as an example of a Met residue that did not show positive map. **B)** Ribbon representation of recFel d 1 monomer mutated *in silico* from 2EJN. Labelled non-mutated and mutated Met side chains are shown as ice blue sticks, SME as short red sticks, and disulfides (not labelled, see Fig. 1B for their identities) as long red sticks. Ca^{2+} interacting with Met49 carbonyl oxygen is shown in grey. **C-D)** Accessibility of SMEs: SME144 is solvent exposed (**C**), while SME49 is buried (**D**). Protein surface is shown as a semi-transparent area over the ribbon diagram. Non-hydrogen atoms of the sulfoxide-containing methionine side chains are shown as spheres with carbon in blue, sulfur in yellow, and oxygen in red.

sulfoxidation levels are not production artefacts or oxidative damage, per se. The methionines in recFel d 1 are potentially prone to very rapid re-oxidation, and/or they cannot be reduced, e.g., due to accessibility issues. One of the Met sulfoxidations is clearly surface exposed (Fig. 4C), while the other is buried, sandwiched between two alpha helices and likely inaccessible to be reduced by MSRs (Fig. 4D). As reversible Met

oxidation has been described as a functional mechanism for various proteins [27–29], it is possible that nFel d 1 also undergoes facile Met sulfoxidation. This poses important questions for future work: is the sensitivity for sulfoxidation related to an uncharacterized function – and to the potent allergenicity of Fel d 1? Either way, Met sulfoxidation of Fel d 1 may have implications for SIT, whether the protein is from a

natural or recombinant source.

4. Conclusions

We show that disulfide-containing recFel d 1 can be produced in a soluble folded form in the cytoplasm of *E. coli* by the aid of eukaryotic folding factors. The produced recFel d 1 had formed three structural disulfides and folded in the cytoplasm as a stable monomer. The expression conditions reported here (*E. coli* K12 strain and chemically defined medium) were chosen to be suitable for future bioreactor production. We observed also high sensitivity for methionine sulfoxidation which may represent an uncharacterized functional mechanism for this important allergen.

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CRediT authorship contribution statement

Chi Zhang: Conceptualization, Investigation, Writing - review & editing. **Rosario Recacha:** Formal analysis, Investigation, Writing - review & editing. **Lloyd W. Ruddock:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Antti Moilanen:** Formal analysis, Data curation, Writing - original draft, Project administration.

Declaration of competing interest

A patent for the production system used to make the protein for this study in the cytoplasm of *E. coli* using a sulfhydryl oxidase and an isomerase is held by the University of Oulu: Method for producing natively folded proteins in a prokaryotic host (patent no. 9238817; date of patent January 19, 2016; inventor: L.W.R.).

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