

The allergen Mus m 1.0102: Dissecting the relationship between molecular conformation and allergenic potency

Elena Ferrari ^a, Emanuela Casali ^a, Samuele E. Burastero ^b, Alberto Spisni ^{c,*}, Thelma A. Pertinhez ^{a,d}

^a Dept. of Biomedical, Biotechnological and Translational Sciences, University of Parma, Parma, Italy

^b Div. Immunology, IRCCS San Raffaele, Milan, Italy

^c Dept. Surgical Sciences, University of Parma, Parma, Italy

^d Transfusion Medicine Unit, ASMN- IRCCS, Reggio Emilia, Italy

ARTICLE INFO

Article history:

Received 26 April 2016

Received in revised form 18 July 2016

Accepted 5 August 2016

Available online 09 August 2016

Keywords:

Conserved free cysteine

Thiol/disulfide exchange

Protein aggregation

Lipocalin fold stability

Unfolding reversibility

Mus m 1 allergen

ABSTRACT

Background: The species *Mus musculus* experiences an obligate proteinuria: predominant are the Major Urinary Proteins (MUPs), that, collectively known as the major mouse allergen Mus m 1, are among the most important aeroallergens for mouse allergic patients. The production of a soluble and stable hypoallergenic form of Mus m 1 is essential for the development of immunotherapeutic protocols to treat allergic symptoms.

Methods: We introduced the substitution C138S in recombinant Mus m 1.0102, an allergenic isoform of Mus m 1. Solubility, conformation, stability and ability to refold after chemical denaturation were investigated with dynamic light scattering, circular dichroism, fluorescence and NMR spectroscopy. An in vitro degranulation assay was used to evaluate the protein allergenic potential, and compare it with Mus m 1.0102 and with an hypoallergenic variant bearing the substitution Y120A.

Results: Mus m 1.0102-C138S retains a native-like fold revealing, however, local conformational alterations that influence some of its physical and allergenic properties: it is monodispersed, thermostable up to 56 °C, able to reversibly unfold and it exhibits an enhanced allergenicity.

Conclusions: The unique free thiol group affects the solution structural stability of the native protein. Because the mutant C138S does not aggregate over time it is a good lead protein to develop diagnostic and therapeutic applications.

General significance: We elucidated the relationship between unfolding reversibility and sulphhydryl reactivity. We ascribed the enhanced allergenicity of the mutant C138S to an increased accessibility of its allergenic determinants, an enticing feature to further investigate the structural elements of the allergen-IgE interface.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Mus m 1.0102 is a native isoform of the mouse Major Urinary Protein complex (MUP), an ensemble of closely related proteins synthesized mainly in the liver under hormonal control, transported via the blood and excreted in the urine. These proteins are involved in pheromonal communication among conspecifics, each mouse expressing a polymorphic pattern of major urinary proteins that differ from each other by a limited number of residues. Acting as pheromone stabilizers and carriers, they provide a mechanism for their slow release into the environment [1–4].

Abbreviations: Allergen-specific immunotherapy, (AIT); Mus m 1.0102-C138S mutant, (MM-C138S); Mus m 1.0102-Y120A mutant, (MM-Y120A); Rat Basophilic Leukemia, (RBL); Dynamic light scattering, (DLS); Static Light Scattering, (SLS); Polydispersity Index, (PDI).

* Corresponding author at: Dpt. of Surgical Sciences, University of Parma, Via Volturmo, 39, 43125 Parma, Italy.

E-mail address: alberto.spisni@unipr.it (A. Spisni).

Besides that, they are among the most important allergens for mouse allergic patients [5–7]. Once excreted, the proteins can easily reach the airways, playing an important role in asthma morbidity. Exposure and sensitization to these allergens are common in occupational settings as well as in inner-city homes [8].

It is well established, by clinical research, that allergen-specific immunotherapy (AIT) is the only available etiologic treatment of respiratory allergies [9]. Moreover, AIT prevents disease progression and the onset of new sensitizations [9,10]. The knowledge of the physicochemical and structural features of allergens is a key factor to unravel the mechanisms of allergenicity, i.e., the intrinsic capabilities of some antigens to act as allergens.

Originally, AIT was based on the administration of extracts, containing complex mixtures of allergen components, obtained from the allergen source(s). However, the use of extracts from natural matrices brings about the risk of potentially severe side effects [11] and the problem of standardization of the allergenic extracts [12]. Moreover, only the availability of a single purified allergen allows to dissect the complex array

of immune cell interactions underlying the establishment and the progression of allergy [13]. Finally, extract-based allergy diagnosis is limited by false positive results due to the IgE reactivity to a variety of other molecules present in allergen sources [14]. In this scenario, recombinant allergens turned out to offer a solution to these problems. Detection for diagnostic purposes of IgE reactivity to single allergen (the so called “Component Resolved Diagnosis”, CRD) not only prevents false positive results, but also allows to distinguish patients' sensitization profiles to the same allergen having different clinical implications [14]. Recombinant allergens can be purified with reproducible quality and even engineered to obtain hypoallergenic proteins. These features favour product standardization and contribute to further basic knowledge on allergen structure with an expected impact on the design of AIT products with higher safety [10,15,16]. We previously identified the point mutation Y120A of Mus m 1.0102, MM-Y120A, which allowed to obtain a protein with reduced allergenicity and preserved immunogenicity [17]. Unfortunately, those positive features were associated to a reduced molecular stability and shelf life of the mutant. Conformational stability in time and resistance to temperature variation are relevant features required for allergens to be used in AIT: these characteristics contributing to increase the commercial sustainability and therapeutic safety of vaccine formulation.

Mus m 1.0102 is a small β -barrel protein belonging to the Lipocalins superfamily: it is composed of eight antiparallel β -strands, forming a hydrophobic core, and one α -helix running alongside the barrel [18]. The structure is stabilized by a disulfide bridge involving residues Cys64 and Cys157. In addition, the protein presents one free cysteine residue at position 138, whose side chain is located at the hydrophobic interface between the β -barrel and the α -helix. The disulfide bond, highly conserved among the Major Urinary Proteins, is part of the hydrophobic core, playing an important role in stabilizing the folded protein. The unique free cysteine residue, highly conserved among mouse urinary proteins and substituted by serine only in the pheromonal protein Darcin [19], instead, is acknowledged to cause inter- or intramolecular thiol/disulfide exchange, an event that is known to interfere with the folding/refolding processes [20,21] and that may lead to protein aggregation. Therefore, to exploit recombinant Mus m 1.0102 for future diagnostic and therapeutic applications and preserve the allergen molecular fold and stability, we investigated the changes in Mus m 1.0102 fold stability and unfolding reversibility upon substitution of Cys138 with the isosteric serine, generating the Mus m 1.0102-C138S mutant (MM-C138S).

Here we discuss the structural features and the capability to induce IgE-dependent basophil degranulation of the mutant MM-C138S and we compare these features with the ones of the native Mus m 1.0102 and with the MM-Y120A mutant, used as model of a hypoallergenic though instable form of the Mus m 1.0102 [17] in functional tests.

2. Material and methods

2.1. Proteins expression and purification

The recombinant allergen Mus m 1.0102 (isoallergen of Mus m 1, UniProtKB accession No. P11589) and its mutants MM-C138S and MM-Y120A were expressed in *Pichia pastoris* as previously described [22]. Uniformly ^{15}N -labeled proteins, used for NMR experiments were prepared growing yeast cultures in Yeast Nitrogen Base medium added with $(\text{NH}_4)_2\text{SO}_4$ (1% final concentration) and buffered at pH 6.6 with potassium phosphate buffer 100 mM.

The culture supernatants containing the secreted proteins were dialyzed against Tris-HCl 10 mM pH 7.2, concentrated and applied to a size exclusion HPLC column (Sephacryl S200, HiPrep 16/60 column), equilibrated and eluted with 10 mM Tris-HCl pH 7.2 and 150 mM NaCl. Protein elution fractions were collected, dialyzed against Tris-HCl 10 mM pH 7.2 and concentrated up to 130–150 μM . Protein concentration was determined spectrophotometrically assuming an extinction

coefficient $\epsilon_{278} = 11,345 \text{ M}^{-1} \text{ cm}^{-1}$ for Mus m 1.0102 and MM-C138S, and $\epsilon_{278} = 9895 \text{ M}^{-1} \text{ cm}^{-1}$ for MM-Y120A [5,17].

2.2. Static and dynamic light scattering

Static and dynamic light scattering (SLS and DLS) measurements were carried out using a Malvern Zetasizer μV instrument. The recombinant Mus m 1.0102 and MM-C138S were dissolved in Tris-HCl 10 mM pH 7.2 and filtered (Anotop siringe filter 0.02 μm , Whatman) before use.

In order to determine the molecular weight and the 2nd virial coefficient (A_2) of the proteins we used the SLS technique. In this case we measured the time-averaged intensity of the scattered light as a function of protein concentration.

Briefly, the intensity of the scattered light, measured at a fixed angle (90°), was determined using solutions containing 0.5, 1.0, 1.3 and 1.74 mg/mL protein and pure solvent as zero protein concentration. The results were compared with the scattering of pure toluene used as reference standard. The intensity values were analysed using the Rayleigh equation describing the intensity of light scattered by a particle in solution:

$$KC/R_\theta = 1/M + 2A_2C \quad (1)$$

where K is an optical constant, C is the molecule concentration, R_θ is the Rayleigh ratio of scattered to incident light intensity, M is the weight-average molecular weight and A_2 is the 2nd virial coefficient. The data were used to generate a standard Debye plot: a linear fit of KC/R_θ versus protein concentration, where the intercept on y-axis is the inverse of the protein molecular weight and the slope is twice the 2nd virial coefficient A_2 .

To detect the onset of the proteins' thermal unfolding process, we used the DLS technique that measures the intensity of the scattered light, detected at a fixed angle (90°) as a function of time. Experiments were carried out using an automated 2.5 $^\circ\text{C}$ incremental temperature ramp in the interval 25–80 $^\circ\text{C}$ (with a 2 min equilibrium time at each measurement temperature) and a protein concentration of 1.87 mg/mL. The Zetasizer algorithm monitors the count rate (scattering intensity, kcps) and, as the measurement progresses, calculates the slope of the line connecting the most recent six points of the intensity trace in the aggregation point graph. The temperature at which the slope exceeds 1 corresponds to the melting onset temperature.

Analysis of the DLS data (Cumulants analysis, for more details see Malvern Zetasizer manual) provided also the Polydispersity Index (PDI), or Dispersity Index ($\bar{D}$), as IUPAC recommends: an estimate of the width of the sizes distribution of the molecules in solution. This parameter, for values smaller than 0.05 indicates a highly monodisperse sample while, for values >0.7 , indicates that the sample has a very broad size distribution and is definitely not suitable for the dynamic light scattering technique [23].

2.3. Circular dichroism and intrinsic fluorescence measurements

Circular dichroism (CD) measurements were carried out with a Jasco spectropolarimeter, model J-720 at 25 $^\circ\text{C}$. Spectra were collected with scan speed of 20 nm/min and response time of 1 s. Each spectrum was the average of three scans. Far-UV CD and near-UV CD spectra were taken at protein concentrations of 1.8 μM and 20 μM with a 1 mm and 0.2/0.5 cm path length cell, respectively. Acquired data were expressed as mean molar residue ellipticity, $[\theta] \text{ deg cm}^2 \text{ dmol}^{-1}$.

Fluorescence measurements were performed on JASCO FP-6200 spectrofluorometer. Tryptophan fluorescence was monitored by exciting at 295 nm, using a band width of 5 nm on both monochromators. The emission spectra were recorded over a 300–550 nm wavelength range, at 25 $^\circ\text{C}$ with a 1 cm path length cell, using a sample concentration of 2 μM . Thermal denaturation curves were obtained plotting the maximum

fluorescence emission wavelength ($\lambda_{\max}$) as a function of temperature (25–90 °C).

2.4. Refolding experiments

Complete proteins unfolding was obtained dissolving the proteins in a solution containing GdmCl 5.5 M and dithiothreitol 4 mM for 48 h. The subsequent protein refolding experiments were carried out by sample 10-fold dilution with Tris-HCl 10 mM pH 7.2 at 4 °C. The refolding reaction was allowed to proceed for 24 h; next, protein samples were extensively dialyzed against the buffer Tris-HCl 10 mM pH 7.2 to assure maximum recovery of the native conformation. The extent of refolding was monitored by far- and near-UV CD and emission fluorescence spectroscopies.

2.5. Allergenicity test: Rat Basophilic Leukemia (RBL) cells degranulation assay

The use of passively sensitized RBL cells for comparing the allergens potency has been extensively exploited being a good model to generate standardized results [24,25]. Here, RBL cells were grown in RPMI medium, supplemented with 10% (v/v) FCS (Gibco-Invitrogen Corporation Italia, Milan, Italy) or autologous plasma. RBL-SX38 cells (a kind gift by M.H. Jouvin and J. P. Kinet, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02,115, USA), stably expressing human FcεRI α , β and γ chains [26], were cultured in complete medium supplemented with 800 μ g/mL Geneticin (Sigma), as previously described [27].

Dose-response test of β -hexosaminidase release was performed by stimulating RBL cells (sensitized with patients' IgE) with allergen concentrations varying from 0.1 to 5.0 μ M. The release of β -hexosaminidase was monitored as described [28], with minor modifications. Briefly, P-NAG (p-nitrophenyl-N-acetyl- β -D-glucosaminide) (Sigma) in 0.1 M citrate buffer (pH 6.2) was added to RBL-SX38 cell supernatants and incubated at 37 °C for 120 min. The reaction was terminated using 0.1 M carbonate buffer (pH 10) and the absorbance measured at 405 nm. The supernatant of non-stimulated cells was considered as a negative control. The maximal stimulation was determined as previously described [29]. Briefly, 100 ng of 4-hydroxy-3-nitro-phenacetyl (NIP)-Bovine Serum Albumine (stoichiometric ratio NIP:BSA > 10) (Biosearch Technologies, Vacaville, CA) was used as an IgE cross-linker, by reacting it with RBL-SX38 cells pre-incubated with a commercially available human recombinant IgE anti-NIP monoclonal antibody [30] (Serotec, Milan, Italy). Negative control values consistently fell in a 15–25% range of the total β -hexosaminidase content, assessed from the 0.05% Triton X-100 (Sigma) lysate of the cell monolayer. The results were calculated as percent of β -hexosaminidase release according to the following equation:

$$100 \times \frac{(A_{405\text{nm}}\text{Sample} - A_{405\text{nm}}\text{Negative Control})}{(A_{405\text{nm}}\text{Maximal Stimulation} - A_{405\text{nm}}\text{Negative Control})} \quad (2)$$

2.6. Western blot

Western blot was performed by electrophoresis of equal amounts of the murine cloned proteins under non-reducing conditions in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie blue staining was used to visualize protein concentration loaded in each lane. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, N.J., USA) and 1:5 diluted sera (0.5 mL) were individually reacted for 60 min at room temperature with the proteins blotted onto single strips. IgE detection was performed with the same volumes of HRP-conjugated rabbit anti-human IgE (DAKO Italia, Milan, Italy). Bound antibodies were revealed

by enhanced chemiluminescence autoradiography (ECL, Amersham Pharmacia Biotech).

2.7. NMR experiments: Chemical shift mapping

^{15}N labeled recombinant Mus m 1.0102 and MM-C138S NMR spectra were acquired at 35 °C on a Varian Inova 600AS spectrometer. Spectral assignment was based on a previous work [4]. Samples for NMR used 120 μ M ^{15}N labeled proteins in 10 mM phosphate buffer at pH 7.2.

Analysis of the ^{15}N and ^1HN chemical shift data used weighted chemical shifts difference, calculated according to the following Eq. (3):

$$\Delta\delta(^1\text{HN}, ^{15}\text{N}) = |\Delta\delta(^1\text{HN})| + 0.2 \times |\Delta\delta(^{15}\text{N})| \quad (3)$$

3. Results

3.1. Protein solubility

Protein solubility is the result of protein-protein and protein-solvent interactions. A qualitative evaluation of the role played by these interactions in the definition of Mus m 1.0102 and MM-C138S solubility was carried out using SLS. Debye analysis of the light scattering data produces the hydrodynamic descriptor A_2 that correlates to protein solubility [31]: A_2 negative values indicate a dominance of attractive forces between protein molecules, while A_2 positive values are diagnostic of proteins that exist preferentially in a solvated state; finally, if A_2 is zero it means that the strength of molecule-solvent and molecule-molecule interactions is equivalent.

The scattered light intensity values, measured at different protein concentrations, were analysed using the Rayleigh Eq. (1) (2.2 subsection) so that the 2nd virial coefficient A_2 and the protein molecular weight could be derived, Table 1 (the Debye plot is presented as Supplementary Figure 1).

As the A_2 value for each protein resulted positive and close to zero, we can conclude that both proteins are expected to have a reduced tendency to aggregate. The extrapolated molecular weight values resulted in agreement with the predicted molecular weight, 18,692 and 18,708 Da for MM-C138S and Mus m 1.0102, respectively.

At the highest tested protein concentration, 1.74 mg/mL, where we could already expect a non-uniform distribution of protein molecular sizes, the obtained DLS intensity data of the two protein samples were analysed by the Cumulants-based algorithm to extract the PDI. The values resulted 0.11 for both MM-C138S and Mus m 1.0102, indicating a narrow spread of the molecular size distribution.

3.2. Conformational characterization by circular dichroism spectroscopy

In native conditions the far-UV and near-UV CD spectra of Mus m 1.0102 and MM-C138S are comparable (Fig. 1A and B), indicating the two proteins have similar secondary structure topology and similar spatial and dynamic organization of the aromatic residues.

3.3. Thermal stability

3.3.1. Intrinsic fluorescence spectroscopy

Recognizing that Trp19 contributes to the structural core of Mus m 1.0102 [18,32,33], we took advantage of its intrinsic fluorescence to

Table 1
Static Light Scattering-derived parameters.

Protein	2nd virial coefficient A_2 , mL mol/g ²	MW, kDa
Mus m 1.0102	0.001880	19.1 $\pm$ 0.87
MM-C138S	0.001525	18.1 $\pm$ 1.70

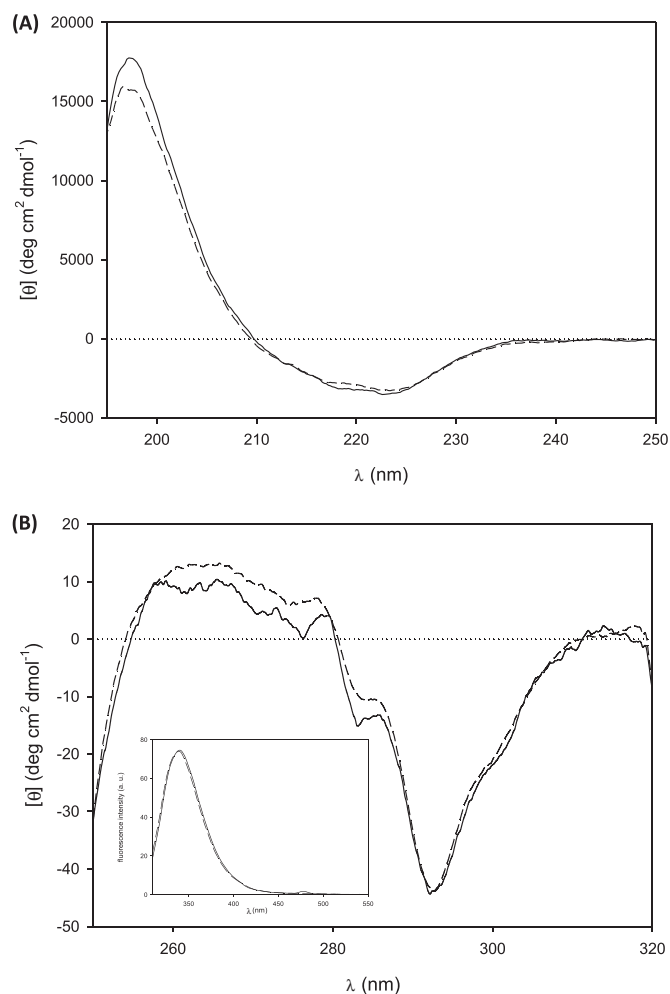


Fig. 1. Circular dichroism and intrinsic fluorescence spectra comparison. CD spectra for Mus m 1.0102 (dashed line) and MM-C138S (solid line) acquired in the far- (A) and near-UV regions (B). Inset of panel B: Tryptophan emission spectra of Mus m 1.0102 (dashed line) and MM-C138S (solid line).

obtain information on the protein conformational modifications induced by temperature or chemical perturbation. As reported in the inset of Fig. 1B, Trp19 exhibits the same fluorescence emission maximum, λ_{max} , 340 nm and 339 nm in MM-C138S and Mus m 1.0102, respectively, indicating its side chain is located in a comparable hydrophobic environment. When increasing the temperature we observe a λ_{max} red shift to 353 nm and 354 nm for MM-C138S and Mus m 1.0102, respectively, Fig. 2.

The λ_{max} red shift indicates that the Trp19 indole ring becomes exposed to a more polar environment, suggesting the proteins unfold. In addition, the sigmoidal profiles indicate that the temperature-induced unfolding occurs in a cooperative manner without accumulation of any intermediate. The calculated transition midpoints 71.9 °C and 75.2 °C for MM-C138S and Mus m 1.0102, respectively, reveal a slightly reduced resistance of the mutant architecture to heat perturbation.

3.3.2. Dynamic light scattering

In the early stage of the unfolding process, the protein conformation is expected to become looser with a concomitant increase of the hydrodynamic radius. As a consequence, the probability of the protein hydrophobic domains to be exposed to the polar external medium increases as well, thus favoring intermolecular hydrophobic interactions that will lead to protein aggregation. Both these events produce a transition to higher light scattering intensities. The temperature at which light scattering intensity begins to increase defines the onset of protein

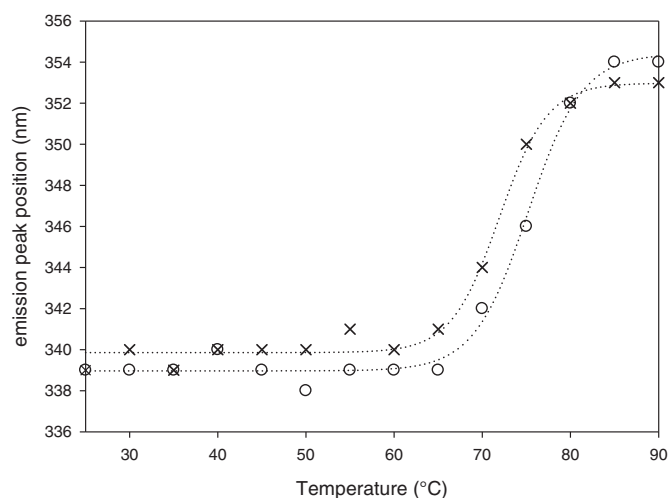


Fig. 2. Thermal denaturation monitored by intrinsic fluorescence spectroscopy excited at 295 nm. Maximum fluorescence emission wavelength (λ_{max}) of Mus m 1.0102 and MM-C138S is presented as a function of temperature. Circles, equilibrium unfolding data of Mus m 1.0102; crosses, equilibrium unfolding data of MM-C138S. Fitting to the fluorescence data points resulted in the two dotted curves whose equations were used to calculate the transition midpoints 75.2 and 71.9 °C for Mus m 1.0102 and MM-C138S, respectively.

unfolding, while the region of steep rise is indicative of aggregates formation.

The temperature dependence of the light scattering intensity of MM-C138S and Mus m 1.0102 samples are reported in Fig. 3. For MM-C138S the onset of the unfolding process is estimated to be at 57.5 °C. As for Mus m 1.0102, the fitting of the data places the onset of the unfolding at 62.5 °C. Above these temperatures, as already mentioned, the exponential increase of the light scattering intensity indicates aggregates formation.

3.4. Reversibility of unfolding after chemical denaturation

3.4.1. Unfolding and refolding monitored by circular dichroism spectroscopy

Both proteins, in the presence of GdmCl 5.5 M and DTT 4 mM, exhibit CD spectra typical of unfolded proteins (Figs. 4 and 5). After tenfold dilution with Tris-HCl buffer, the far-UV CD spectrum of Mus m 1.0102 indicates a not complete recovery of the native conformation,

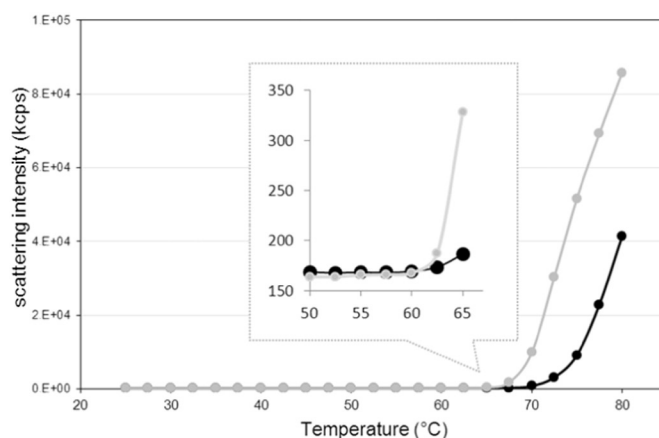


Fig. 3. Thermal denaturation monitored by dynamic light scattering. Scattering intensities of Mus m 1.0102 and MM-C138S samples are presented as a function of temperature. The black and grey traces are generated by the scattering intensity parameter (kilocounts per sec, kcps in scientific *e* notation) of Mus m 1.0102 and MM-C138S, respectively. The graph inset was generated only with the data relative to the melting onset temperatures.

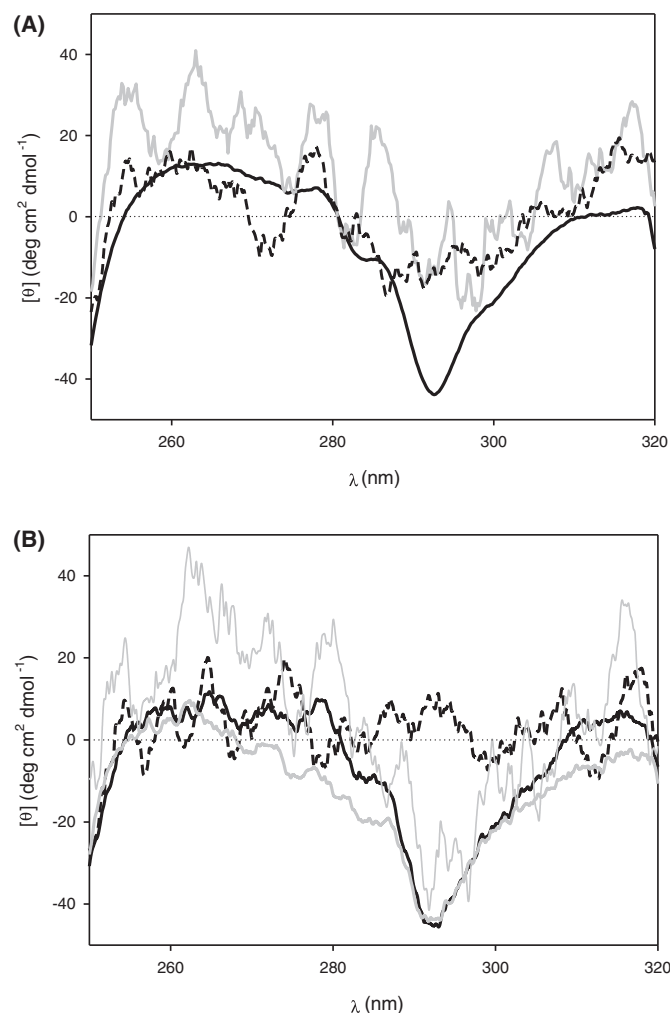
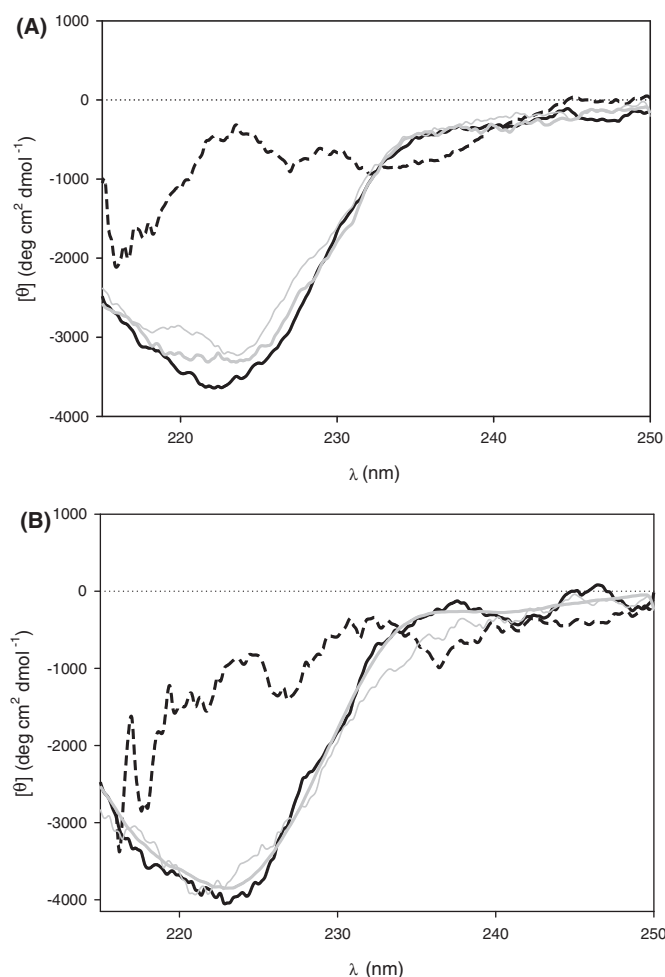


Fig. 4. Protein refolding monitored by far-UV CD spectroscopy. Far-UV spectra of Mus m 1.0102 (A) and MM-C138S (B): native proteins spectra are represented by solid black lines, the denatured ones by dashed lines and the refolding ones are in grey. The thin grey spectra were registered 24 h after tenfold dilution of the denaturant agent, while the thick grey ones were acquired after dialysis.

Fig. 4A, while the mutant exhibits a native-like far-UV CD profile, Fig. 4B. A similar result is obtained after extensive dialysis, Fig. 4A and B.

As for the aromatic side chain spatial organization, in spite of the poor signal to noise ratio of the near-UV spectra due to the combined negative effect of the low optical activity of the protein in that spectral region and low protein concentration, it is evident that even after extensive dialysis Mus m 1.0102 does not recover its native tertiary fold, Fig. 5A. The near-UV CD spectrum of MM-C138S, instead, already after tenfold dilution of the denaturation mix shows a clear recovery of the tertiary structure (in the same conditions Mus m 1.0102 does not show any recovery, data not shown). After extensive dialysis, the recovery of the elements of tertiary structure is complete, Fig. 5B.

3.4.2. Unfolding and refolding monitored by fluorescence spectroscopy

Differently from the results obtained previously with the mutants MM-Y120A and MM-Y120F, where mutation induced an increase of the fluorescence emission [17], Fig. 1B (inset) shows that Trp19 exhibits the same fluorescence intensity in both proteins. Upon addition of the denaturation mix (GdmCl 5.5 M and dithiothreitol 4 mM), we observe a red shift of the emission maximum to 356 nm for both proteins and a decrease of the fluorescence intensity, consistent with their unfolding, Fig. 6A and B.

Nonetheless, in the case of MM-C138S, we measure a minor decrease of the fluorescence intensity when compared with the native protein. This result indicates that the chemical environment experienced by Trp19, in

Fig. 5. Protein refolding monitored by near-UV CD spectroscopy. Near-UV spectra of Mus m 1.0102 (A) and MM-C138S mutant (B): native proteins spectra are represented by solid black lines, the denatured ones by dashed lines and the refolding ones are in grey. The thin grey spectrum was registered 24 h after tenfold dilution of the denaturant agent, while the thick grey ones were acquired after dialysis.

the two unfolded proteins, is not the same. After renaturation, the fluorescence spectrum presents the emission λ_{max} at 337 nm and 344 nm, instead of 340 and 339 nm, for MM-C138S and Mus m 1.0102, respectively. As for the fluorescence maximum intensity, in the case of MM-C138S it returns to a value quite similar to the one measured in the native state, while for Mus m 1.0102 we detect a marked increase, indicative that Trp19 does not recover the native chemical environment.

3.5. Allergenicity test

To assess the allergenicity of the selected proteins, the IgE-mediated degranulation was quantified using a Rat Basophil Leukemia (RBL) cell line, permanently transfected with the human high-affinity receptor for the Fc region of IgE (Fc ϵ RI).

The cells were preliminarily reacted with sera from 10 mouse-allergic individuals characterized by Mus m 1.0102-specific IgE levels ranging from 0.60 to 65.39 kU/L, Table 2. Subsequently equimolar amounts of either Mus m 1.0102 or MM-C138S or MM-Y120A were added and the triggered Fc ϵ RI-dependent degranulation was measured (Fig. 7).

All the proteins reacted with the IgE from mouse allergic patients and caused a dose dependent degranulation response. The degranulation induced by MM-Y120A was consistently lower with respect to Mus m

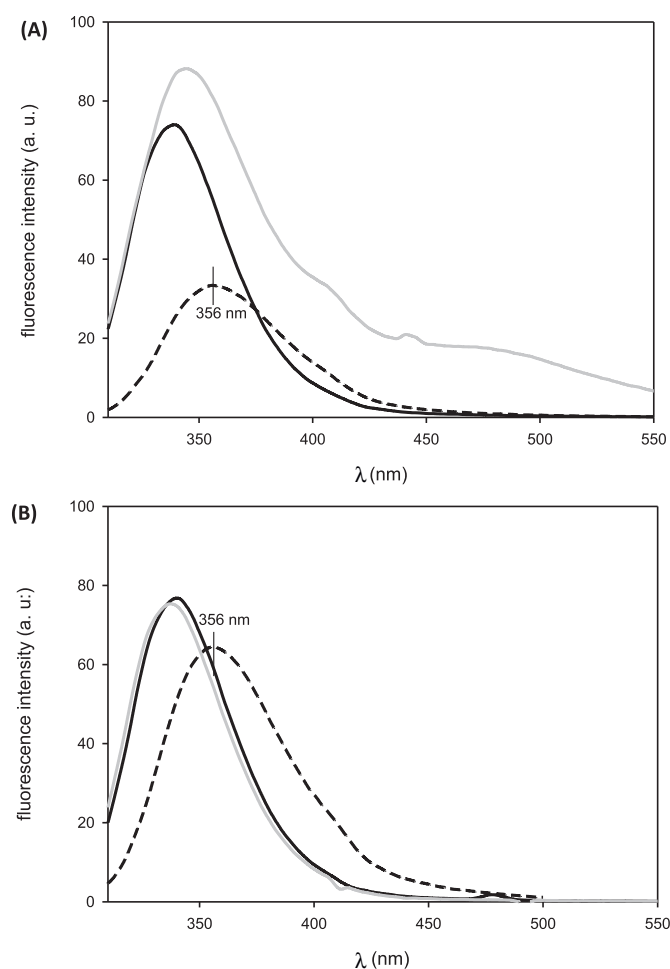


Fig. 6. Protein refolding monitored by intrinsic fluorescence spectroscopy. Tryptophan emission spectra of Mus m 1.0102 (A) and MM-C138S (B): native proteins spectra are represented by solid black lines, the chemically denatured ones by dashed lines and the refolding ones, obtained after dialysis, are in grey.

1.0102 (exception was patient 8 for which, at the moment, we have no explanation), confirming our previous observation [17]. As for MM-C138S, in all tested patients the mutant exhibited a higher capability to induce degranulation than the wild-type protein (Fig. 7).

Table 2
Details of the allergic patients.

	Age ^a	Gender ^b	Diagnosis ^b	Years from diagnosis	Total IgE ^c	Mus m 1 ^{d,e}	Fel d 1 ^{d,e}	Equ c 1 ^{d,e}	Can f 1 ^{d,e}
1	25	f	RC	5	90	2.02	5.19	nd	11.45
2	24	f	RCA	3	132	1.32	0.78	8.54	31.33
3	40	m	RC	3	213	0.91	0.00	0.00	0.00
4	42	f	RC	11	456	61.82	22.06	nd	22.64
5	34	m	RCA	4	88	65.39	17.93	nd	59.41
6	23	f	RC	4	85	25.30	4.49	0.00	0.00
7	22	f	RC	4	876	11.00	2.16	nd	0.00
8	22	f	RC	3	109	0.60	2.02	0.00	0.00
9	21	f	RC	2	98	7.80	40.30	8.07	0.00
10	24	f	RC	4	143	0.66	14.44	38.23	0.00
11	32	m	RC	na	222	0.00	34.00	45	nd

na: not available, nd: not determined.

^a Numbers indicate age in years, F or M indicate gender.

^b The clinically prevalent diagnosis is indicated (RC: rhinoconjunctivitis, A: asthma).

^c Total IgE titer is expressed in International Units/ml (IU/ml).

^d Mus m 1, Fel d 1, Equ c 1 and Can f 1 are, respectively, mouse, cat, horse and dog allergens.

^e Mus m 1, Fel d 1, Equ c 1 and Can f 1-specific IgE are reported in ISAC Standardized Units (ISU, by ImmunoCAP ISAC Immunoassay, Phadia, Uppsala).

No degranulation was induced by challenging the proteins with the cell line that had been treated with the serum from patient 11 who was allergic to cat and horse dander proteins, but not to mouse urine proteins, indicating the Mus m 1-specificity of the tests.

Then we used western blotting to confirm those results. Representative results are depicted in Fig. 8. In all sera containing IgE to Mus m 1.0102, a relatively higher binding signal was observed in lanes containing the MM-C138S, as compared to the wild type Mus m 1.0102. Serum from patient 11, containing high total IgE levels and high titers of specific IgE to cat and horse allergens, Fel d 1 and Equ c 1, did not show any reactivity to any of the considered Mus m 1.0102 recombinant proteins. As a further control, serum 6 was reacted with MM-Y120A, which confirmed the previously described pattern, i.e. a lower reactivity with respect to Mus m 1.0102.

Western blot analysis of Mus m 1.0102 and MM-C138S consistently displayed a pattern characterized by doublet bands. We justify this fact assuming that, though the experiment is carried out in non-reducing denaturant conditions, some sulfur bridge can be reduced anyway: in this event, the slower band could be due to the proteins without the disulfide bridge that adopt a more extended conformation.

3.6. NMR structural analysis: chemical shift perturbation mapping

Weighted chemical shift differences between the ¹H and ¹⁵N chemical shifts, $\Delta\delta(^1\text{H}, ^{15}\text{N})$, of Mus m 1.0102 and MM-C138S, obtained by the analysis of ¹⁵N/¹H HSQC NMR spectra of the uniformly ¹⁵N-labeled proteins, are reported as a bar plot, Fig. 9A. In Fig. 9B, using the cartoon of the NMR-solution structure of Mus m 1.0102 (PDB ID: 1DF3) [16], where Trp19 and Cys138 side chains are in sticks, the residues whose $\Delta\delta(^1\text{H}, ^{15}\text{N})$ presents an alteration larger than 0.15 ppm are highlighted in red. It is evident that, except for Glu123 and Ala160, the affected residues are close, either in sequence or in space, to the site of mutation.

4. Discussion

Allergic respiratory diseases are chronic pathologies including rhinitis and asthma, with an incidence, among young and adult population, second only to hypertension, but prevalent on such common non-communicable chronic diseases as hypercholesterolaemia and diabetes. Allergic asthma is a Th2-driven inflammatory pathology of the bronchial mucosa induced by environmental allergens and triggered by the allergen-specific IgE response [34].

Allergen-specific Immunotherapy (AIT) is the only available therapeutic intervention to treat the immunological unbalance underlying these conditions [35], and allergen structure is the starting point to increase the knowledge of the allergic process in the perspective of potential clinical applications, up to the rational design of hypoallergenic derivatives [36].

We previously described that the single-point mutation Y120A produced a protein with a reasonably reduced allergenicity and preserved immunogenicity [17]. When we designed the mutant MM-Y120A, the mutagenesis target was the core residue Tyr120, positioned in the protein hydrophobic pocket; therefore the substitution with Ala was expected to perturb the ensemble of noncovalent interactions that contribute to the stabilization of the protein architecture. Indeed, we observed a significant perturbation of the spatial arrangement of the aromatic core residues [17], with a consequent alterations of conformational epitopes that impaired their recognition by patients' Mus m 1-specific IgE molecules. Unfortunately, the substitution drastically destabilized the protein architecture, thus impairing its molecular stability in solution and its shelf life; a serious limitation when designing molecules for immunotherapy, as the pharmacological efficacy is obviously compromised by folding instability, poor solubility and aggregation.

Aiming to the preservation of the allergen molecular fold and recognizing that the free Cys in position 138 is responsible for protein aggregation especially upon temperature variation, we regarded

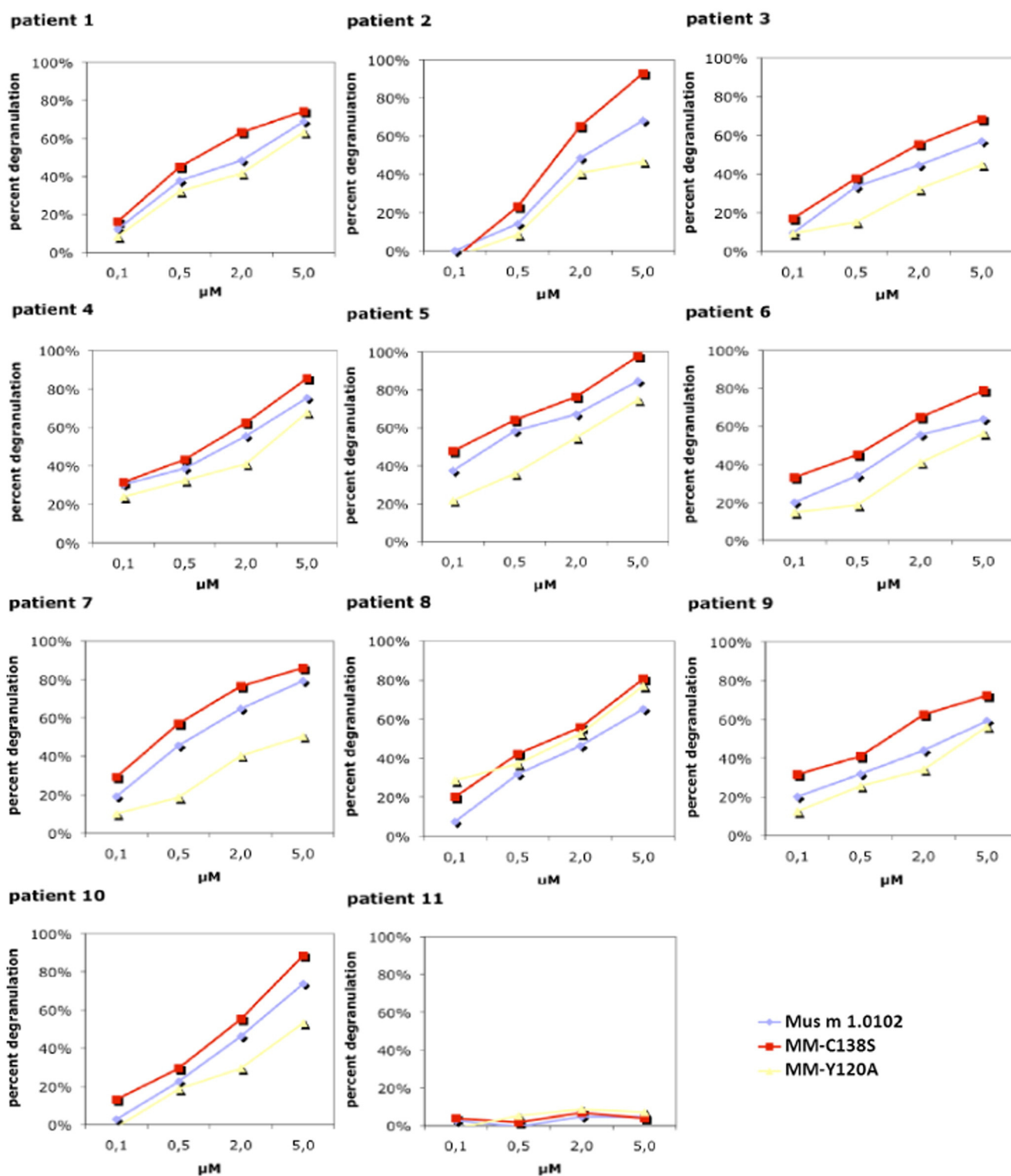


Fig. 7. IgE mediated degranulation. IgE mediated degranulation of human Fc ϵ RI-expressing RBL-SX38 cells that were reacted in vitro with pooled sera from ten allergic individuals and challenged with either native Mus m 1.0102 or MM-C138S or MM-Y120A mutant. Degranulation was measured as percent of maximum β -hexosaminidase release (y-axis) upon incubation with different antigen concentrations (x-axis). MM-Y120A mutant is fully described in ref. Ferrari 2012, and its degranulation data are here reported for a thorough comparison of available Mus m 1.0102 variants data. Mus m 1.0102-specificity of the results was confirmed testing the serum of patient 11 who was allergic to cat and horse dander proteins, but not to mouse urinary proteins.

the C138S mutation as a promising option to overcome these problems.

Circular Dichroism and fluorescence spectroscopies indicate that MM-C138S assumes an overall conformation comparable with the native protein. In addition, DLS data analysis did not evidence macro-aggregates,

yet not excluding the occurrence, over time, of small aspecific oligomers (dimers and trimers).

Thermal denaturation monitored by tryptophan fluorescence and DLS measurements revealed that MM-C138S is slightly less stable than Mus m 1.0102. We hypothesize that the substitution of Cys with the

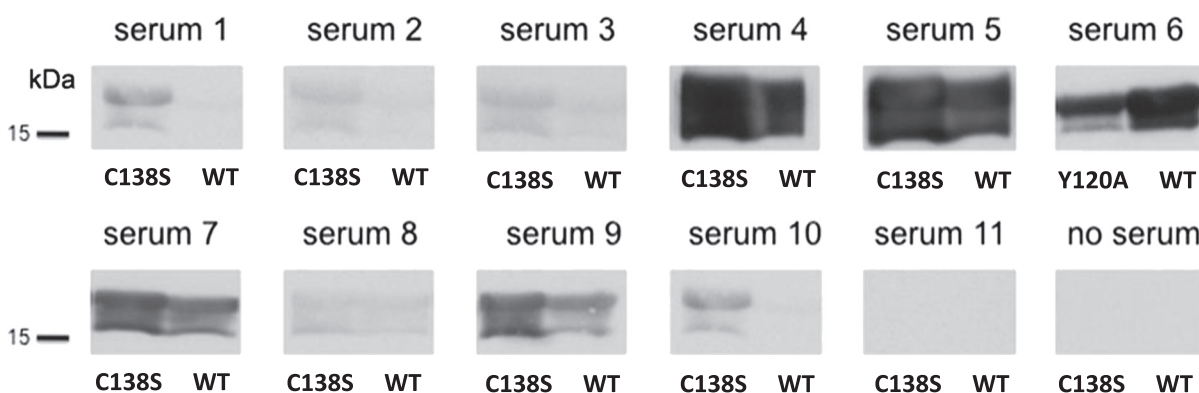


Fig. 8. Western blot analysis. IgE reactivities of Mus m.1.0102, MM-C138S and MM-Y120A mutants, blotted onto single strips, were tested using eleven allergic patients' sera, whose details are reported in Table 2. WT, C138S and Y120A are abbreviations of Mus m.1.0102, MM-C138S and MM-Y120A protein samples, respectively.

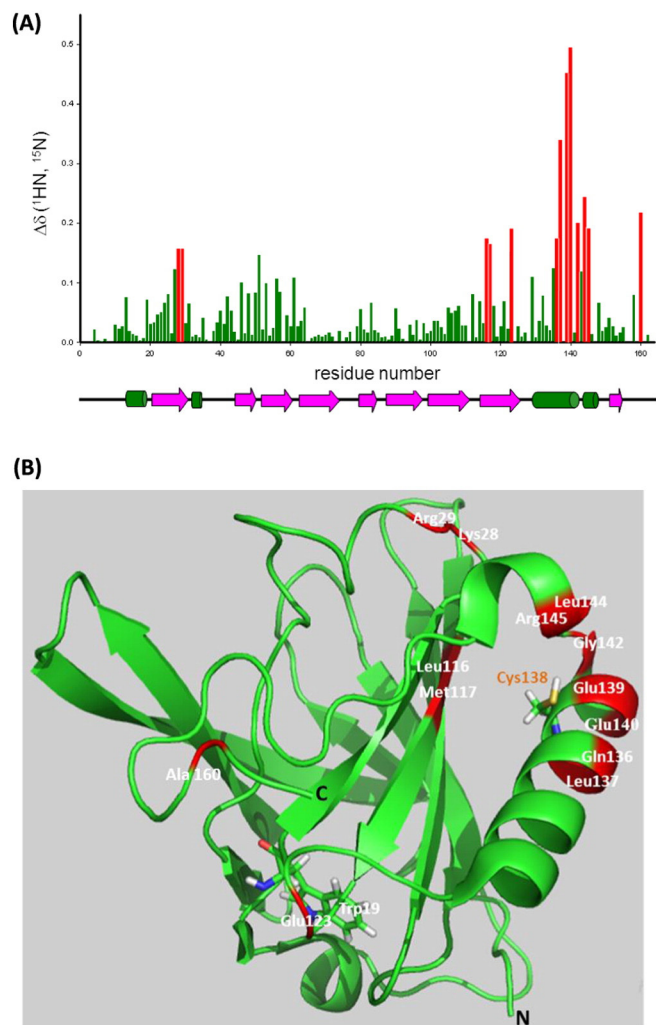


Fig. 9. NMR chemical shift mapping of MM-C138S. A, weighted chemical shift differences between Mus m.1.0102 and the mutant MM-C138S, plotted as a function of amino acid sequence. The scheme of the elements of secondary structure is indicated below the residues string. The red bars correspond to residues presenting a chemical shift higher than 0.15 ppm. B, structural representation of chemical shift perturbations: NMR structure of Mus m.1.0102 (PDB ID: 1DF3, model 1 in cartoon representation, with Cys138 and Trp19 side chains evidenced in sticks) was used to highlight in red the mutant residues with significant chemical shift alteration (>0.15 ppm).

more polar Ser residue may alter the hydrophobic interactions at the interface between the α -helix and the β -barrel, favoring the access to water molecules and thus increasing temperature sensitivity.

GdmCl unfolding in a reducing environment and subsequent refolding of MM-C138S and Mus m.1.0102 confirmed the existence of structural differences between the two proteins. In the unfolded state, though in both proteins Trp19 exhibits a typical red shift of the fluorescence maximum indicative of an increased polarity of its microenvironment, we observe that the fluorescence intensity of Mus m.1.0102 is significantly quenched as normally happens when tryptophan is exposed to water molecules, while the fluorescence intensity of MM-C138S is only slightly reduced. This observation suggests that, in MM-C138S, the increased polarity of the microenvironment sensed by the indole is probably due to a spatial reorganization of nearby polar residues that exert a less efficient quenching action than the water molecules. A successful refolding is obtained only with the mutant. In fact, after extensive dialysis, MM-C138S regains the initial elements of secondary structure (Fig. 4B) and repositions the Trp19 residue in a nearly native hydrophobic environment, as confirmed by the recovered native-like fluorescence spectrum (Fig. 6B). The near-UV CD spectra confirm this fact (Fig. 5B). In the case of Mus m.1.0102 the increased intrinsic emission fluorescence observed after dialysis (Fig. 6A), together with the red shifted emission peak, when compared with the native state features, have been ascribed to the impossibility to recover the cation- π interaction involving the pair Trp19/Arg122 that, in native conditions, acts as an internal quenching mechanism [17,18].

Overall, we summarize these results suggesting that while C138S mutation does not alter the protein architecture, it introduces changes in the intramolecular interactions that lead the mutant to an unfolded state where Trp19 experiences a different microenvironment than in the unfolded Mus m.1.0102 and this fact may justify the diverse refolding capability.

In vitro allergenicity test showed that MM-C138S has an enhanced capability to induce degranulation when compared to Mus m.1.0102, suggesting that its new conformational features increase the efficacy of IgE binding. Western blotting confirmed that result. In fact, nine out of nine human sera, with widely spanning titers of specific IgE to murine allergen, displayed a higher reactivity to MM-C138S than to Mus m.1.0102. Notably, this occurrence does not depend on aspecific binding to IgE because the proteins did not react with sera containing IgE to dander lipocalins from two different species, namely *Felis domesticus* and *Equus caballus*. Finally, in one subject sensitized to mouse urine, we verified, by in vivo prick test, with an end-point dilution approach, that higher reactivities were elicited by MM-C138S as compared to Mus m.1.0102 in a dose dependent manner (Burastero et al., in preparation).

NMR chemical shift mapping revealed local conformational alterations of the backbone primarily positioned near, in sequence and

space, to the region of the mutation. However it is worth noting that also Glu123 experience a modification of its local environment. Recognizing that point mutations can produce modifications in the spatial organization and dynamics of protein regions far from the mutation site [37], we suggest that the mutation, by inducing a repositioning of the negatively charged Glu123, close in space to Trp19, may be responsible for the observed fluorescence results of the denatured samples. In fact, while this residue may contribute to increase the polarity of the microenvironment experienced by Trp19, it is known to be a much less efficient fluorescence quencher than water. Further studies are needed to clear this point.

5. Conclusions

Summarizing, the C138S mutation produces localized structural modifications that provide the mutant with new physico-chemical and functional properties. We envisage that Ser, though isosteric to Cys, by presenting a more polar side chain, perturbs the hydrophobic helix-barrel interface (Fig. 9B): an event responsible, not only for the reduced thermal stability of the mutant, but likely also for an increased flexibility and/or accessibility of allergenic determinants available for binding to allergen specific IgE from patients allergic to mouse urine. These new features, recognized to be relevant for the key residues of a conformational epitope [38], might justify the observed enhanced affinity for the specific IgE.

Overall, MM-C138S, though it exhibits an enhanced allergenicity, because it is prevalently monodispersed and it possesses unfolding reversibility and thermostability up to 56 °C, appears as:

- a good lead protein suitable for diagnostic tests,
- a promising “starting block” to develop, through an additional mutagenesis event, a stable in time and effective molecule for immunotherapy applications,
- an ideal model to investigate the structural features that characterize the allergen-IgE interface.

Finally, observing that the acquired unfolding reversibility of MM-C138S has been obtained by removing an evolutionary conserved residue, we speculate that the free Cys138 may constitute a structural feature of the MUP family finalized to direct these proteins toward an irreversible unfolded state, thus laying the molecular basis for the modulation of their pheromonal communication function and turnover.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2016.08.003>.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgement

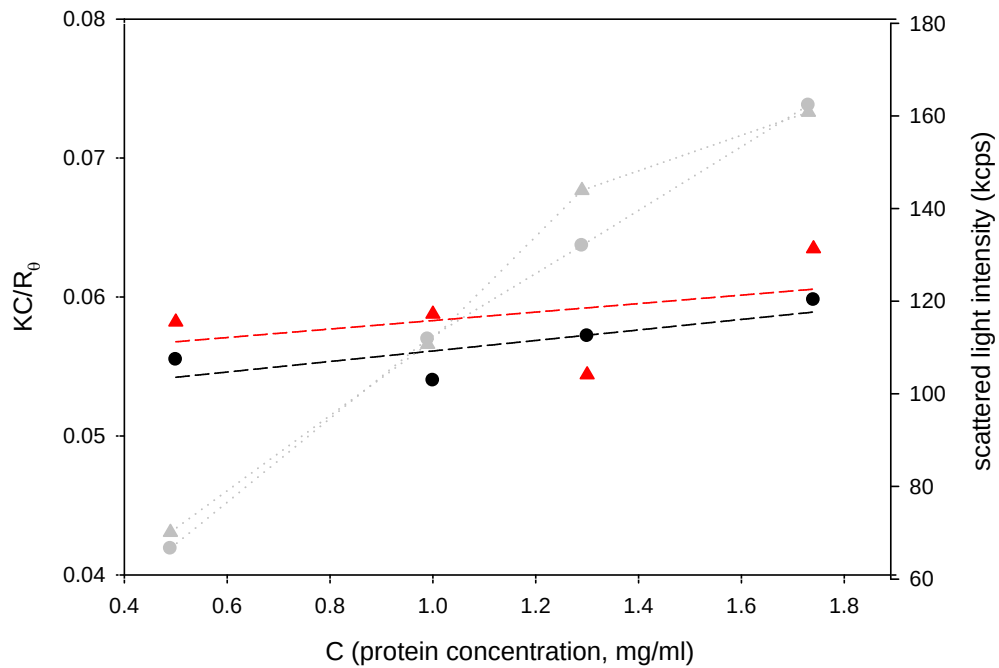
We thank the Centro Interdipartimentale Misura “Giuseppe Casnati” of the University of Parma for the use of the CD spectropolarimeter and NMR spectrometer.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

- [1] S.D. Sharrow, J.L. Vaughn, L. Zidek, M.V. Novotny, M.J. Stone, Pheromone binding by polymorphic mouse major urinary proteins, *Protein Sci.* 11 (2002) 2247–2256, <http://dx.doi.org/10.1110/ps.0204202>.
- [2] R.J. Beynon, J.L. Hurst, Urinary proteins and the modulation of chemical scents in mice and rats, *Peptides* 25 (2004) 1553–1563, <http://dx.doi.org/10.1016/j.peptides.2003.12.025>.
- [3] S.D. Armstrong, D.H. Robertson, S.A. Cheetham, J.L. Hurst, R.J. Beynon, Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone, *Biochem. J.* 391 (2005) 343–350, <http://dx.doi.org/10.1042/BJ20050404>.
- [4] T.A. Pertinhez, E. Ferrari, E. Casali, J.A. Patel, A. Spisni, L.J. Smith, The binding cavity of mouse major urinary protein is optimised for a variety of ligand binding modes, *Biochem. Biophys. Res. Commun.* 390 (2009) 1266–1271, <http://dx.doi.org/10.1016/j.bbrc.2009.10.133>.
- [5] E. Ferrari, A. Tsay, P.A. Eggleston, A. Spisni, M.D. Chapman, Environmental detection of mouse allergen by means of immunoassay for recombinant Mus m 1.0102, *J. Allergy Clin. Immunol.* 114 (2004) 341–346, <http://dx.doi.org/10.1016/j.jaci.2004.04.028>.
- [6] E.C. Matsui, R.A. Wood, C. Rand, S. Kanchanaraks, L. Swartz, P.A. Eggleston, Mouse allergen exposure and mouse skin test sensitivity in suburban, middle-class children with asthma, *J. Allergy Clin. Immunol.* 113 (2004) 910–915, <http://dx.doi.org/10.1016/j.jaci.2004.02.034>.
- [7] E.C. Matsui, P.A. Eggleston, P.N. Breyse, C.S. Rand, G.B. Diette, Mouse allergen-specific antibody responses in inner-city children with asthma, *J. Allergy Clin. Immunol.* 119 (2007) 910–915, <http://dx.doi.org/10.1016/j.jaci.2006.12.663>.
- [8] E. Zahradnik, M. Raulf, Animal allergens and their presence in the environment, *Front. Immunol.* 5 (2014), <http://dx.doi.org/10.3389/fimmu.2014.00076> (art.76).
- [9] M. Jutel, I. Agache, S. Bonini, A.W. Burks, M. Calderon, W. Canonica, L. Cox, P. Demoly, A.J. Frew, R. O’Hehir, J. Kleine-Tebbe, A. Muraro, G. Lack, D. Larenas, M. Levin, B.L. Martin, H. Nelson, R. Pawankar, O. Pfaar, R. van Ree, H. Sampson, J.L. Sublett, K. Sugita, G. Du Toit, T. Werfel, R. Gerth van Wijk, L. Zhang, M. Akdis, A. CA, Position paper – international consensus on allergy immunotherapy, *J. Allergy Clin. Immunol.* 136 (2015) 556–568, <http://dx.doi.org/10.1016/j.jaci.2015.04.047>.
- [10] R. Valenta, R. Campana, M. Focke-Tejkl, V. Niederberger, Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future, *J. Allergy Clin. Immunol.* 137 (2016) 351–357, <http://dx.doi.org/10.1016/j.jaci.2015.12.1299>.
- [11] J. Bousquet, R. Lockey, H.J. Malling, Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper, *J. Allergy Clin. Immunol.* 102 (1998) 558–562, [http://dx.doi.org/10.1016/S0091-6749\(98\)70271-4](http://dx.doi.org/10.1016/S0091-6749(98)70271-4).
- [12] M.A. Calderón, D. Larenas, J. Kleine-Tebbe, L. Jacobsen, G. Passalacqua, P.A. Eng, E.M. Varga, E. Valovirta, C. Moreno, H.J. Malling, E. Alvarez-Cuesta, S. Durham, P. Demoly, European Academy of Allergy and Clinical Immunology task force report on ‘dose-response relationship in allergen-specific immunotherapy’, *Allergy* 66 (2011) 1345–1359, <http://dx.doi.org/10.1111/j.1398-9995.2011.02669.x>.
- [13] T. Basinski, C. Ozdemir, C. Sackesen, P.Y. Mantel, I. Barlan, M. Akdis, M. Jutel, C.A. Akdis, Highlights in cellular and molecular mechanisms of allergic diseases. XXVth Congress of the European Academy of Allergy and Clinical Immunology in Vienna, *Int. Arch. Allergy Immunol.* 142 (2) (2007) 91–98, <http://dx.doi.org/10.1159/000096113>.
- [14] G.W. Canonica, I.J. Ansotegui, R. Pawankar, P. Schmid-Grendelmeier, M. van Hage, C.E. Baena-Cagnani, G. Melioli, C. Nunes, G. Passalacqua, L. Rosenwasser, H. Sampson, J. Sastre, J. Bousquet, T. Zuberbier, WAO-ARIA-GA²LEN Task Force, K. Allen, R. Asero, B. Bohle, L. Cox, F. de Blay, M. Ebisawa, R. Maximiliano-Gomez, S. Gonzalez-Diaz, T. Haahela, S. Holgate, T. Jakob, M. Larche, P.M. Matricardi, J. Oppenheimer, L.K. Poulsen, H.E. Renz, N. Rosario, M. Rothenberg, M. Sanchez-Borges, E. Scala, R. Valenta, A WAO – ARIA – GA²LEN consensus document on molecular-based allergy diagnostics, *World Allergy Organ. J.* 6 (1) (2013) 1–17, <http://dx.doi.org/10.1186/1939-4551-6-17>.
- [15] G. Pauli, T.H. Larsen, S. Rak, F. Horak, E. Pastorello, R. Valenta, A. Purohit, M. Arvidsson, A. Kavina, J.W. Schroeder, N. Mothes, S. Spitzauer, A. Montagut, S. Galvain, M. Melac, C. André, L.K. Poulsen, H.J. Malling, Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis, *J. Allergy Clin. Immunol.* 122 (2008) 951–960, <http://dx.doi.org/10.1016/j.jaci.2008.09.017>.
- [16] R. Valenta, The future of antigen-specific immunotherapy of allergy, *Nat. Rev. Immunol.* 2 (2002) 446–453, <http://dx.doi.org/10.1038/nri824>.
- [17] E. Ferrari, D. Breda, R. Longhi, L. Vangelista, C.R. Nakaie, L. Elviri, E. Casali, T.A. Pertinhez, A. Spisni, S.E. Burastero, In search of a vaccine for mouse allergy: Significant reduction of Mus m 1.0102 allergenicity by structure-guided single-point mutations, *Int. Arch. Allergy Immunol.* 157 (2012) 226–237, <http://dx.doi.org/10.1159/000327551>.
- [18] C. Lücke, L. Franzoni, F. Abbate, F. Löhner, E. Ferrari, R.T. Sorbi, H. Rütersjans, A. Spisni, Solution structure of a recombinant mouse major urinary protein, *Eur. J. Biochem.* 266 (1999) 1210–1218, <http://dx.doi.org/10.1046/j.1432-1327.1999.00984.x>.
- [19] M.M. Phelan, L. McLean, J.L. Hurst, R.J. Beynon, L.Y. Lian, Comparative study of the molecular variation between ‘central’ and ‘peripheral’ MUPs and significance for behavioural signaling, *Biochem. Soc. Trans.* 42 (2014) 866–872, <http://dx.doi.org/10.1042/BST20140082>.
- [20] D. Jayat, J.C. Gaudin, J.M. Chobert, T.V. Burova, C. Holt, I. McNae, L. Sawyer, T. Haertlé, A recombinant C121S mutant of bovine beta-lactoglobulin is more susceptible to peptic digestion and to denaturation by reducing agents and heating, *Biochemistry* 43 (2004) 6312–6321, <http://dx.doi.org/10.1021/bi0362469>.
- [21] L. Jiafu, L. Kejiang, G. Chenyun, G. Hongchang, Y. Yihe, L. Donghai, Expression and purification of cysteine mutation isoforms of rat lipocalin-type prostaglandin D synthase for nuclear magnetic resonance study, *Acta Biochim. Biophys. Sin.* 40 (2008) 489–496, <http://dx.doi.org/10.1111/j.1745-7270.2008.00426.x>.
- [22] E. Ferrari, T. Lodi, R.T. Sorbi, R. Tirindelli, A. Cavaggioni, A. Spisni, Expression of a lipocalin in *Pichia pastoris*: secretion, purification and binding activity of a recombinant mouse major urinary protein, *FEBS Lett.* 401 (1997) 73–77, [http://dx.doi.org/10.1016/S0014-5793\(96\)01436-6](http://dx.doi.org/10.1016/S0014-5793(96)01436-6).
- [23] Malvern Instruments White Paper, Dynamic light scattering – Common terms defined.
- [24] S. Kaul, D. Luttkopf, B. Kastner, L. Vogel, G. Holtz, S. Vieths, A. Hoffmann, Mediator release assays based on human or murine immunoglobulin E in allergen standardization, *Clin. Exp. Allergy* 37 (2007) 141–150, <http://dx.doi.org/10.1111/j.1365-2222.2006.02618.x>.

- [25] A.H. Nowak-Węgrzyn, R. Bencharitiwong, J. Schwarz, G. David, P. Eggleston, P.J. Gergen, A.H. Liu, J.A. Pongracic, S. Sarpong, H.A. Sampson, Mediator release assay for assessment of biological potency of German cockroach allergen extracts, *J. Allergy Clin. Immunol.* 123 (2009) 949–955, <http://dx.doi.org/10.1016/j.jaci.2009.01.070>.
- [26] T.W. Wiegand, P.B. Williams, S.C. Dreskin, M.H. Jouvin, J.P. Kinet, D. Tasset, High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor 1, *J. Immunol.* 157 (1996) 221–230.
- [27] L. Vangelista, M. Cesco-Gaspere, R. Lorenzi, O. Burrone, A minimal receptor-Ig chimera of human Fc epsilon RI alpha-chain efficiently binds secretory and membrane IgE, *Protein Eng.* 15 (2002) 51–57, <http://dx.doi.org/10.1093/protein/15.1.51>.
- [28] L. Vangelista, E. Soprana, M. Cesco-Gaspere, P. Mandiola, G. Di Lullo, R.N. Fucci, F. Codazzi, A. Palini, G. Paganelli, O.R. Burrone, A.G. Siccardi, Membrane IgE binds and activates Fc epsilon RI in an antigen-independent manner, *J. Immunol.* 174 (2005) 5602–5611, <http://dx.doi.org/10.4049/jimmunol.174.9.5602>.
- [29] S.E. Burastero, C. Paolucci, D. Breda, G. Monasterolo, R.E. Rossi, L. Vangelista, Unreliable measurement of basophil maximum leukotriene release with the Buhlmann CAST 2000 enzyme-linked immunosorbent assay kit, *Clin. Vaccine Immunol.* 13 (2006) 420–422, <http://dx.doi.org/10.1128/CVI.13.3.420-422.2006>.
- [30] M.S. Neuberger, G.T. Williams, E.B. Mitchell, S.S. Jouhal, J.G. Flanagan, T.H. Rabbitts, A hapten-specific chimaeric IgE antibody with human physiological effector function, *Nature* 314 (1985) 268–270.
- [31] A. George, W.W. Wilson, Predicting protein crystallization from a dilute solution property, *Acta Cryst D50* (1994) 361–365.
- [32] L.H. Greene, E.D. Chrysina, L.I. Irons, A.C. Papageorgiou, K.R. Acharya, K. Brew, Role of conserved residues in structure and stability: tryptophans of human serum retinol-binding protein, a model for the lipocalin superfamily, *Protein Sci.* 10 (2001) 2301–2316, <http://dx.doi.org/10.1110/ps.22901>.
- [33] H.L. Greene, D. Hamada, J.S. Eyles, K. Brew, Conserved signature proposed for folding in the lipocalin superfamily, *FEBS Lett.* 553 (2003) 39–44, [http://dx.doi.org/10.1016/S0014-5793\(03\)00925-6](http://dx.doi.org/10.1016/S0014-5793(03)00925-6).
- [34] M. Schatz, L. Rosenwasser, The allergic asthma phenotype, *J. Allergy Clin. Immunol. Pract.* 2 (6) (2014) 645–648, <http://dx.doi.org/10.1016/j.jaip.2014.09.004>.
- [35] M. van Hage, G. Pauli, New vaccines for mammalian allergy using molecular approaches, *Front. Immunol.* 5 (2014), <http://dx.doi.org/10.3389/fimmu.2014.00081> (art.81).
- [36] V. Niederberger, F. Horak, S. Vrtala, S. Spitzauer, M.T. Krauth, P. Valent, J. Reisinger, M. Pelzmann, B. Hayek, M. Kronqvist, G. Gafvelin, H. Grönlund, A. Purohit, R. Suck, H. Fiebig, O. Cromwell, G. Pauli, M. van Hage-Hamsten, R. Valenta, Vaccination with genetically engineered allergens prevents progression of allergic disease, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 14677–14682, <http://dx.doi.org/10.1073/pnas.0404735101>.
- [37] D. Gagné, R.L. French, C. Narayanan, M. Simonović, P.K. Agarwal, N. Doucet, Perturbation of the conformational dynamics of an active-site loop alters enzyme activity, *Structure* 23 (2015) 2256–2266, <http://dx.doi.org/10.1016/j.str.2015.10.011>.
- [38] F. Dall'Antonia, T. Pavkov-Keller, K. Zangger, W. Keller, Structure of allergens and structure based epitope predictions, *Methods* 66 (2014) 3–21, <http://dx.doi.org/10.1016/j.ymeth.2013.07.024>.



Supplementary Figure Debye plots. Debye plots (KC/R_q versus C) of Mus m 1.0102 (black circles) and MM-C138S (red triangle) were created by measuring the scattered light at four sample concentrations. The scattered light intensity values were analysed using the Rayleigh equation (1), presented in the Material and Methods section; the dashed lines are generated by linear regression of the data points and are coloured according to protein symbols. The slope of the fitting lines is twice the 2nd virial coefficient A_2 , while the intercept at zero concentration is the inverse of the protein molecular weight. The scattered light intensity values of Mus m 1.0102 (grey circles) and MM-C138S (grey triangle) are plotted as a function of protein concentration.