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Relationship between the magnitude of IgE production in mice and conformational stability of the house dust mite allergen, Der p 2

Hitomi Nakamura^{a,b,d}, Takatoshi Ohkuri^{a,b,d}, Takanori So^c, and Ueda Tadashi^{a*}.

^aGraduate School of Pharmaceutical Sciences, Kyushu University

^bFaculty of Pharmaceutical Sciences, Sojo University

^cGraduate School of Medicine, Tohoku University

^d These authors contributed equally to this work

*Corresponding author at: Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
TEL, +81-92-642-6662; FAX, +81-92-642-6667;

E-mail address: ueda@phar.kyushu-u.ac.jp

Abbreviations: Der p, *Dermatophagoides pteronyssinus*; HEL, hen egg lysozyme; CD, circular dichroism.

ABSTRACT

Background: Protein antigens are degraded by endosomal protease in antigen presentation cell. T cells recognize peptides derived from antigen proteins bound to class II major histocompatibility complex molecules. We previously reported that an increase in the conformational stability of an antigen depressed its immunogenicity. However, there is little information on antigens with differences in molecular properties such as net charges, molecular weight and so on.

Methods: Denaturation experiments against guanidine hydrochloride. The serum IgE levels to protein antigens at 35 days after the first immunization analyzed using ELISA.

Results: The Der p 2 mutations in which Ile13 is mutated to Ala (I13A) and Ala122 is mutated to Ile (A122I) were shown to have lower and higher conformational stability than the wild-type, respectively, by denaturation experiments. The amount of IgE production by the less stable I13A mutant was higher and that of the stable A122I mutant was lower than that of the wild-type.

Conclusion: Our results suggest that the increased conformational stability of Der p2 depressed the IgE production in mice.

General significance: These findings should provide a milestone for the engineering of allergen vaccines.

1. Introduction

Various therapeutic proteins—including erythropoietin, insulin proteins, growth hormones and cytokines—have been shown to provide unique and effective treatments for human diseases [1,2]. In addition, several therapeutic immunoglobulins are currently proving to be blockbuster drugs in the pharmaceutical industry. Nonetheless, repeated administration of protein therapeutics, whether natural or recombinant, often leads to the induction of undesirable anti-drug antibodies (ADAs), which can neutralize the effect of the drug [3-6]. In the mammalian immune system, antigen-specific CD4⁺ T-cells recognize antigen-derived peptides. The antigen peptides are produced by lysosomal protease and efficient lysosomal degradation favors the production of peptides for MHC class II molecules [7-11]. Proteins in solution usually exist in equilibrium between a native and denatured state. Proteases preferentially digest proteins in an unfolded state rather than those in a folded state [12-15]; thus, the conformational protein stability may be related to intracellular antigen processing. In previous studies using modified hen egg lysozyme (HEL) as a model protein antigen, we found that the T-cell triggering response was governed by the degree of conformational stability of the protein antigen [16,17]. Moreover, it was demonstrated that the amounts of IgG production against HEL with different stabilities in mice were inversely

and linearly correlated with the free-energy changes of protein unfolding [18]. These findings were supported by other studies using snake toxin [19].

There are many antigens with differences in molecular properties, such as net charges, molecular weight and so on. Therefore, we still need to verify the relation between the conformational stability of protein antigens and the immune response using various antigens. House dust mites are a major source of aeroallergens for patients with allergic asthma [20]. Der p 2, which has a molecular weight of about 14 kDa, is one of the major allergens of *Dermatophagoides pteronyssinus* (Der p). Since it has structural homology with MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signaling complex [21-22], Der p 2 induced allergy in MD-2-deficient mice [23]. In this study, we examined whether the relationship between the magnitude of IgE production and conformational stability was applicable to the house dust mite allergen, Der p 2.

2. Materials and Methods

2.1. Construction of the expression vector

The Der p 2 gene was obtained by PCR using long primers and was

inserted between the NdeI and EcoRI sites of the pET22b vector (Novagen, Madison, WI), just downstream of the T7 promoter. Site-directed mutagenesis of the Der p 2 gene was performed by PCR, according to previously described methods [24]. Mutations were confirmed by DNA sequence analysis.

2.2. Expression, refolding, and purification of Der p 2

Der p 2 was overexpressed in *Escherichia coli* BL21 cells harboring a recombinant plasmid constructed using a pET22b expression vector (Novagen). The transformant cells were grown at 37°C in 1L of LB medium containing 100 µg/ml ampicillin. The culture was allowed to grow until mid-log phase ($OD_{600}=0.5-1.0$) and the expression of Der p 2 was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 3 h. The cultured cells were harvested by centrifugation for 7 min at 8,000 rpm. The pellets were suspended in 50 ml of 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM EDTA, and then sonicated twice for 2 min each time in an ice-water bath. The mixture was centrifuged for 20 min at 12,000 rpm. The precipitates were resuspended in 7 ml of 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM EDTA. After addition of DNase I (final concentration of 5 µg/ml), RNase A (final concentration of 20 µg/ml) and $MgCl_2$ (final concentration of 10

mM), the solution was incubated at 40°C for 1 h to decompose nucleic acids. Then sodium chloride was added to the solution to a final concentration of 0.8 M, and the mixture was centrifuged. The precipitates were suspended in 160 ml of 6 M guanidine solution (0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM EDTA and 6 M guanidine hydrochloride) and incubated at 4 °C for 12 h. The folding of Der p 2 was performed by dialyzing against 4L of 20 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA without guanidine hydrochloride at 4 °C, three times for 4 h each. After the refolding reaction, the protein solution was dialyzed against 50 mM sodium acetate buffer, pH 4.0, and applied to a cation exchange (SP-Toyopearl) column (2 cm x 25 cm). The column was eluted with a gradient of 300 ml of 50 mM sodium acetate buffer, pH 4.0, and 300 ml of the same buffer containing 1.5 M NaCl. The protein fraction was collected and lyophilized after dialysis against 1% acetic acid.

2.3. CD spectra of the folded wild-type and mutant Der p 2

The folded and purified wild-type and mutant Der p 2 were dialyzed against ultrapure water and prepared at a concentration of 0.1 mg/ml with ultrapure water. Then, the CD spectra were obtained with a Jasco-J 720 spectropolarimeter.

2.4. Measurement of the stabilities of the wild-type and mutant Der p 2 against guanidine hydrochloride

Measurement of the stabilities of Der p 2 against guanidine hydrochloride was performed with a Hitachi F2000 fluorescence spectrophotometer thermostated at 37 °C. Der p 2 was incubated overnight at 37 °C in 50 mM sodium acetate buffer, pH 4.0, due to slow relaxation in the unfolding-refolding process of Der p 2. The tryptophyl fluorescence of Der p 2 at 330 nm and 370 nm was measured by using 280 nm light for excitation with various concentrations of guanidine hydrochloride. The protein concentration was 0.2 µM.

2.5. Protease digestion of the wild-type and mutant Der p2

The wild-type and mutant Der p 2 dissolved 1% aqueous acetic acid were exhaustively dialyzed against 50 mM phosphate buffer at pH 7.5. To these protein solutions (final concentration of 1 mg/ml), 10 micrograms of subtilisin BPN' (Sigma-Aldrich, St. Louis, MO) were added three times every 15 minutes. Meanwhile, respective solutions were incubated at 37°C. After 15 minutes of the final addition of subtilisin BPN', in which total incubation time at 37°C is 1 h, 10% aqueous acetic acid was added to the protein solutions in order to stop respective protease reactions.

Respective reactants was applied to the cation-exchange resin (1 x 3 cm, CM-Toyopearl) equilibrated with 50 mM acetate buffer, and then eluted with the buffer containing 1.0 M NaCl. Each elution was monitored by absorbance at 280nm. On the other hand, the respective proteins (1 mg/mL) unreacted with protease were eluted as was the same. Protease digestion ratios for the wild-type and mutant Dep 2 were obtained by the ratio of respective absorbances from the wild-type and mutant Dep 2 reacted with subtilisin BPN' to those unreacted with subtilisin BPN'.

2.6. Immunization protocol

Six-week-old female BALB/c mice were obtained from SLC Inc. (Japan). Five mice were immunized by repeated intraperitoneal injections (i.p.) with 0.1 ml of phosphate buffered saline (PBS) emulsified 1:1 in Imject Alum adjuvant (Thermo Scientific, Rockford, IL) containing the wild-type Der p 2 or Der p 2 mutants (50 µg/mouse) on day 0, 7, 21, and 28. Blood samples were drawn from the orbital sinus into capillary tubes.

2.7. Measurement of serum IgE levels

Antigen-specific IgE antibody titers were measured by sandwich ELISA. 96-well ELISA plates were coated with anti-mouse IgE (10 µg/ml)

(LO-ME-2; Technopharm Biotechnology, Paris, France) in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4°C. Plates were washed three times with wash solution (0.02% Tween 20, 0.15M NaCl, 0.02% NaN₃; TBS-Tween) and blocked for 1 h with blocking buffer (TBS-Tween containing 2% non-fat dry milk) at room temperature. After washing, serum diluted 1/50 in blocking buffer was added and the plates were incubated overnight at 4°C. Der p 2 modified with biotin (10 µg/ml) (Sulfo-NHS-LC Biotin; Thermo Scientific) was then added to each well followed by incubation overnight at 4°C. Binding of the biotinylated Der p 2 was subsequently probed by the avidin-biotin alkaline phosphatase complex method using a Vectastain ABC-AP Standard Kit (Vector Laboratories, Burlingame, CA). p-nitrophenyl phosphate was used as a substrate for alkaline phosphate and, following development, the absorbance at 405 nm of each well was recorded using an ImmunoMini NJ-2300 plate reader (InterMed, Tokyo, Japan).

3. Results

3.1. Preparation of the functional Derp2

Recombinant Der p 2 was overexpressed in *E. coli* and the expression of the protein was assessed by SDS-PAGE as shown in Fig.1. Der p 2 was

produced as an inclusion body and cell debris was collected by centrifugation after sonication of the harvest cells. The refolding of Der p 2 from the inclusion body was performed according to the method described previously [25]. Mueller *et al.* reported that the inclusion body was dissolved in one-fifth of the culture volume of 6 M guanidine-HCl and dialyzed against 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, with the result that Der p 2, which has three disulfide bonds (Cys21-Cys27, Cys73-Cys78, Cys8-Cys119), could be refolded without oxidizing and reducing reagents. Unfortunately, however, by this method most of the Der p 2 was aggregated during the dialysis in the refolding step even in the presence of additives such as arginine because the aggregation was depressed. Under these circumstances, because we extended the denaturation time of Der p 2 in 6 M guanidine hydrochloride solution to 12 h, the aggregation during the refolding was drastically decreased, with the result that there were no other proteins but Der p 2 in the supernatant after the dialysis, as shown in Fig. 2. After the refolding reaction, Der p 2 was purified by cation-exchange chromatography on a SP-Toyopearl column (Fig. 3A). The main eluted peak was found to be homogeneous with a molecular weight similar to that of Der p 2, based on SDS-PAGE analysis (Fig. 3B). The CD spectrum of the purified wild-type Der p2 was also measured (Fig. 3C). The spectrum showed a typical beta-sheet like that in the crystal structure of Der p 2 [22]. The yield of refolded Der p 2 was 38 mg from 1 l culture.

3.2. Preparation of Der p 2 mutants and their stabilities

Next, we attempted to modify the conformational stability of Der p 2. Hydrophobic interaction is one of the main factors in the stabilization of protein conformation. Generally, there are cavities within the protein cores, especially in large proteins [26]. It has been reported that an increase in cavity size within the hydrophobic cores of proteins leads to destabilization [27-29]. On the other hand, the cavity-filling mutations with hydrophobic residues have improved the protein stabilities [30-32]. The X-ray structure of Der p 2 consists of two anti-parallel β -plated sheets and shows a cavity $\sim 9\text{\AA}$ wide, $15\text{\AA}$ long and $3.5\text{\AA}$ deep between the two β -sheets [22]. It has been reported that the internal cavity line with several hydrophobic residues and I13 or A122 is one of these residues [22], as shown in Fig. 4. To alter the conformational stability, I13A mutation or A122L mutation was introduced into Der p 2. These mutations and protein expressions were carried out according to the descriptions in the Materials and Methods. The preparation of tertiary structures of mutant Der p 2 was done in a manner similar to that described above for the wild-type. And the CD spectra of these Der p 2 mutants were identical to that of the wild-type shown in Fig. 3C. The stabilities of these mutants were evaluated by guanidine-induced equilibrium as a change of tryptophyl

fluorescence of Der p 2 (Fig. 5). The folding-unfolding of the wild-type Der p2 against guanidine hydrochloride denaturation was almost reversible (data not shown). The unfolding curve of the I13A mutant was shifted to a lower guanidine concentration relative to that of the wild-type, indicating that the I13A mutant was more unstable than the wild-type. The destabilization of the I13A mutant was probably due to the decrease in favorable interactions in the internal cavity of Der p 2 by the mutation. On the other hand, the unfolding curve of the A122L mutant was shifted to a higher guanidine concentration relative to that of the wild-type, indicating that the A122L mutant was more stable than the wild-type. This result raises the possibility that the stabilization of the A122L mutant was caused by cavity-filling of Der p 2. Thus, we succeeded in the preparation of Der p 2 mutants possessing different conformational stabilities.

3.3. Protease resistance of the wild-type and mutant Der p 2

To evaluate protease resistance of the wild-type and mutant Der p 2, we employed trypsin and chymotrypsin in vain due to their higher stabilities. Then, we digested them at pH 7.5 using considerable amounts of subtilisin BPN' (Fig. 6). As a result, under the condition where residual amount of the wild-type Der p2 was about 10%, that of A122I was about 5% whereas that of I13A was about 20%, resulting that more stable protein

among the wild-type and two mutant Der p2 has a tendency resistant to subtilisin BPN' digestion. The similar tendency was obtained in the protease digestions using proteinase K (data not shown).

3.4. Immunogenicities of the wild-type and mutant Der p 2 against mice

To determine the effect of protein conformational stability on IgE production, Balb/c mice were immunized with the wild-type or Der p 2 mutants, which were emulsified in alum adjuvant. The reactivities of these antisera to the wild-type and respective Der p 2 mutants were analyzed using ELISA. The serum IgE levels to each protein antigen at 35 days after the first immunization are shown in Fig. 7 as relative absorbance according to the previous results [18]. We confirmed that each value of absorbance was proportional to the serum IgE concentration. The serum IgE levels of the unstable I13A mutant were higher both than that of the wild-type and that of the stable A122I mutant with higher conformational stability. As described above, Der p 2 mutants had the same secondary-structure content as the wild-type based on circular dichroism analysis. Additionally, it has been reported that the amino acid residues I13 and A122 were not included in T cell epitopes of Der p 2 in BALB/c mice (H-2^d haplotype) [33]. In this study, therefore, it was also found that conformational stability of the antigen, Der p 2, was involved in

the IgE production.

4. Discussion

Antigen peptides are produced by lysosomal proteolysis. Although a globular protein is in equilibrium between the native and denatured state, the protease preferentially digests proteins in a denatured state rather than those in a native state [13,14,34]. The conformational stability of antigens is an important factor regulating antigen-processing efficiency and thus the T-cell triggering response is governed by the conformational stability of protein antigens [16]. It has been reported that the conformational stability of HEL was inversely correlated not only to the magnitude of the *in vivo* Th1 response but also to the magnitude of the *in vivo* Th2 response, which is closely related to IgE production [17]. Moreover, in our previous studies, it was shown that a decrease in IgG production could also occur through increased stabilization of Phl p 7, which is the grass pollen allergen, as well as through increased stabilization of HEL [18]. On the other hand, the conformational stability in solution is not always the main factor in immunogenicity [35,36]. Given these facts, we further examined the relationship between the conformational stability of Der p 2 and the immunogenicity. In addition, because the IgE production is known to be controlled by a class-switch from IgG to IgE due to cytokine IL-4, we also

examined the IgE production in mice in response to antigens with different conformational stabilities.

At first, we could prepare Der p 2 mutants whose conformational stabilities were higher or lower than the wild-type Der p 2, respectively. Second, the protein with higher conformational stability among the wild-type and mutant Der p2 was shown to have a tendency resistant to protease digestions such as subtilisin BPN[®] and proteinase K. Then, the wild-type and respective Der p 2 mutants were immunized to mice so that antibodies of IgE class could be produce using PBS emulsified 1:1 in alum adjuvant. As a result, we demonstrated that there was a relationship between the magnitude of IgE production and conformational stability. This finding was consistent with previous studies in which peptides showed minimal IgE reactivity, confirming that the epitopes on Der p 2 are dependent on the tertiary structure of the protein [37-39]. Moreover, we also showed using Der p 2 mutants that the increase in conformational stability with resistance to protease digestion has a tendency to depress the antigen immunogenicity. Recently, there have been reports on the conformational stability of proteins used in the preparation of vaccines [40,41]. Therefore, these findings should provide a milestone for the engineering of allergen vaccines.

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(Figure legends)

Fig. 1. SDS-PAGE analysis of expression of the wild-type Der p 2 from *E.coli*. Lanes 1 and 2, the total extract *E. coli* cells harboring the pET22b/Der P 2 gene, uninduced (lane 1) and induced with IPTG (lane 2).

Fig. 2. SDS-PAGE analysis of folding of the wild-type Der p 2. Lane 1, the supernatant after dialysis. Lane 2, the precipitant after the dialysis.

Fig. 3. Purification of the wild-type Der p 2. A) Cation-exchange

chromatography of the supernatant after the dialysis refolding. The column (SP-Toyopearl; 2 cm × 25 cm) was eluted with a gradient of 300 ml of 50 mM acetate buffer, pH 4.0, and 300 ml of the same buffer containing 1.5 M NaCl at 4°C. B) SDS-PAGE analysis of the eluted fraction on the chromatography in Fig. 3A. C) CD spectra of the wild-type and mutant Der p2 (0.1 mg/ml) with a Jasco-J 720 spectropolarimeter.

Fig. 4. Three-dimensional structure of the wild-type Der p 2 (from PDB entry, 1NEP). The mutated amino acids are shown as sticks. Lower figure is from top view of upper figure.

Fig. 5. Fluorescence intensity ratio (350 nm / 330 nm) of the wild-type Der p 2 (open circles) and I13A Der p 2 mutants (closed squares), or A122L Der p 2 mutants (closed triangles) at the various concentrations of guanidine chloride. The wild-type Der p 2 and the mutants were incubated at 37 °C in 50 mM sodium acetate buffer, pH 4.0, and various concentrations of guanidine hydrochloride; the protein concentrations were 0.2 µM.

Fig. 6. Protease digestion of the wild-type and mutant Der p2.

The wild-type and mutant Der p 2 (1 mg/ml) were digested with subtilisin BPN' (total amount of 30 micrograms) for 1 h (total incubation time) (see

Materials and Methods in detail). Residual percentages of the wild-type and mutant Der p2 were evaluated by dividing the wild-type and mutant Der p2 without subtilisin BPN' digestion into the amounts of the wild-type and mutant Der p2 after subtilisin BPN' digestion.

Fig. 7. Measurement of serum IgE levels by ELISA. Three groups of five mice each were immunized with the wild-type Der p 2, I13A Der p 2 mutants, or A122L Der p 2 mutants. The data represent IgE levels in the serum after day 35 and were expressed relative to the value of absorbance at 405 nm in the wild-type. Statistically significant differences by Student's *t*-test are indicated with asterisks: * $p < 0.05$; ** $p < 0.01$.

Fig.1

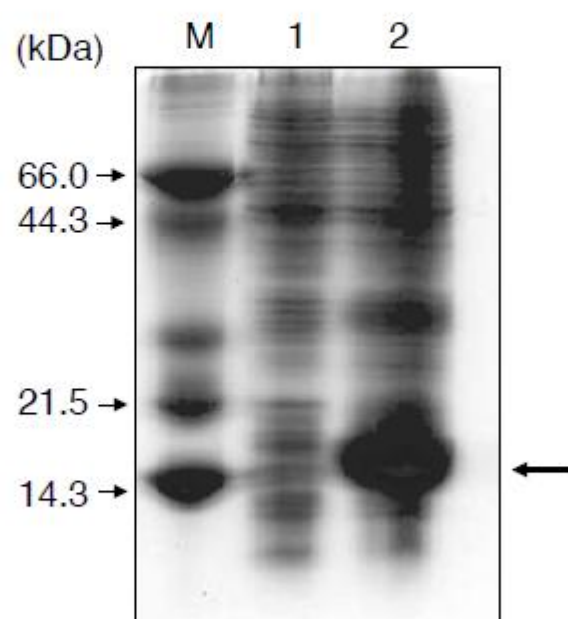


Fig.2

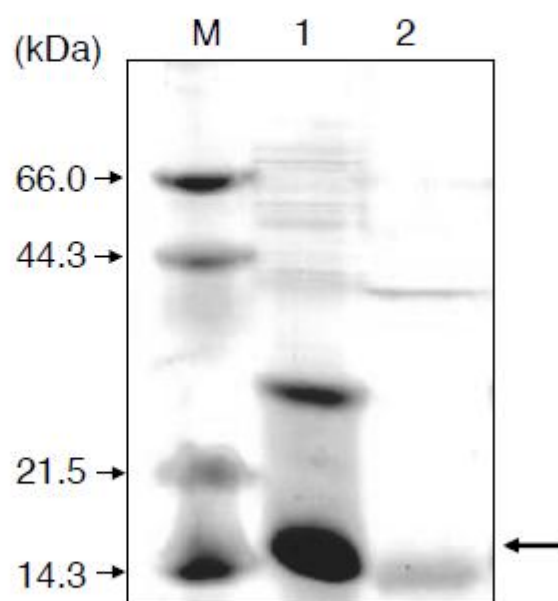


Fig.3

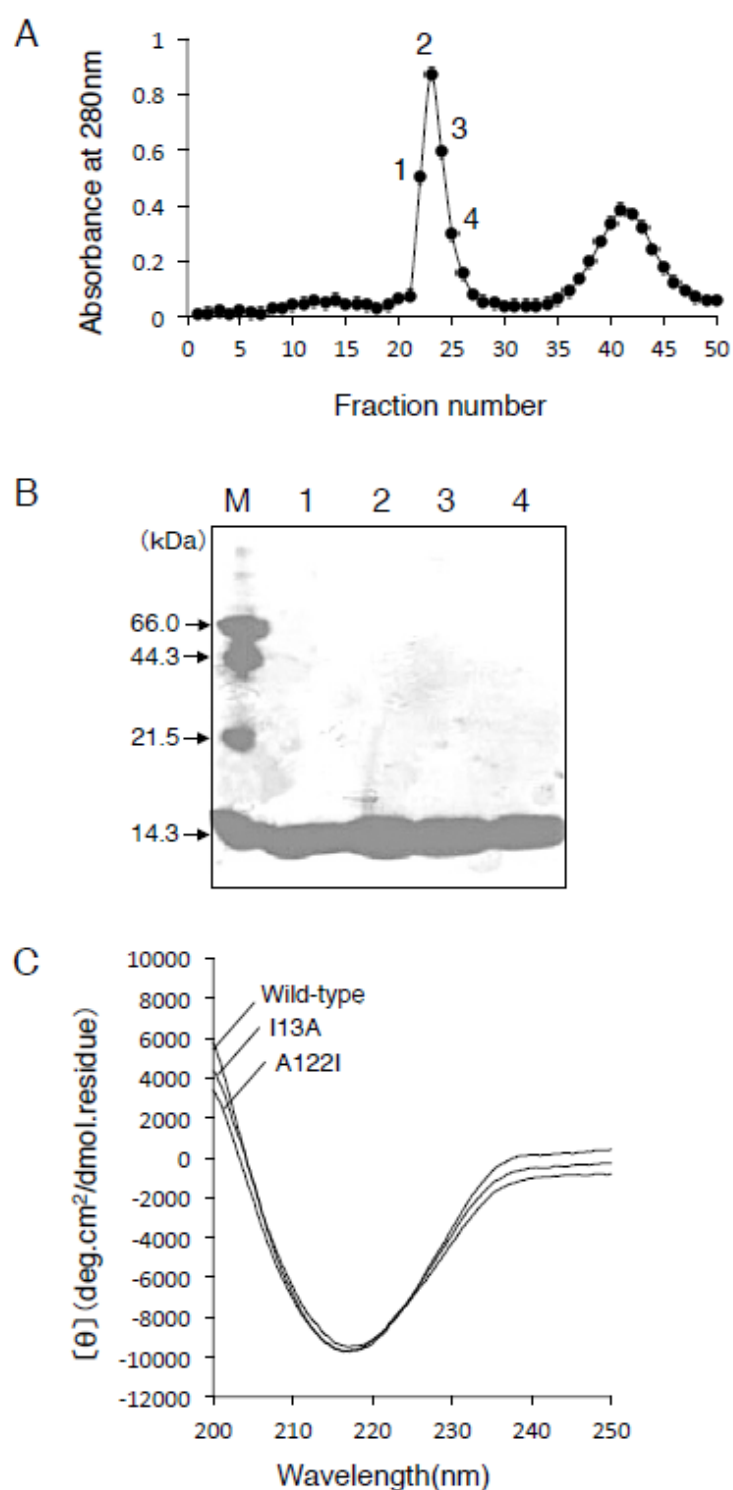


Fig.4

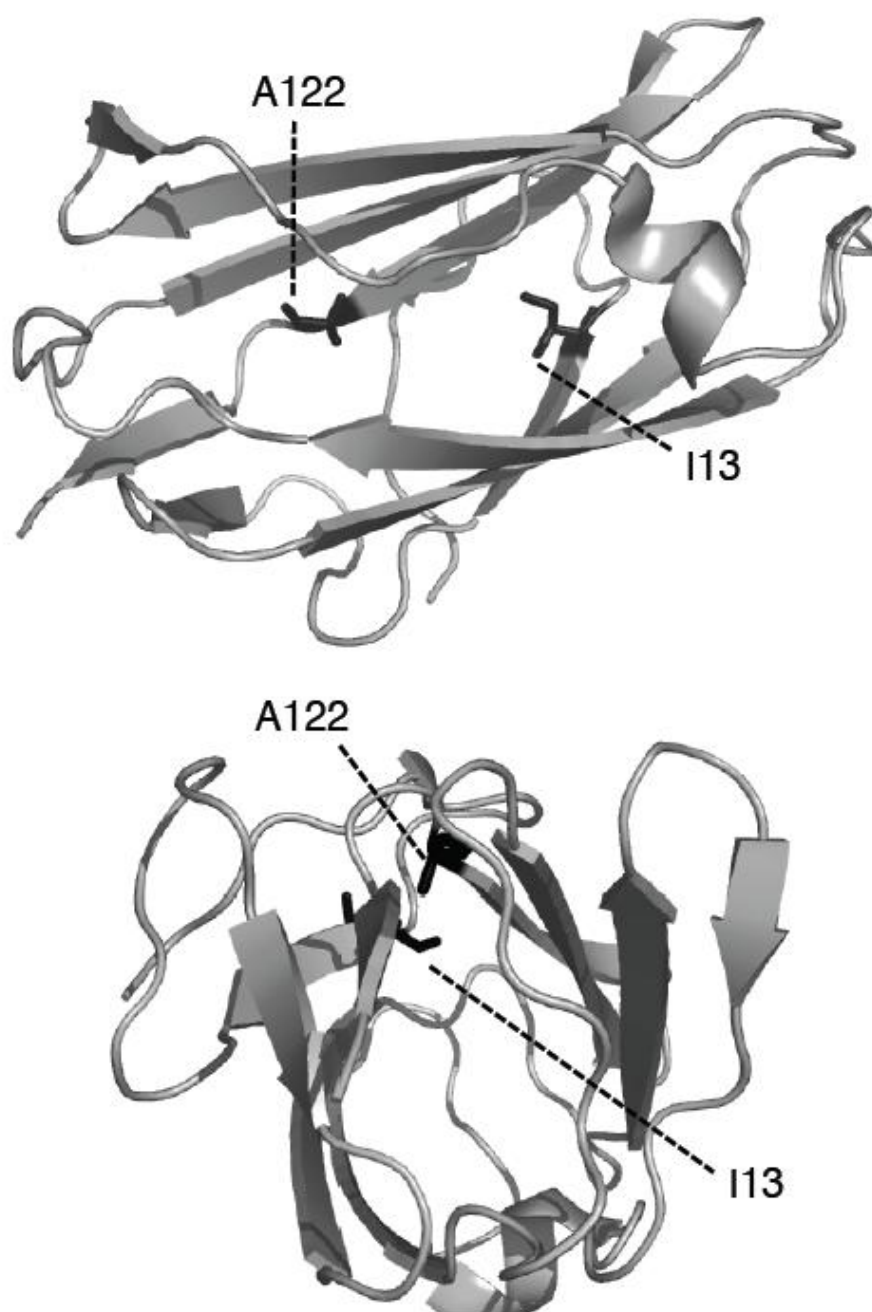


Fig.5

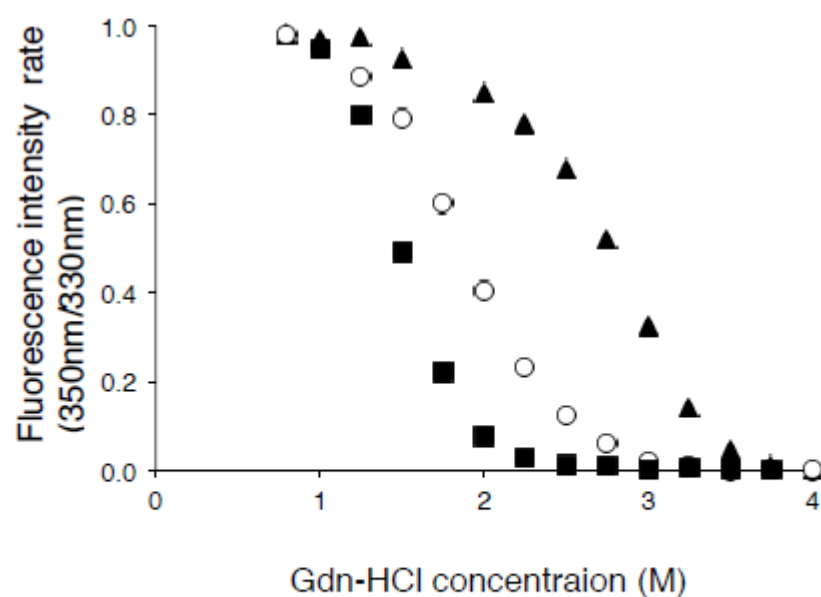


Fig.6

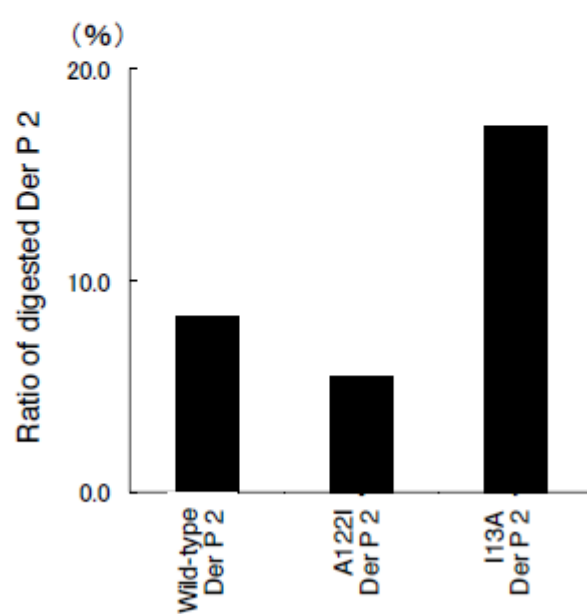
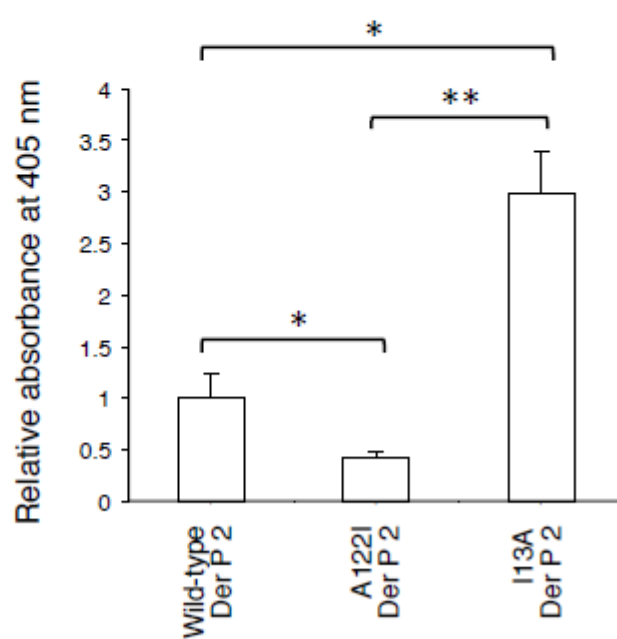


Fig.7



Highlights

- Conformational stability of Der p2 relates to its immunogenicity
- Stabilization of Der p2 depresses the IgE production in mice
- Conformational stability of a protein may be closely involved in preparing vaccines