

Characterization of Dog Allergens Can f 1 and Can f 2

2. A Comparison of Can f 1 with Can f 2 Regarding Their Biochemical and Immunological Properties

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Key Words

Can f 1 • Can f 2 • Cross-reaction • Dimer structure •
Disulfide bridge • Dog allergens • Non-covalent association

Abstract

Background: The major dog allergens, Can f 1 and Can f 2, are members of the lipocalin protein family. The characterization of both dog allergens is still not complete. Their deduced amino acid sequences indicate the presence of three cysteine residues, probably connected with a disulfide bridge. We compared the biochemical and immunological properties of Can f 1 with those of Can f 2 using gel filtration, electrophoresis, and immunological assays. **Methods:** The rCan f 1, rCan f 2 and dog salivary proteins containing natural Can f 1 and Can f 2 were analyzed by HPLC gel filtration. The recombinant Can f 1 (rCan f 1) and rCan f 2 were analyzed by native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with or without reduction. The binding ability of rabbit IgG purified by protein G affinity chromatography from the antiserum against rCan f 1 and rCan f 2 was examined after a reduction in the recombinant allergens. The immunological cross-reaction between rCan f 1 and rCan f 2 was examined by an enzyme-linked immunosorbent assay (ELISA) using the rabbit IgG against rCan f 1

and rCan f 2. The cross-reaction of human IgE in the serum of a patient with dog allergy between rCan f 1 and rCan f 2 was also analyzed by competitive ELISA. **Results:** The molecular weights of rCan f 1 and of rCan f 2 were 18 and 21 kDa, respectively, using SDS-PAGE under reducing conditions, but the natural Can f 1 and Can f 2 were separated by HPLC gel filtration into fractions containing proteins of 31 and 34 kDa, respectively. rCan f 1 and rCan f 2 migrated as multiple bands (30–100 kDa) in native PAGE in the presence or absence of a reductant. The molecular weights of natural Can f 1 and of Can f 2 were 20 and 23 kDa, respectively, in SDS-PAGE under reducing conditions. The ability of rabbit IgG to bind to rCan f 1 and rCan f 2 increased after the reduction of the recombinant allergens. The rabbit IgG against rCan f 1 bound to rCan f 2. Cross-reaction of human IgE was observed between rCan f 1 and rCan f 2. **Conclusions:** In the native and recombinant forms, Can f 1 and Can f 2 possessed a dimer structure under natural (non-reduced) condition. The dimers of Can f 1 and of Can f 2 were not built with a disulfide bridge but by non-covalent association. Cleavage of a disulfide bond of rCan f 1 and rCan f 2 increased the ability of binding of rabbit IgG to the allergens. The cross-reactivity of rabbit IgG and human IgE between rCan f 1 and rCan f 2 indicates that the same epitope(s) was present in Can f 1 and Can f 2.

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Introduction

Major dog allergens, Can f 1 and Can f 2, are present in dog hair/dander extracts [1–4]. Dog saliva is also a strong source of allergen [1]. Konieczny et al. [5] cloned the genes of Can f 1 and Can f 2 from the mRNA of dog salivary glands and prepared the recombinant Can f 1 (rCan f 1) and rCan f 2 derived from the recombinant proteins with histidine tag. Saarelainen et al. [6] studied rCan f 1 and rCan f 2 from the recombinant proteins produced by the yeast, *Pichia pastoris*. In the previous paper, we also cloned the Can f 1 and Can f 2 genes and prepared rCan f 1 and rCan f 2 derived from the recombinant proteins with the glutathione S-transferase tag [7]. On sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), the native Can f 1 and Can f 2 in dog saliva possessed molecular weights of 20 and 23 kDa, respectively, but the molecular weights of our rCan f 1 and rCan f 2 were 18 and 20 kDa, respectively.

Can f 1 and Can f 2 are members of the lipocalin protein family [5, 8]. Can f 1 consists of 148 amino acids and Can f 2 of 161 [5, 7]. Regarding homology, the amino acid sequence of Can f 1 showed a 24.5% identity to that of Can f 2 [7]. Three cysteine residues exist in both Can f 1 and Can f 2. The number of the cysteine residues, namely three, suggests the possibility of the presence of an intra- or intermolecular disulfide bridge. A disulfide bridge in the proteins of the lipocalin group, such as human tear lipocalins, affects their molecular structure, thus resulting in their appropriate functioning [9].

Can f 1 is more allergenic than Can f 2: 70% of the patients showed positive allergenic signals to Can f 1, but only 20% of the patients reacted to Can f 2 [1]. A recent research study in Finland showed that such an allergenic dominance of Can f 1 was not found [6]. In a previous study of patients with dog allergy, specific serum IgE against Can f 1 was more frequently detected compared to Can f 2 [7]. Using semiquantitative RT-PCR analysis, expression of mRNA of Can f 2 was increased compared to Can f 1 in the dog salivary gland and skin [7]. Can f 1 was detected in the saliva at 5.3 ng/ μ g protein and Can f 2 at 19.4 ng/ μ g protein. There is still no explanation as to why Can f 2 is less allergenic than Can f 1, even though the concentration of Can f 2 is higher in the saliva.

So far, no detailed comparison has been made between Can f 1 and Can f 2 regarding their biochemical and immunological properties. The distinct characteristics of Can f 1 and Can f 2 may thus provide an answer as to why Can f 1 has a greater allergic potential. We herein report

the findings of an analysis of the dog allergens in saliva, a native allergen source, using electrophoresis and a gel filtration technique. We also examined the binding ability of anti-Can f 1 or anti-Can f 2 IgG toward Can f 1 and Can f 2. In addition, the immunological cross-reaction of rabbit IgG and human IgE between Can f 1 and Can f 2 was analyzed.

Materials and Methods

rCan f 1, rCan f 2 and Their Antibodies

Both rCan f 1 and rCan f 2 were prepared as described previously [7]. The polyclonal antibodies against rCan f 1 and rCan f 2 were raised in rabbits using immunizing procedures reported previously [7]. The IgG fraction was purified from rabbit serum by protein G (Amersham Bioscience, Piscataway, N.J., USA) affinity chromatography. The protein concentrations of dog saliva, the recombinant allergens and antibodies were determined by Bradford's [10] method.

HPLC-Gel Filtration of the Dog Allergens

A TSK-G2000SW (Toso, Tokyo, Japan) column was equilibrated in 0.05 M phosphate buffer, pH 7.0, containing 0.3 M NaCl. An equal volume of 25% trichloroacetic acid was added to dog saliva collected from an outbred dog (beagle). The precipitated protein was dissolved in the above buffer. After filtration of the saliva protein solution by a 0.2- μ m filter, the solution (5 μ g protein in 10 μ l) was loaded onto the column at 1 ml/min; rCan f 1 and rCan f 2 were loaded in the same way. The eluted proteins were then monitored by absorbance at 220 nm. Lysozyme (14.3 kDa) and elastase (26 kDa) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Arabinanase TS (35.4 kDa) was prepared in our laboratory [11]. Statistical approximation was done with respect to the retention time and the molecular weight of these standard proteins by the Cricket Graph software program (Apple Macintosh). This software program gives us an approximation formula as a calculation curve to obtain the molecular weight of proteins. The R^2 value of the objective formula shows its credibility; a higher R^2 value indicates a higher credibility: a R^2 value of 0.999 is the maximum. Depending on the calculation curve, eluates of the dog salivary proteins were divided into three fractions: the S fraction covered molecular weights from 10 to 30 kDa, M from 30 to 50, and L from 50 to 70 kDa. Each designated fraction (2 ml) was concentrated 10 times by ultrafiltration with a membrane having a molecular cutoff weight of 10 kDa (Centricon-10[®], Millipore, Bedford, Mass., USA). Then 2 μ l of the concentrated fraction were dotted onto a nitrocellulose membrane (Nihon Millipore, Tokyo, Japan). The dot blot assay described below was performed using rabbit polyclonal IgG purified by protein-G affinity chromatography from antiserum against rCan f 1 and rCan f 2.

Electrophoresis of the Dog Allergens

The recombinant protein was diluted in 0.02 M Tris-HCl buffer, pH 6.8, containing various amounts of dithiothreitol and 20% glycerol in the presence or absence of SDS. The mixture was incubated at 37°C for 30 min in the absence of SDS and for

3 min in boiling water in the presence of SDS. The mixtures (1–2 µg of the recombinant allergens per lane) were loaded onto an acrylamide gel (15%) and then electrophoresed in the absence (native PAGE) or presence of SDS (SDS-PAGE) at 20 mA per slab gel. The procedures for SDS-PAGE were based on Laemmli's [12] method. Native PAGE was performed in the absence of SDS. The proteins in the gels were stained with brilliant blue R 250.

Dot Blot Assay

A dot blot was done using a Bio-Dot Apparatus (Bio-Rad Laboratories, Hercules, Calif., USA). The recombinant protein was diluted to 100 µg/ml in PBS containing 10 mM dithiothreitol. The reduction treatment was carried out for 1 h at room temperature, and the solution was then diluted to various protein concentrations in PBS. Two microliters of the diluted recombinant allergens were dotted onto a nitrocellulose membrane, and the membrane was dried at room temperature for 30 min and then blocked for 1 h in 10% skim milk (Becton, Dickinson, Sparks, Mo., USA). The membrane was incubated for 1 h at room temperature in 10 µg/ml of the IgG purified by protein-G affinity chromatography from rabbit anti-rCan f 1 or anti-rCan f 2 serum. After washing the membrane for 10 min in PBS-0.1% Tween 20, the membrane was incubated for 1 h at room temperature with peroxidase-labeled goat anti-rabbit IgG (3,000-fold dilution, Bio-Rad), washed, and the reaction products were visualized in a substrate solution (0.4 mg/ml diaminobenzidine and 0.006% hydrogen peroxide). After washing and drying the membrane, the colored spots were scanned and scored using NIH Image, public domain software, available for free over the Internet. This software program is able to count the black/white density from a photograph.

Enzyme-Linked Immunosorbent Assay for the Cross-Reaction of Rabbit IgG between rCan f 1 and rCan f 2

The recombinant allergen was diluted to 10 µg/ml in PBS. Each well of a 96-well microplate (Iwaki, Funahashi, Japan) was coated with 100 µl of the recombinant protein. The wells were washed with 0.05% Tween 20 in PBS. After blocking the unreacted sites of each well with 1% bovine serum albumin (Sigma), the affinity-purified rabbit IgG of anti-rCan f 1 or anti-rCan f 2 diluted serially was added to the wells. After 1 h of incubation at 37°C, the wells were washed as described previously. Peroxidase-labeled goat anti-rabbit IgG antibody (3,000-fold dilution, Bio-Rad) was added and incubated for 1 h at 37°C. After incubation and washing, a substrate solution (0.4 mg/ml *o*-phenylenediamine and 0.006% hydrogen peroxide) was added at 150 µl/well. The reaction was terminated after a 30-min incubation by the addition of 1 N H₂SO₄ at 50 µl/well. Absorbance of the reaction products was then monitored at 490 nm.

Cross-Reaction of Human IgE between rCan f 1 and rCan f 2 Analyzed by Competitive ELISA

The wells of the ELISA plate (Iwaki) were coated with 1 µg/ml of rCan f 1 or rCan f 2. After incubation, the wells were washed, and the unreacted sites were blocked by treatment with 1% skim milk. Independently, serum of a patient (H-1 in the previous paper [7], RAST score = 6) diluted tenfold was mixed with an equal volume of a competitor, serially diluted rCan f 1 or rCan f 2, respectively. The mixture was incubated for 1 h at 37°C and

overnight at room temperature, and added to the well of the ELISA plate after washing. After washing, goat anti-human IgE (ε) labeled with biotin (1 µg/ml; Kirkegaard & Perry Laboratories, Washington, D.C., USA) was added, and the plate was incubated. After washing, peroxidase-labeled streptavidin (1 µg/ml, Sigma-Aldrich) was added. The sequential procedures of ELISA were described above. Each determination was done in triplicate. The absorbance value of the diluted serum in the absence of a competitor was defined as 100% of the binding of human IgE.

Results

HPLC Gel Filtration of rCan f 1, rCan f 2, and Dog Saliva Proteins

rCan f 1 and rCan f 2 showed one single sharp peak on HPLC gel filtration (fig. 1). The retention time of rCan f 1 was 9.724 min and that of rCan f 2, 9.305 min. The dog salivary proteins showed many peaks under the same HPLC gel filtration conditions, but no peaks were observed at positions of 9.305 and 9.724 min.

The retention times of lysozyme (14.3 kDa), elastase (26 kDa), and arabinanase TS (35.4 kDa) were 11.972, 10.619, and 8.992 min, respectively. From these data, an approximation line (fig. 2) gave the following formula and R² value:

$$Y (\text{molecular weight}) = -7,073 \times X (\text{retention time}) + 99,317 \\ R^2 = 0.987$$

Because of the high R² value, the approximation line or the formula shows high credibility. The molecular weights of rCan f 1 and of rCan f 2 were then estimated to be 31 and 34 kDa, respectively.

An eluted solution of the dog saliva proteins was analyzed to see which fraction showed a positive signal against the rabbit IgG against rCan f 1 and rCan f 2. As shown in figure 3, only the M fraction was positive. This indicates that the natural Can f 1 and Can f 2 in dog saliva are proteins with a molecular weight of 30–50 kDa.

Electrophoretic Properties of rCan f 1 and rCan f 2

The purified rCan f 1 migrated as three bands (30, 60, and 70 kDa) in native PAGE in the absence of dithiothreitol. After treatment with dithiothreitol, rCan f 1 migrated as four bands, although the additional band (100 kDa) was faint (fig. 4). On the other hand, the reduced rCan f 1 showed a single band of 18 kDa on SDS-PAGE, but the non-reduced rCan f 1 migrated as two bands on the gel; one major band with a molecular weight

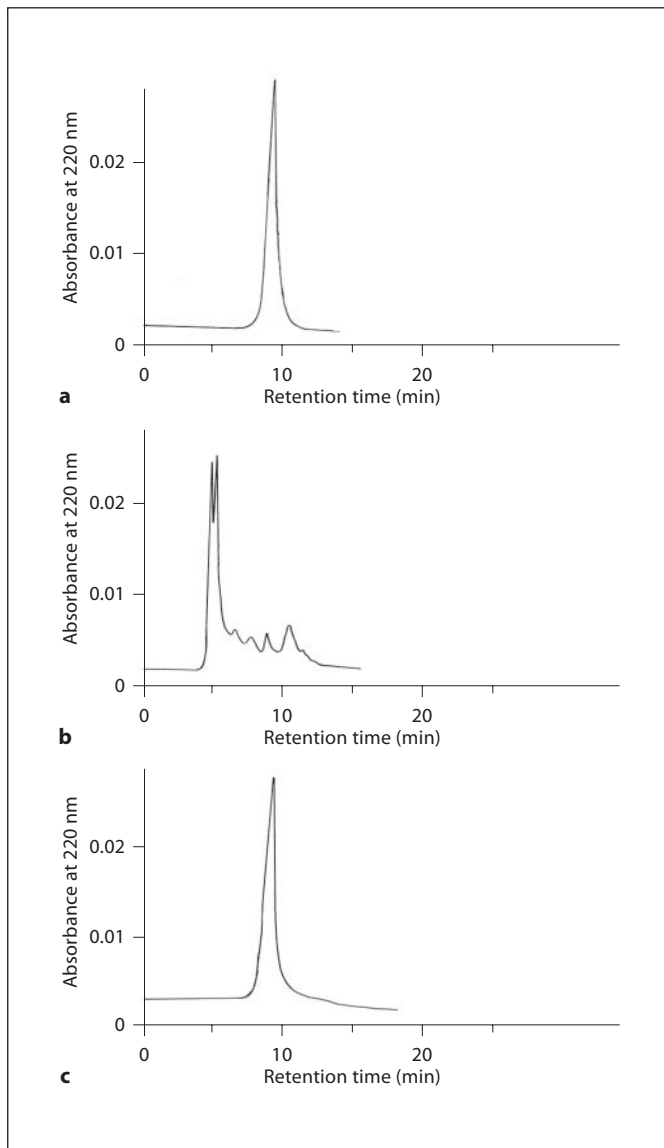


Fig. 1. HPCL gel filtration of the recombinant dog allergens, Can f 1 and Can f 2, and dog salivary proteins on a TSK-G2000SW column. The rCan f 1 (**a**), rCan f 2 (**c**) and dog salivary proteins (**b**) were loaded onto the column, and the eluted proteins were monitored by absorbance at 220 nm.

of 16 kDa, and the other with a molecular weight of 32 kDa.

The purified rCan f 2 with or without reduction treatment migrated on the gel as two bands in native PAGE: one major band with a molecular weight of 10 kDa and one minor band with a molecular weight of 17 kDa (fig. 5). On SDS-PAGE, the reduced rCan f 2 migrated as a single band with a molecular weight of 21 kDa, but the non-

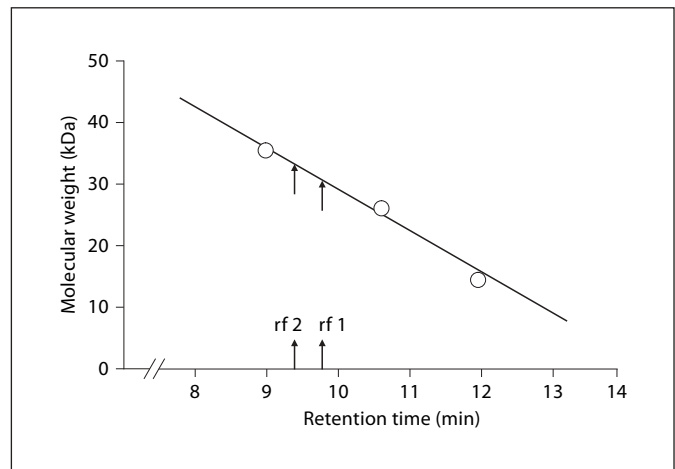


Fig. 2. The calculation curve to determine the molecular weights of the recombinant dog allergens by TSK-G2000SW column HPLC gel filtration. Standard proteins (open circles) are: arabinanase TS (35.4 kDa), elastase (26.0 kDa), and lysozyme (14.3 kDa). rf 1 = rCan f 1; rf 2 = rCan f 2. An approximation line was obtained by a computer software program (CriketGraph) based on the data of the standard proteins.

reduced rCan f 2 migrated as four bands (17.5, 18, 18.5, and 19 kDa).

Effects of a Reduction to rCan f 1 and rCan f 2 on Their SDS-PAGE Migrating Pattern

Purified rCan f 1 and rCan f 2 were treated with dithiothreitol at various concentrations (0.05–25 mM), and their SDS-PAGE patterns were then observed. Multiple bands of each non-reduced recombinant allergen were focused into a single band, thus corresponding to the concentration of dithiothreitol (fig. 6). This shift from the multiple bands to the single band of both recombinant allergens was attained at 6.2 mM of dithiothreitol (lane 3 in fig. 6), thus suggesting that the disulfide bridge(s) of both recombinant allergens possesses the same degree of sensitivity to dithiothreitol.

Effects of a Reduction of rCan f 1 and rCan f 2 on the Binding Ability of the Antibodies

After treating the recombinant allergens with 10 mM of dithiothreitol, a dot blot assay was performed to determine the ability of the antibody to bind to the recombinant allergens. As shown in figure 7, the reduced rCan f 1 bound to the antibody to a greater extent than the non-reduced one. An increase in the binding ability was similarly observed in rCan f 2 (after reduction).

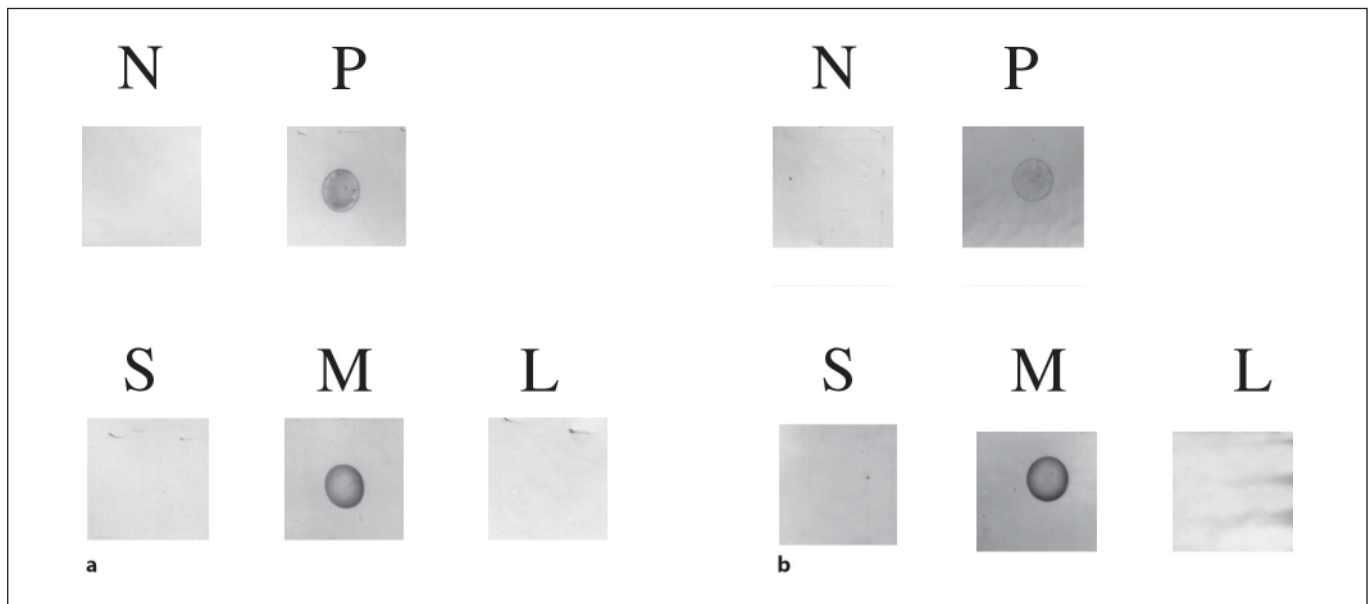


Fig. 3. Dot blot analysis of the fraction of dog saliva proteins separated from TSK-G2000SW gel filtration using rabbit IgG purified from the serum of anti-rCan f 1 (**a**) and anti-rCan f 2 (**b**) by protein-G affinity chromatography. N = PBS as negative control; P = rCan f 1 or Can f 2 as positive control (50 ng); S, M, and L = the pooled fractions that contain proteins with molecular weights of 10–30, 30–50, and 50–70 kDa, respectively.

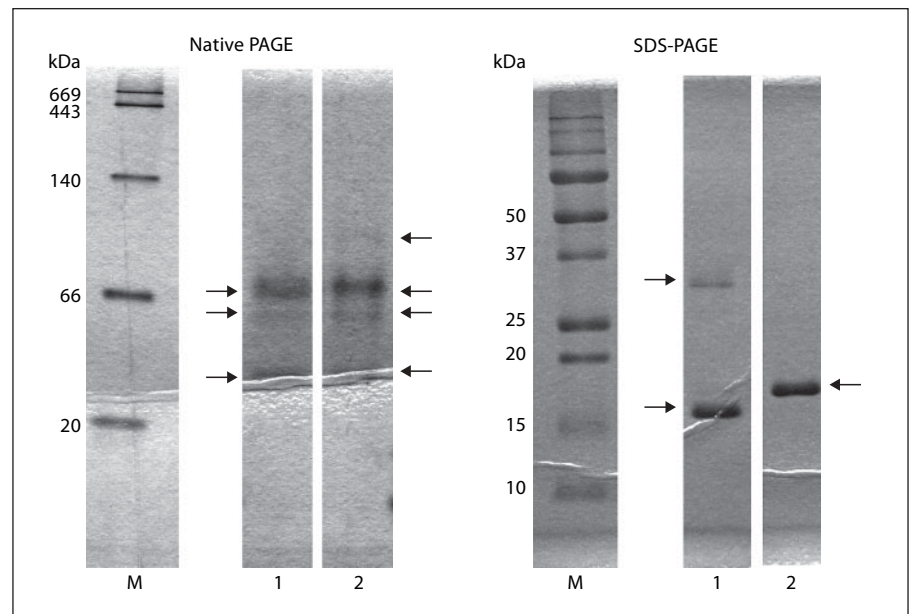


Fig. 4. Migrating pattern of rCan f 1 on native and SDS-PAGE in the presence or absence of a reducing reagent. Proteins were stained with brilliant blue R 250. Lane M = Marker proteins; lane 1 = in the absence of dithiothreitol; lane 2 = in the presence of 50 mM dithiothreitol.

Immunological Cross-Reaction of Rabbit IgG between rCan f 1 and rCan f 2

Rabbit IgG to rCan f 1 bound to rCan f 1 and rCan f 2, but anti-rCan f 2 did not bind to rCan f 1 but to rCan f 2

(fig. 8). The binding ability of anti-Can f 1 IgG to rCan f 2 was almost half of that of anti-Can f 1 IgG to rCan f 1 (fig. 8). However, the binding ability of anti-Can f 2 to rCan f 1 was almost zero.

Fig. 5. Migrating pattern of rCan f 2 on native and SDS-PAGE in the presence or absence of a reducing reagent. The proteins were stained with brilliant blue R 250. Lane M = Marker proteins; lane 1 = in the absence of dithiothreitol; lane 2 = in the presence of 50 mM dithiothreitol.

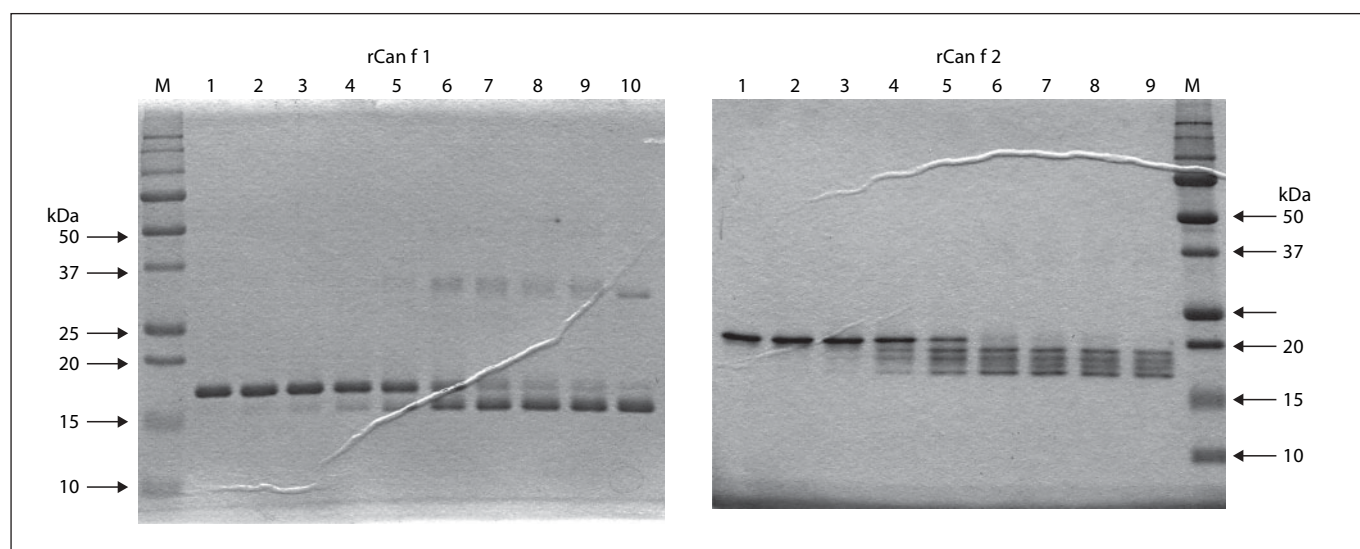
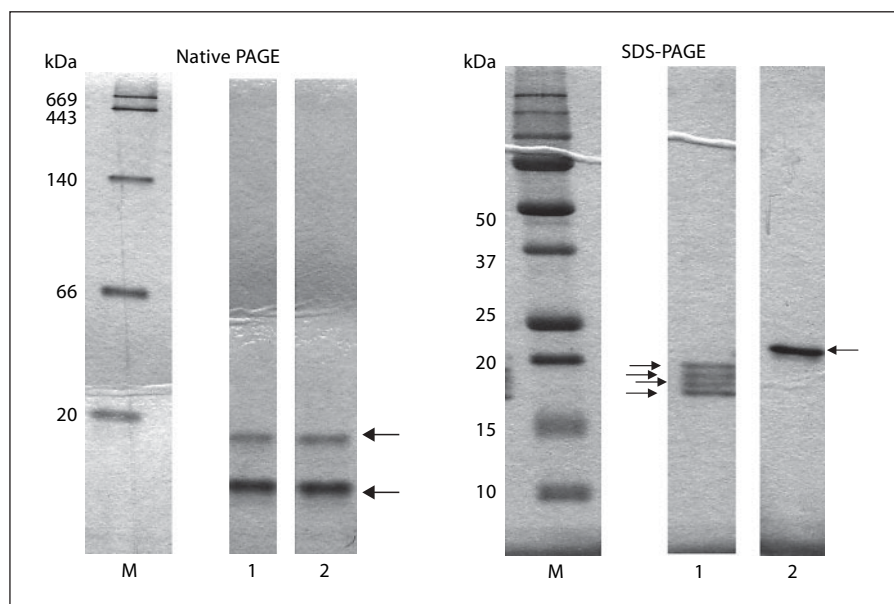


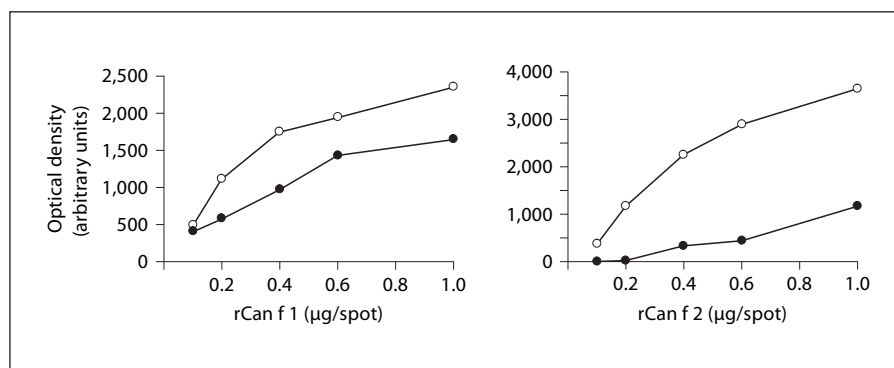
Fig. 6. Effects of reduction of rCan f 1 and rCan f 2 on the SDS-PAGE migrating pattern. The proteins were stained with brilliant blue R 250. Lane M = Marker proteins; lanes 1–10 = in the presence of 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.1, and 0.05 mM of dithiothreitol, respectively.

Immunological Cross-Reaction of Human IgE between rCan f 1 and rCan f 2

The human IgE in the serum of the patient with dog allergy bound to rCan f 1 and rCan f 2 immobilized on a well of an ELISA plate (fig. 9). Preincubation of the serum in the presence of rCan f 1 caused a decrease in the binding of IgE to the immobilized rCan f 1, preincubation of

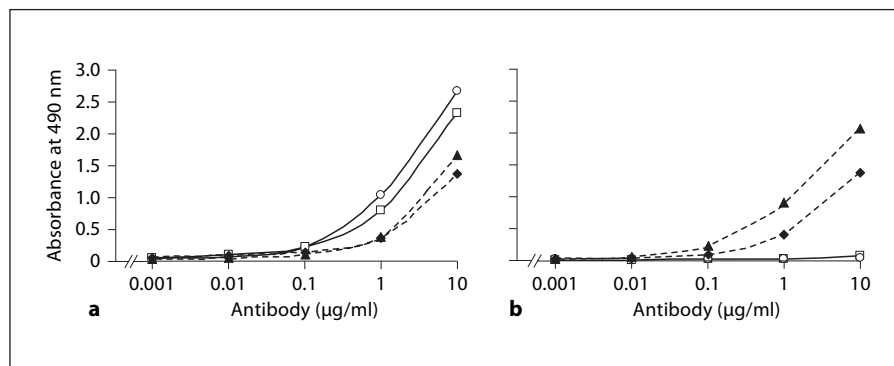
the serum with rCan f 2 resulted in a decrease in the binding of IgE to the immobilized rCan f 1. Similarly, the binding of IgE to the immobilized rCan f 2 was competed by the preincubation of the serum with rCan f 2 and rCan f 1 (fig. 9). These findings clearly indicate that cross-reaction of human IgE is present between rCan f 1 and rCan f 2.

Fig. 7. Effects of reduction of rCan f 1 and rCan f 2 on their ability for antibody binding determined by dot blot analysis. The polyclonal IgG was purified by protein-G affinity chromatography from rabbit anti-serum against rCan f 1 and rCan f 2. rCan f 1 and rCan f 2 were treated with (○) or without 50 mM dithiothreitol (●) before determination.



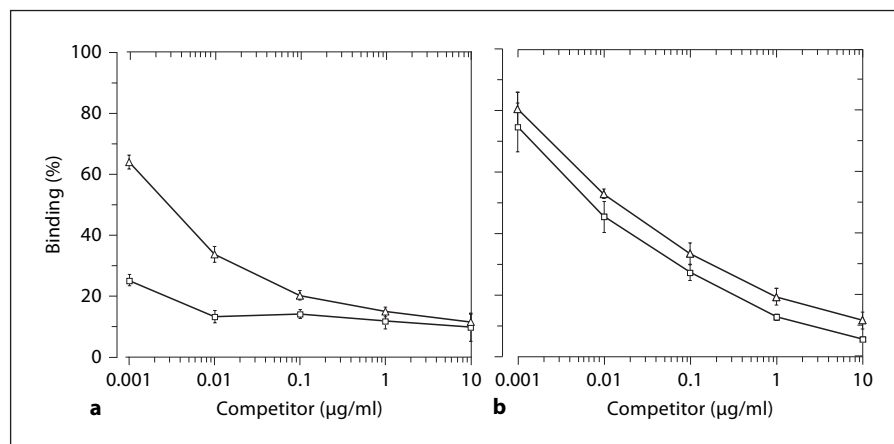
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Fig. 8. Cross-reaction of rabbit IgG between rCan f 1 and rCan f 2. The ELISA plate was coated with rCan f 1 (a) and rCan f 2 (b). After washing the wells, anti-Can f 1 antibody (IgG) purified from rabbits 1 and 2 (○, □) or anti-Can f 2 antibody purified from rabbits 3 and 4 (▲, ◆) was allowed to react at 1 µg/ml. The individual antibody (IgG) was purified by protein-G affinity chromatography from the antiserum of rabbits immunized with rCan f 1 or rCan f 2. The degree of binding of the antibodies to rCan f 1 and rCan f 2 was shown as a titer of absorbance at 490 nm.



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Fig. 9. Cross-reaction of human IgE between rCan f 1 and rCan f 2 in the serum of a patient with dog allergy detected by competitive ELISA. The IgE in the serum of a patient (RAST score = 6) bound to the immobilized rCan f 1 (a) and rCan f 2 (b). rCan f 1 (□) and rCan f 2 (△) were used as competitors. The diluted serum was mixed with the serially diluted rCan f 1 or rCan f 2, respectively. The mixture was added to the well of an ELISA plate coated with rCan f 1 or rCan f 2. The sequential procedures are described in the text. The absorbance value of the diluted serum in the absence of competitor was defined as 100%.



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Discussion

In our previous paper, we demonstrated that rCan f 1 and rCan f 2 were biologically and immunologically active [7]. This fact suggests that the recombinant proteins are good materials for analyzing the properties of dog allergen proteins.

rCan f 1 and rCan f 2 reduced with a higher concentration of dithiothreitol showed a single band with a molecular weight of 18–20 kDa on SDS-PAGE. These 'puri-

fied' and reduced recombinant allergens migrated as multiple bands on native PAGE. The non-reduced recombinant allergens migrated as multiple bands on SDS-PAGE. These findings suggest that the Can f 1 and Can f 2 molecules are connected in the native (no SDS) condition. Because a reduction did not alter the migrating pattern of both recombinant allergens on native PAGE, a disulfide bridge(s) must not be involved in such an association. The gel filtration experiments clearly showed that the molecular weights of both recombinant allergens

were about 30 and 34 kDa in the native (no SDS) condition, indicating that the recombinant allergens have a homo-dimer structure.

Regarding the native dog allergens in saliva, the signals corresponding to the anti-Can f 1 and anti-Can f 2 antibodies were found in the 30- to 50-kDa fraction, but not in the 10- to 30-, or the 50- to 70-kDa fractions in the gel filtration experiments. This finding showed that native Can f 1 and Can f 2 are homo-dimers. As described, rCan f 1 and rCan f 2 are connected, but not via a disulfide bridge(s). Therefore, non-covalent bindings turn each allergen protein into a homo-dimer structure. From the sequence of deduced amino acids, each allergen protein possesses three cysteine residues [5, 7]. A reduction of the allergen proteins altered their migration patterns on SDS-PAGE. This finding suggests that a disulfide bridge must thus be present, and such a disulfide bridge may be located intramolecularly.

The disulfide bridge(s) of Can f 1 and Can f 2 has a similar sensitivity to dithiothreitol, but their patterns on SDS-PAGE were clearly different. Reduction of rCan f 1 and rCan f 2 increased the binding ability of the antibodies to the allergen proteins. The cleavage of a disulfide bond would cause alterations in the molecular shape of

the allergens, thus resulting in increased binding ability of the antibodies to the allergens. Increasing the ability of binding of the antibody was first reported in this communication. The preparation of monoclonal antibodies against Can f 1 and Can f 2 [1] and the determination of the amounts of Can f 1 and Can f 2 have been reported previously [13–15], but no precise experiments have yet been done concerning the binding ability with or without reduction. The antibodies against Can f 1 and Can f 2 should therefore be reevaluated.

The cross-reaction of rabbit IgG and human IgE between rCan f 1 and rCan f 2 was observed. This finding suggests that a common IgG/IgE epitope(s) is present between Can f 1 and Can f 2. In the future, we plan to perform the competitive ELISA shown in this communication on many patients with dog allergy to confirm such cross-reaction of human IgE. Can f 2 is less allergenic than Can f 1 [1, 7], but hair/dander extracts as a source of dog allergen possess a higher concentration of Can f 2 than Can f 1 [unpubl. data]. Some questions still remain, but continuing efforts to analyze the character of these dog allergens should eventually allow us to treat and prevent dog allergy in the future.

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