

Identification of Cyclophilin as an IgE-Binding Protein from Carrots

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Key Words

Allergen · Carrot · IgE · Cyclophilin · Food allergy · Birch pollen

Abstract

Background: Plant food allergies have been associated with pollenosis, although most of the causative allergens are as yet undefined. It is important to elucidate the properties of plant food allergens in order to minimize a patient's risks in food selection. The purpose of the present study was to examine and characterize the IgE-binding proteins in carrots as possible allergens by using patients' sera. **Method:** IgE-binding proteins in carrot extract were screened by an immunoblot technique using sera of patients with atopic dermatitis (selected based upon a case history of food allergies). An allergenic protein was purified from carrot extract by chromatographic procedures. The N-terminal amino acid sequence of allergenic protein was determined and subjected to a computer homology search. Cross-reactivity between carrot and birch allergens was examined by immunoblotting. **Results and Conclusion:** A unique, approximately 20-kDa allergenic protein that reacted with about 14% of patients' sera was isolated and characterized. The N-terminal amino acid sequence of the purified protein was found to be homologous with those of plant cyclophilins. This allergen exhibited a peptidyl-prolyl *cis-*

trans isomerase activity, which was inhibited by the conjugation of cyclosporin A. These properties of the allergenic protein isolated from carrot identified it as a cyclophilin, a possible plant food allergen. No cross-reactivity between this 20-kDa carrot allergen and Bet v 7, a birch pollen cyclophilin, was observed.

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Introduction

Recently, IgE-mediated type 1 allergies caused by plant-derived allergens, such as foods, latex and pollens associated with oral allergy syndrome, have been reported [1]. While the series of immune responses underlying hypersensitivity reactions in allergic patients, from the initial sensitization with allergens to the release of chemical mediators, has been widely studied and elucidated [2, 3], the common structures and functions present in allergenic proteins have not been well characterized. Recently, proteins in certain classes have been shown to participate as allergens [4–6]. For instance, cross-reactivity between Bet v 1, a major allergen from birch pollen, and many fruits and vegetables has been demonstrated, suggesting that Bet v 1 and the proteins in these plants might share a homologous structure [4]. On the other hand, Fernandez-Rivas et al. [7] reported that some patients allergic to Rosaceae fruits (including apple, peach and pear) did not

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suffer from pollenosis. The latest reports have identified recently discovered allergens from peach [8] and apple [9] as members of the lipid transfer protein family, a group related to the defense proteins (or pathogenesis-related proteins) [10] which were recognized only by sera of patients allergic to these fruits in the absence of birch pollenosis.

Many efforts have been made to resolve the relationship between the structure and functions of proteins and their ability to induce IgE production. In order to identify common features of allergenic proteins, we have isolated and identified the allergenic proteins from various plant foodstuffs that are recognized by a patient's IgE. Although findings obtained by *in vitro* tests often conflict with the signs derived from *in vivo* tests (such as skin prick test) or with their relevance to the patient's manifestations, the more precise *in vitro* examinations, such as immunoblotting analyses of proteins binding patient's IgE antibodies, will still be important in characterizing unknown causative allergens in diverse plant foodstuffs. In this study, immunoblot screening of IgE-binding proteins in carrot was carried out. It had been reported that a 16-kDa protein, designated Dau c 1, has been identified as a major allergen from carrot and demonstrated to possess a structure homologous to Bet v 1 [11]. We found a unique IgE-binding protein with a molecular mass of about 20 kDa in carrot extract by immunoblotting with sera of patients that were suspected to suffer from food allergies and had been diagnosed with atopic dermatitis, with the incidence of reactivity observed to be about 14%. In this report, we describe the purification and identification of a new 20-kDa allergenic protein from carrot as a plant cyclophilin [12–14].

Materials and Methods

Materials

Fresh carrot (*Daucus carota*) used in this study was obtained from a local market. TSK gel Super Q-Toyopearl 650M, TSK gel Hi-trap SP Toyopearl and TSK gel G3000SW HPLC columns were purchased from TOSOH (Tokyo, Japan). Centricon 10 and Microcon-10 were procured from Amicon (Beverly, Mass., USA). Immobiline isoelectric focusing (IEF) gels (pH 3–10), PD-10 columns, rainbow markers, Coomassie brilliant blue R-350 and the ECL Western blotting kit were from Amersham-Pharmacia Biotech (Uppsala, Sweden). Protein assay kit and nitrocellulose membranes were from Bio-Rad (Richmond, Calif., USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE was acquired from KPL (Gaithersburg, Md., USA). Suc-Ala-Leu-Pro-Phe-pNA as a substrate of peptidyl-prolyl *cis-trans* isomerase (PPIase) assay came from the Peptide Institute (Osaka, Japan). Cyclosporin A (CsA) and GS Birch Mix (protein extract of birch pollen mixture) were purchased from Wake Pure

Chemicals (Osaka, Japan) and From Greer Laboratories (Lenoir, N.C., USA), respectively. All other chemicals were of reagent grade and obtained from commercial sources.

Patients' Sera

Serum samples were prepared from 361 patients with atopic dermatitis (mean age 6.1 years, ranging from 3 months to 23 years) who were asked to donate blood samples for testing at the National Kagawa Children's Hospital and Takamatsu Heiwa Hospital [15]. According to case histories, the food components were suspected allergens in all subjects, but no strict *in vivo* diagnostic tests, such as double-blind placebo-controlled food challenges, were performed because the subjects received treatment as outpatients only. When a randomly selected subgroup of 86 patients (about 25% of patients who donated blood samples) was examined simultaneously by RAST using disks of five major allergenic food items in Japan, the frequency of positive responses to individual disks was estimated as follows: egg white (26.7%), soybean (14.0%), wheat (12.8%), milk (11.6%) and rice (8.1%). Moreover, about 19% of the patients tested showed a positive response to several of the above-mentioned food disks. 272 randomly selected patients' sera were subjected to the screening by the immunoblotting techniques in the present study. An additional pooled serum sample obtained from 5 nonallergic healthy individuals served as a negative control.

Purification of a Carrot Allergen

Fresh carrot roots were homogenized in 30 mM Tris-HCl (pH 8) using a Polytron homogenizer. The homogenate was filtered through three layers of cheesecloth, and then the filtrate was centrifuged at 6,500 *g* for 40 min at 4°C to remove fibers and cell debris. The proteins in the supernatant were recovered by precipitation with 70% saturated ammonium sulfate. The precipitate was collected and dissolved in 30 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.0, buffer A), followed by dialysis against the same buffer for 20 h at 4°C. The dialysate was applied on a Super Q 650 column previously equilibrated with buffer A. The allergen appeared in the flowthrough fraction. The protein was recovered by precipitation with 70% saturation of ammonium sulfate. This precipitate was dissolved in 20 mM 2-(*N*-morpholino) ethanesulfonic acid buffer containing 1 mM EDTA (pH 6.0) (buffer B), desalted using a PD-10 column, and then applied on a Hi-trap SP column (5 ml) which was previously equilibrated with buffer B. Elution was performed with a linear gradient of NaCl (0–1 *M*) in buffer B at a flow rate of 0.5 ml/min. The fractions containing the IgE-binding protein were pooled and concentrated using Centricon-10. The concentrated material was chromatographed using HPLC with a G3000 SW column (7.5 mm × 60 cm) previously equilibrated with 20 mM Tris-HCl buffer containing 0.15 *M* NaCl and 1 mM EDTA (pH 7.5) at a flow rate of 0.4 ml/min. Fractions containing the protein recognized by patients' sera were pooled and concentrated by Microcon-10. Protein concentration was determined by the method of Bradford [16] with the protein assay kit using bovine γ -globulin as a standard.

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.5% polyacrylamide gels according to the method of Laemmli [17]. For immunoblotting analyses, the proteins separated on an SDS-PAGE were electrotransferred onto a nitrocellulose membrane under semidry conditions [18]. The transferred membrane was incubated in 10 mM sodium phosphate-buff-

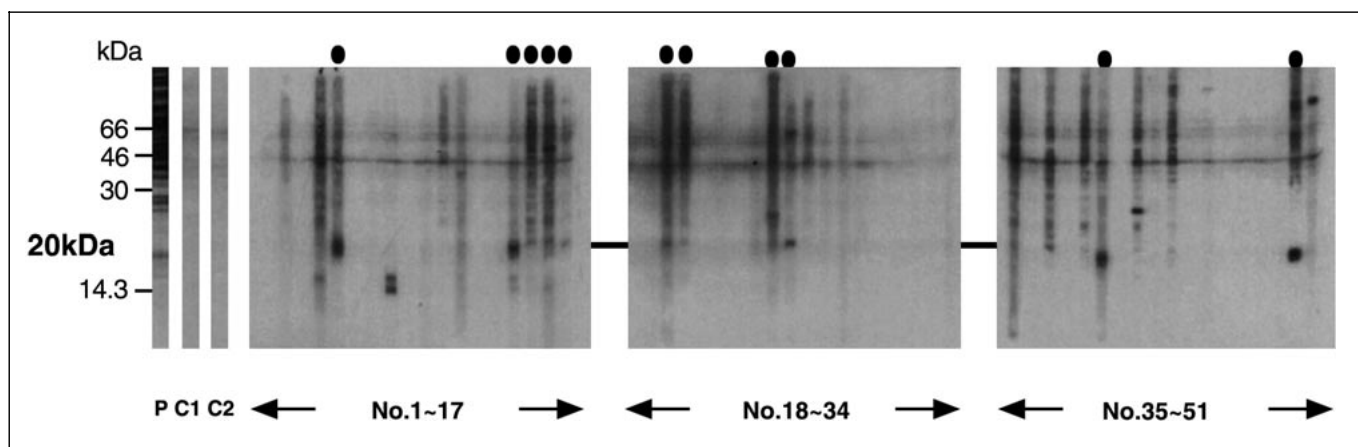


Fig. 1. IgE-binding proteins in carrot extract. The ECL fluorograms of the immunoblots obtained from 51 randomly selected patients' sera (see Materials and Methods). The lanes indicated by closed circles represent IgE antibodies from the patients' sera recognizing the carrot protein with a molecular mass of about 20 kDa. Molecular masses of the standard proteins are indicated on the left. Lane P

represents carrot proteins stained with Coomassie brilliant blue, R-350. Lanes C1 and C2 show immunoblots with pooled serum from nonallergic healthy volunteers and buffer instead of serum as control, respectively. Lanes 1–51 represent immunoblots with sera of the allergic patients with atopic dermatitis.

ered saline (PBS, pH 7.5) containing 0.1% Tween 20 and 2% skim milk for blocking. The membrane was incubated overnight with patients' sera diluted 1:6 in PBS. Bound IgE antibodies were detected using HRP-conjugated goat anti-human IgE and an ECL-Western blotting kit. Proteins on a gel were stained by Coomassie brilliant blue R-350.

Two-Dimensional PAGE

IEF was performed using Immobiline precast gel strips, according to the manufacturer's instructions. For the second dimension, SDS-PAGE was carried out under reducing conditions.

Analysis of N-Terminal Amino Acid Sequence

The partially purified allergen was subjected to two-dimensional PAGE as described above and separated proteins were electrotransferred onto a polyvinylidene difluoride membrane and detected by staining with 0.2% Ponceau S. The band corresponding to the allergen was cut off and subjected to N-terminal amino acid sequencing using an Applied Biosystems 492 protein sequencer (Perkin Elmer, Foster City, Calif., USA).

PPIase Assay

The PPIase activity of the isolated allergen was measured according to the method of Fischer et al. [19]. Briefly, the allergen and the substrate, Suc-Ala-Leu-Pro-Phe-*p*NA, at 100 μ M final concentration were mixed into the assay buffer, 40 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes), containing 80 mM NaCl (pH 7.8), and the reaction was initiated by the addition of chymotrypsin (4 μ M final concentration). The reactions were carried out on ice. The PPIase activity was determined by measuring the catalysis of the *cis* to *trans* interconversion of the substrate. Chymotrypsin is able to cleave the C-terminal amide bond of the substrate with *trans* X-Pro conformer. The hydrolytic product, *p*-nitroaniline, was monitored by the absorbance at 390 nm using a spectrophotometer. An

inhibition assay was performed in the same manner as described above except the reaction mixture was preincubated for 10 min with 10 μ M CsA before adding chymotrypsin.

Dot Blotting Analysis

The allergenic protein (0.3 μ g/ μ l) was incubated in the presence or absence of 10 μ M CsA for 10 min on ice. After incubation, 1 μ l of the protein sample was dot-blotted onto a nitrocellulose membrane. Immunoblotting of the dotted proteins with patient's serum was carried out as described above.

Cross-Reactivity

The carrot 20-kDa allergen and birch pollen protein extract (including Bet v 7, a birch cyclophilin) were subjected to SDS-PAGE and the fractionated proteins were transferred onto a nitrocellulose membrane. The binding capability of specific IgEs from patient's serum was determined by immunoblotting. The sera used in this experiment were selected from the patients' sera positive for carrot 20-kDa cyclophilin (fig. 1).

Results

Screening of Carrot Allergen Using Patients' Sera

As a first step, the proteins recognized by sera of the allergic patients were examined in carrot extract. Individual sera from 272 randomly selected patients with atopic dermatitis were screened. A protein with a molecular mass of around 20 kDa was strongly recognized by patients' sera with an incidence of 14% (38 serum samples from 272 patients). The immunoblotting profiles of pa-

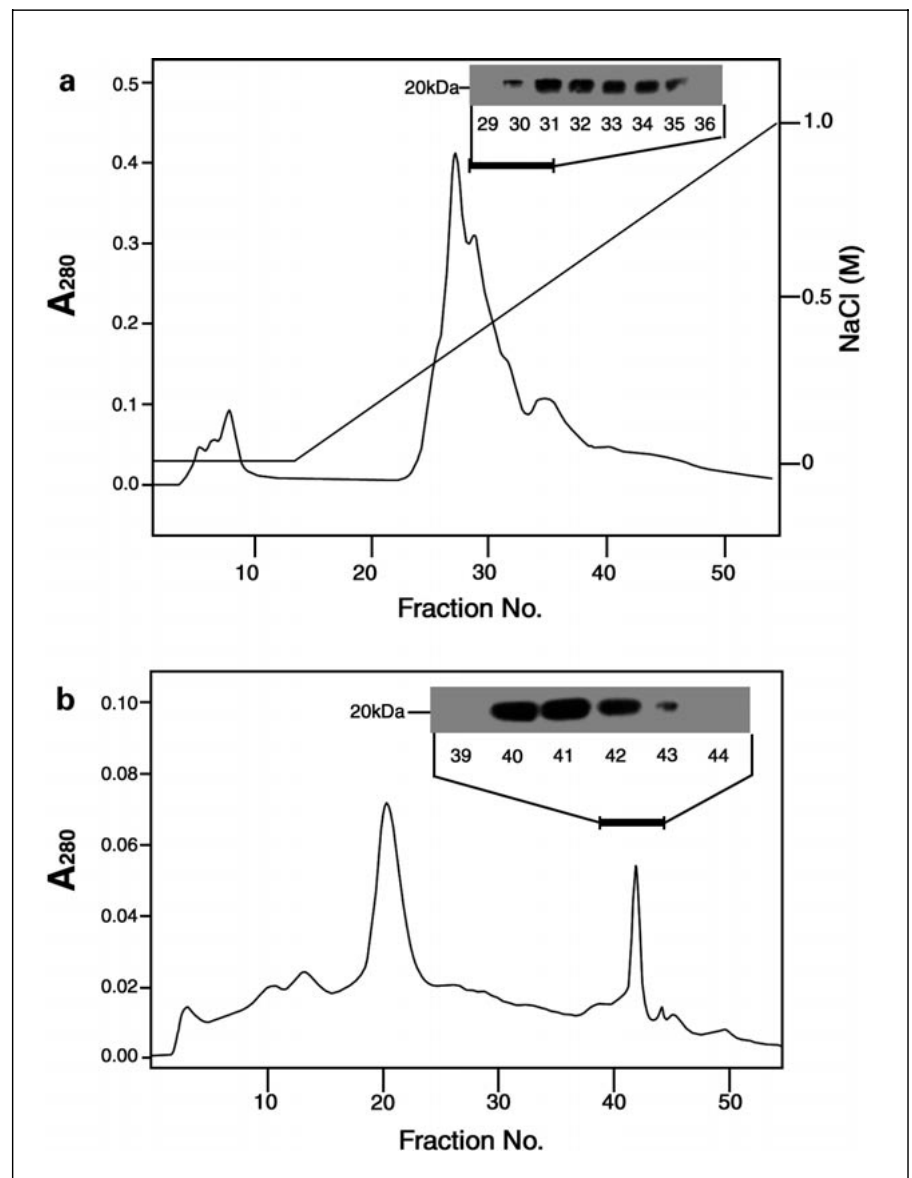


Fig. 2. HPLC chromatograms of the carrot 20-kDa allergen. **a** Chromatogram on Hi-trap SP column. **b** Chromatogram on G3000SW column. Inset figure represents immunoblot profiles obtained from patient serum. Experimental details are described in Materials and Methods.

tients 1–51 are presented in figure 1. Furthermore, proteins with masses of approximately 27 and 12–14 kDa were also shown to be recognized by several sera (fig. 1).

Purification of 20-kDa IgE-Binding Protein

The 20-kDa IgE-binding protein was fractionated by chromatography on a Super Q anion exchange column. The allergen was found in the flow-through fraction. It was absorbed on a Hi-trap SP cation exchange column and eluted by buffer B with a linear gradient of 0.3–0.5 M NaCl (fig. 2a). Two major peaks appeared on HPLC with G3000SW column and the 20-kDa allergenic protein was

found in the later peak (fig. 2b). On the two-dimensional PAGE of this fraction, it was confirmed that the major protein spot (indicated by an arrow) on the membrane corresponded to the 20-kDa allergen recognized by patients' sera with a broad pI value of 8.3–9.1 (fig. 3a, b). This preparation of the 20-kDa allergen was subjected to further experiments.

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the 20-kDa allergen was TNPKVFFDMTVGGKP-, which matched well the N-termini of plant cyclophilins by computer

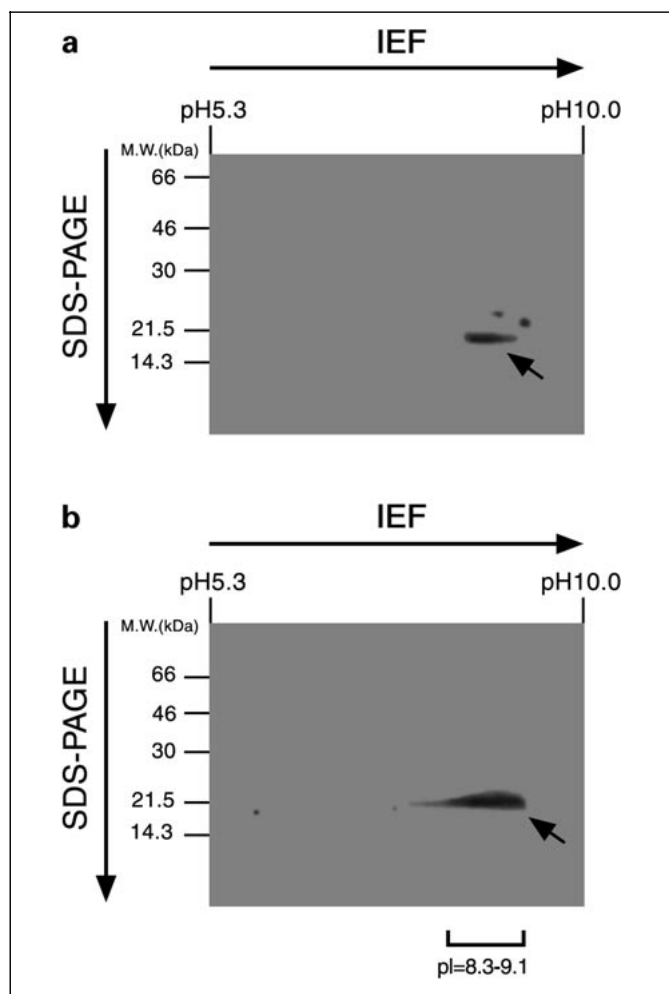


Fig. 3. Two-dimensional PAGE of carrot allergen. The pooled fractions of figure 2b (fraction No. 40–43) were concentrated and subjected to two-dimensional PAGE using Immobiline IEF gels as described in Materials and Methods. **a** Staining with Coomassie brilliant blue R-350. **b** Immunoblot using patients' sera. Arrows indicate 20-kDa allergen.

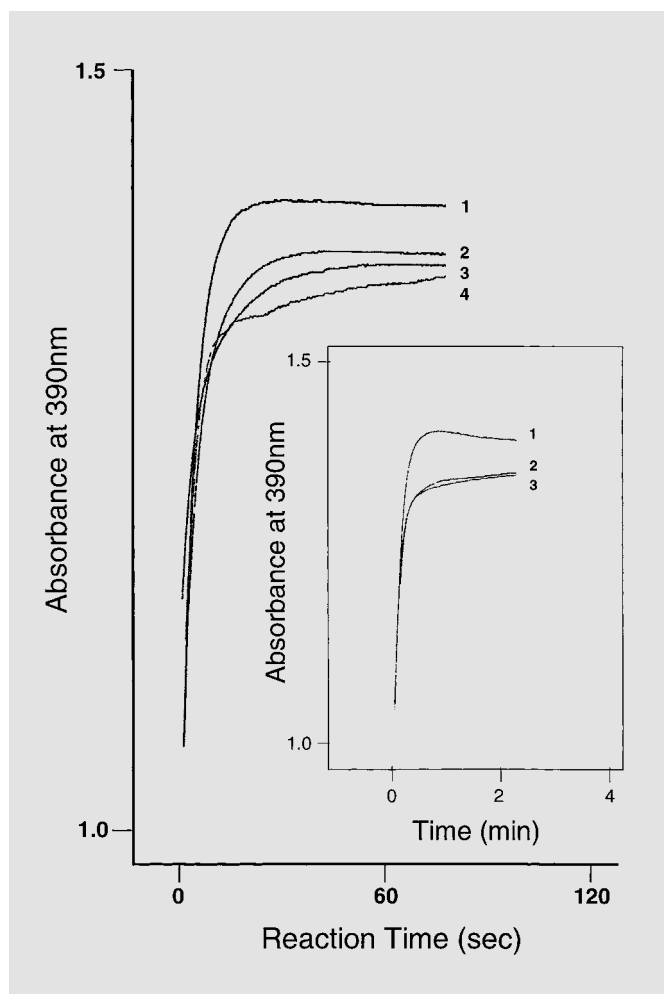


Fig. 4. Determination of PPIase activity. Assay procedure of the PPIase activity is described in Materials and Methods. Dose-dependent PPIase activity of 20-kDa allergen: 1 = 4- μ g protein; 2 = 2- μ g protein; 3 = 1- μ g protein, and 4 = buffer only. Inset represents inhibition of PPIase activity of 20-kDa allergen by CsA: 1 = 11.4- μ g protein in the absence of CsA; 2 = 11.4- μ g protein in the presence of 10 μ M CsA, and 3 = buffer containing 10 μ M CsA. The production of *p*-nitroaniline was measured by monitoring absorbance at 390 nm.

homology search using BLASTP. Alignment of N-terminal amino acid sequence of carrot 20-kDa allergen and related plant cyclophilins is shown below (bold print represents homologous residues).

Carrot 20-kDa allergen	TNPKVFFDMTVGGK P
Cyclophilin (<i>Arabidopsis thaliana</i>)	TNPKVYFDMTVGGK
Cyclophilin (<i>Digitalis lanata</i>)	SNPKVFFDMTIGGQ P
Cyclophilin (<i>Phaseolus vulgaris</i>)	NPKVFFDMTIGGQ P
Cyclophilin (<i>Zea mays</i> ; maize)	NPRVFFDMTVGGAP

PPIase Activity of Carrot 20-kDa Allergen

Cyclophilins are generally known to have PPIase activity, which is inhibited by CsA [20, 21]. We examined whether the purified 20-kDa allergen has a PPIase activity. Under the conditions employed in this experiment, it was assumed that almost all substrates were in the *trans* conformation in equilibrium. Therefore, the initial burst of absorbance at 390 nm arose due to the hydrolytic product, *p*-nitroaniline, from the *trans* conformers (fig. 4). The slow increment of absorbance followed by the initial burst was attributed to the PPIase activity of the 20-kDa aller-

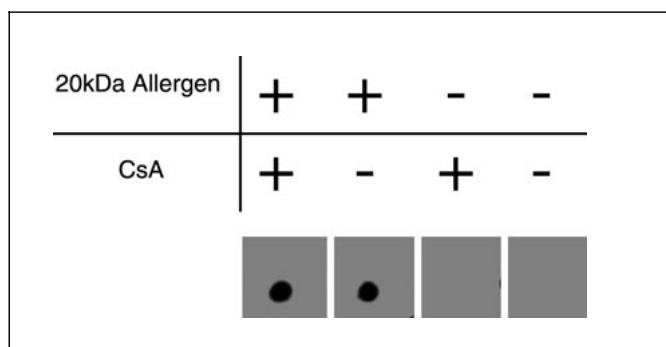


Fig. 5. Effect of CsA binding on immunoreactivity of carrot 20-kDa allergen. The reaction mixture with or without allergen (0.3 μ g) was preincubated in the presence or absence of 10 μ M CsA, respectively. An aliquot (1 μ l) of the reaction mixture was dot-blotted onto a nitrocellulose membrane. Results of four cases of the reactions are shown. The symbols + or - refer to presence or absence of CsA, respectively.

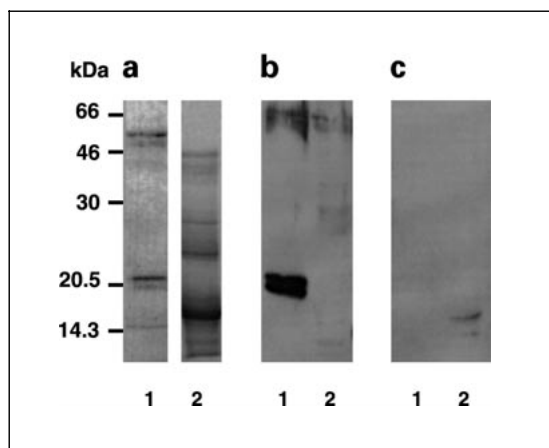


Fig. 6. Cross-reactivity assay against carrot cyclophilin and birch pollen proteins using the patients' sera positive for carrot cyclophilin. Molecular masses of marker proteins are indicated on the left. **a** Proteins stained with Coomassie brilliant blue R-350. **b** Immunoblots with patients serum. **c** Immunoblots with buffer instead of serum (control). Lane 1 represents purified carrot cyclophilin, lane 2 represents birch pollen mixture (protein extracts).

gen. As shown in figure 4, the allergen exhibited PPIase activity in a dose-dependent manner, and the activity was almost completely inhibited by the addition of 10 μ M CsA.

Influence of CsA on Immunoreactivity of 20-kDa Allergen

The effect on the immunoreactivity with patients' IgE of CsA binding the 20-kDa allergen was examined by dot

blotting. No difference in the recognition of the 20-kDa allergen by patients' IgE was shown in the presence or absence of CsA (fig. 5).

Cross-Reactivity

Bet v 7, a minor 20-kDa allergenic protein found in birch pollen, was identified as a cyclophilin by Cadot et al. [22]. We examined whether Bet v 7 could be recognized by the patients' sera binding this 20-kDa carrot allergen (cyclophilin). The representative immunoblot is shown in figure 6. None of the sera examined was demonstrated to recognize the birch pollen cyclophilin.

Discussion

In this study, an allergenic protein from carrot with a molecular mass of about 20 kDa was detected by IgE binding from carrot and purified for characterization. The N-terminal amino acid sequence was homologous with the group of cyclophilins. Cyclophilin was originally isolated by Handschumacher et al. [23] as a protein from bovine thymocytes that binds to the immunosuppressive drug, CsA. It was demonstrated that cyclophilin was PPIase [20, 21], an enzyme catalyzing the *cis-trans* isomerization of X-Pro peptide bonds [18]. The PPIase activity is inhibited by binding of CsA to cyclophilin. The 20-kDa carrot allergen likewise showed a PPIase activity and it was inhibited by a binding of CsA. Moreover, the high pI value of the isolated carrot protein is similar to those reported from other plants [24]. Based on the above observations, it was confirmed that the carrot 20-kDa allergenic protein is a member of plant cyclophilins [12–14]. In addition, it was also shown that the immunoreactivity of the 20-kDa allergen against a patient's IgE antibodies was not influenced by a binding of CsA to the allergen, indicating that the IgE epitope regions of this protein are distinct from the CsA-binding site. Several cyclophilins have also been reported as allergens, including Pci c 2 [25], Asp f 11 [26] and Mal f 6 [27], from molds. Recently, Cadot et al. [22] described that a minor allergen from birch pollen, Bet v 7 could be classified as a plant cyclophilin. The present report is the first evidence that a cyclophilin is a suspected allergen from a plant food item. Cyclophilins are proteins widely conserved from prokaryotes to mammals [28] and several investigators have described that the production of cyclophilins in maize and beans was enhanced by stress factors such as heat, chemical agents and infection by pathogens [29, 30]. It is assumed that their primary structures are highly homologous to one another, so that it is of interest

whether cross-reactivity occurs among these allergens and plant cyclophilins. Cross-reactivity between the purified 20-kDa carrot allergen and birch pollen mixture extract including Bet v 7 was examined by the immunoblotting assay using representative patients' sera. No IgE cross-reactivity was observed for carrot cyclophilin and birch pollen protein with the use of sera of the patients positive for carrot cyclophilin, suggesting that the patients might be sensitized at least by carrot proteins as food items, but not birch pollen.

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