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Antigenic Analyses of Sugi Basic Protein by Monoclonal Antibodies: II. Detection of Immunoreactive Fragments in Enzyme-cleaved *Cry j I*

Key Words

Cry j I
Epitope
Amino acid sequence
Enzymatic cleavage
Synthetic peptide
Monoclonal antibodies

Abstract

The 4 anti-*Cry j I* mAbs showing an epitope specificity different from each other, 046, 029, 026 and 027, were selected to analyze the structure of the antigenic determinant for each mAb on a *Cry j I* molecule. Immunoreactive fragments in enzyme-cleaved *Cry j I* were detected by means of the adsorption on the mAb column and of the binding to the mAbs on Elisa. The mAb 026 was found to be reactive to the fragments containing a *Cry j I* N-terminal region obtained by V8 protease or pepsin digestion, but not to those by lysylendopeptidase digestion. The mAb 027 was found to be capable of binding to the fragments containing a linear structure of Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys, which were generated by V8 protease, lysylendopeptidase or pepsin digestion. Furthermore, the synthetic peptide Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-Arg could bind to 027, but not to 026, and could inhibit the binding of 027 to *Cry j I* or to its immunoreactive fragments. No fragments capable of reacting to the mAbs 046 and 029 could be found in this study, suggesting that 046 and 029 recognize a conformationally constituted epitope of *Cry j I* molecule which is destroyed by enzymatic cleavage. The epitope recognized by the mAbs 027 or 026 was found to be located in conformationally hidden parts of the molecule which was exposed to react to the mAbs only after the physicochemical or enzymatic treatment.

Introduction

In the preceding paper [1], we stated that the anti-*Cry j I* mAbs obtained in our laboratory could be classified into four groups by the epitope specificity and that some of these mAbs recognize the epitopes identical with or similar to those for the human IgE antibodies from Japanese cedar pollen allergic individuals. Therefore, the structural

analysis of these epitopes would be very important for understanding the mechanisms of the allergic disease and IgE antibody responses. Mapping of allergenic epitopes on some allergens by using mAbs has been well done as reported on grass pollen allergens [2-7], house dust mite allergens [8-14], cod fish allergen M [15] and some insect allergens [16, 17]. Recently, the complete primary structures of a white-face hornet venom allergen [17], a house dust

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mite allergen *Der p I* [18, 19] and of the IgE-binding protein from Kentucky bluegrass pollen [20] were determined. However, in a few cases, the information on the structures of the epitopes on these allergens is available [5, 15, 16, 20], but not in Japanese cedar pollen allergens.

In this study, we found that one of the epitopes on *Cry j I* is contained in the peptide Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys, and another one is located in the enzyme-cleaved fragments containing a *Cry j I* N-terminal region. Details are presented and discussed.

Materials and Methods

Antigens and Antibodies

The same *Cry j I* and anti-*Cry j I* monoclonal antibodies were used as those in the preceding study [1].

Treatment of *Cry j I* with Guanidine

A constant dose of *Cry j I* was mixed with various concentration of HCl guanidine (adjusted pH at 7.5), so that the final concentration of *Cry j I* became 2 µg/ml and these concentrations of guanidine were 4 and 1 M, and 250, 60 and 15 mM, respectively. The mixtures were incubated at room temperature for 1 h, then 50 µl of each mixture were coated on the wells of microplates.

Enzymatic Cleavage of *Cry j I*

Pepsin Digestion. One milliliter of 1.67 mg/ml *Cry j I* in H₂O, 185 µl of 0.1 N HCl, 480 µl of H₂O and 3.2 µl of 1 mg/ml pepsin (from porcine stomach mucosa, Sigma, USA) were mixed together. The mixture was incubated at room temperature for 20 h. To stop the enzymatic reaction, 1 drop of pyridine was added to the mixture.

V8 Protease Digestion. One milliliter of 1.5 mg/ml *Cry j I* in H₂O was incubated in boiling water for 5 min, after cooling, followed by addition of 1 ml of 0.2 M ammonium acetate in 10 mM EDTA, pH 5.0, and 50 µl of 1 mg/ml V8 protease (from *Staphylococcus*, Sigma). The mixture was incubated at 37°C for 48 h. During the incubation, the same amount of the enzyme was added twice to the mixture at intervals of 8–18 h. To stop the reaction of the enzyme, the mixture was incubated in boiling water for 10 min.

Lysylendopeptidase Digestion. 1.5 mg of *Cry j I* in 1 ml of 0.01 M phosphate buffer in 0.3 M NaCl was mixed with 1 ml of 0.2 M Tris-HCl buffer, pH 8.5, then the mixture was incubated in boiling water for 5 min. After cooling, 15 µl of 1 mg/ml lysylendopeptidase (from *Achromobacter*, Seikagaku Kogyo, Japan) were added to the mixture. The reaction was carried out for 15 h at 37°C, then stopped by incubation in boiling water for 10 min.

Reversed-Phase HPLC Analysis of Enzyme-Cleaved *Cry j I*

80–800 µg of enzyme-cleaved *Cry j I* were loaded to an RP318 column (Bio-Rad, USA) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid. Peptide maps were obtained by monitoring the absorbance of the eluate at 214 nm, and the eluted peptide fractions were manually collected for amino acid sequencing or Elisa. For Elisa, the solvent of each fraction was evaporated by centrifugation at 50°C, then the dried fragment was dissolved with H₂O.

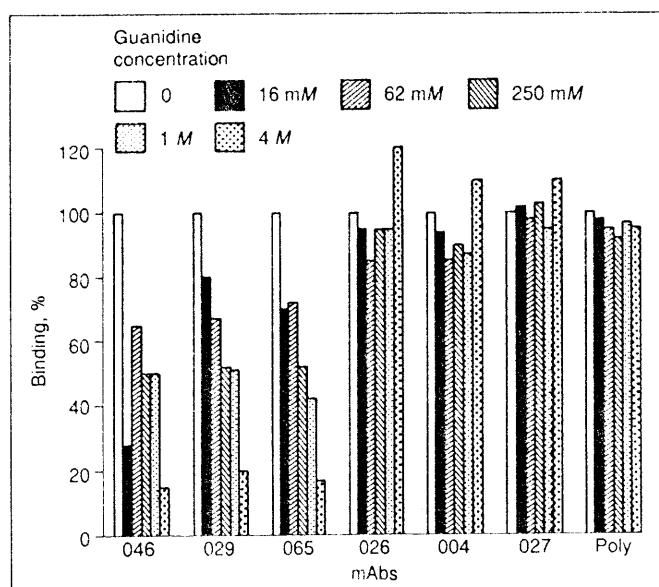


Fig. 1. Effect of guanidine-treatment of *Cry j I* on the binding of the anti-*Cry j I* mAbs. *Cry j I* treated with various concentrations of guanidine was coated on microplates. A constant dose of mAbs (5 µg/ml) was added to the wells. Reactions were detected with HRPO-labeled anti-mouse immunoglobulin and substrate.

Elisa for the Detection of mAb Binding to Peptides

96 wells of microplates (CovaLink, Nunc) were treated with 100 µl of 0.1 mg/ml Bis(sulfosuccinimidyl)suberate (BS3, Pierce, USA) at 37°C for 2 h, then a peptide or a fraction of enzyme-cleaved *Cry j I* was coated on the plates. Elisa was performed in the usual procedures as described in the preceding paper [1].

Determination of the Amino Acid Sequence

Analyses of the amino acid sequence of the peptide was performed by automated degradation with an Applied Biosystem 470A sequencer (Applied Biosystems, USA). The amino acid composition of the peptide was analyzed by System 6300 (Beckman, USA).

Peptide Synthesis

Peptides were synthesized by an Excell Peptide Synthesizer (Milligen Bioresearch, Millipore, USA) using Fmoc-protected amino acids and Fmoc reactor column resin. The synthesized peptides were cleaved from resin according to the manual of the synthesizer, purified by reversed-phase HPLC using a C18 column and analyzed the amino acid composition.

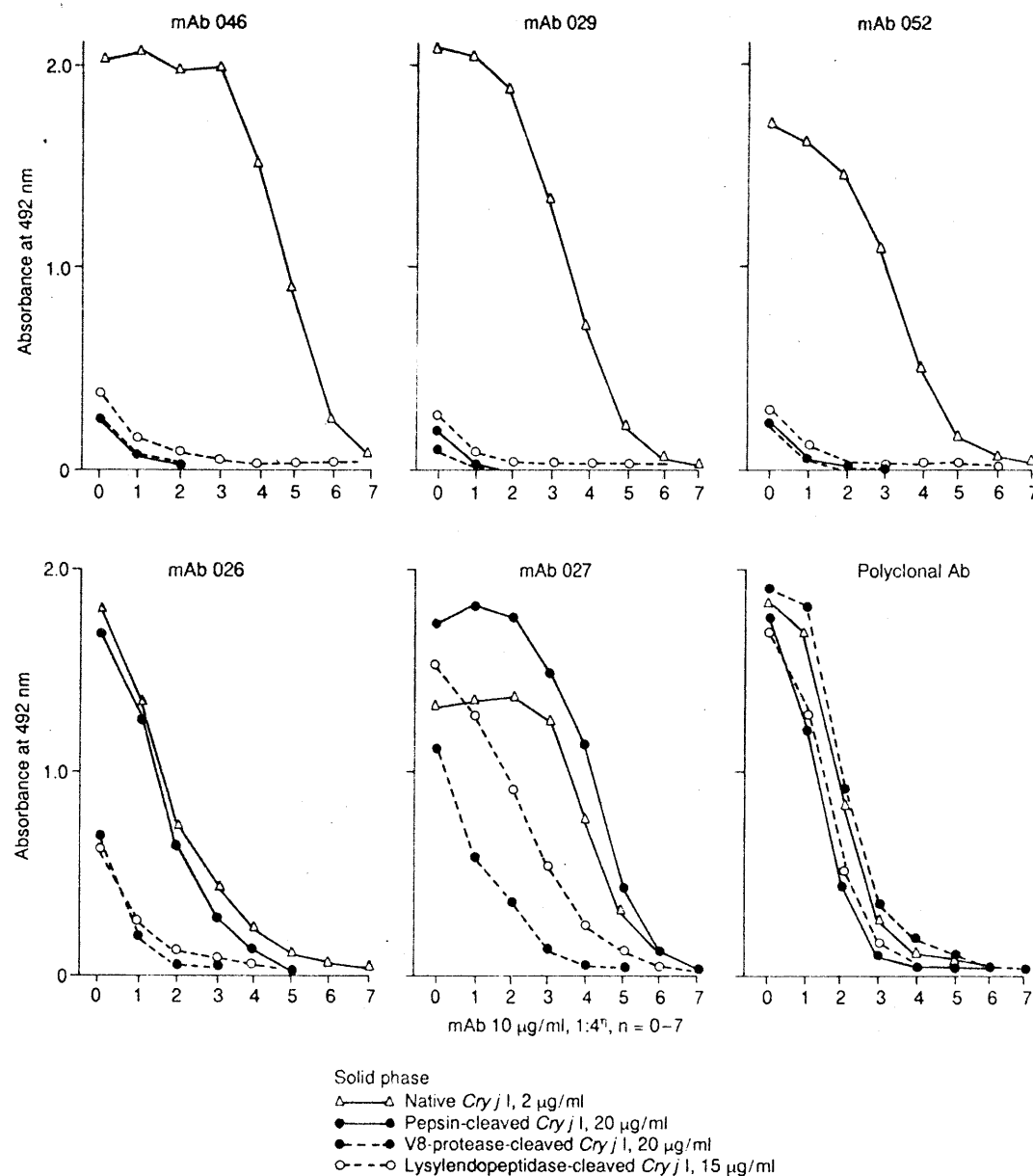


Fig. 2. Binding profiles of the mAbs against native or enzyme-cleaved *Cry j I*. Native or enzyme-cleaved *Cry j I* was coated on microplates at the concentrations indicated. Serial 4-fold dilutions of the mAbs (10 µg/ml) were added to the wells. The reactions were visualized by HRPO-labeled anti-mouse immunoglobulin and substrate.

Results

Binding Abilities of Anti-Cry j I mAbs against Native, Denatured and Enzyme-Digested Cry j I

Six mAbs, 046 (group 1), 029 and 065 (group 2), 026 and 004 (group 3) and 027 (group 4) were selected and tested in Elisa for the binding activities against native, guanidine-

denatured or enzyme-digested *Cry j I*. Figure 1 shows the percent binding of each mAb to the *Cry j I* treated with various concentration of HCl guanidine. The binding of the treated *Cry j I* to the mAbs 046, 029 and 065 decreased as the concentration of guanidine increased, whereas the treatment of *Cry j I* with guanidine exerted little influence on the binding to the mAbs 026, 004 and 027, and to poly-

clonal antibodies. Figure 2 displays the binding profiles of these mAbs to the native and enzyme-digested *Cry j I*. The mAbs 046 (group 1), 029 and 052 (group 2) did not give any positive reactions against the digested antigen. On the other hand, 026 and 027 (groups 3 and 4) as well as polyclonal antibodies clearly could bind to the digested antigen, although the strength of the binding was decreased to some extent, as shown in figure 2. Further, pepsin-cleaved *Cry j I* could inhibit the mAbs 026 and 027, but not 046, 029 and 052 from the binding to solid-phase *Cry j I* (data not shown). The results suggest that mAbs 046, 029, and 052 recognize some conformational surface structures of *Cry j I* molecules which are destroyed by chemical denaturation or enzymatic cleavage, whereas 027, 026 or 004 recognize an epitope stable for the treatments.

Detection of Enzyme-Cleaved *Cry j I* Fragments Responsible for the Binding of *Cry j I* to Anti-*Cry j I* mAbs

The enzyme-cleaved fragments were examined by two methods for their immunoreactivities. One is based on the adsorption of the enzyme-cleaved *Cry j I* onto the mAb column [Ikegami et al., in preparation], and the other is based on Elisa detecting the binding of mAbs to the peptides covalently coupled to microplate wells.

V8-protease-cleaved *Cry j I* (80 µg) was loaded to the mAb 026 column (500 µl gel). After 1 h of incubation at room temperature, 1 ml of PBS was added to the column. The passed-through fraction was collected and subjected to reversed-phase HPLC analysis to obtain a peptide map. A control map was prepared under the same condition using an irrelevant mAb column (117, anti-DNP mouse mAb). Comparing the two maps to each other (fig. 3a, b), two peaks, V46 and V55, were found to disappear in the map of the 026 column passed-through fraction (fig. 3b). V31, V37 and V42 were found to be missed in the map of the 027 column passed-through fraction (fig. 3c). The results suggest that the fragments V46 and V55 contain the binding epitope for 026, and V31, V37 and V42 that for 027.

To confirm further the specific binding of these fragments to the mAbs, each fraction of V8-protease-cleaved *Cry j I* obtained by reversed-phase HPLC was subjected to Elisa as described in Materials and Methods. Figure 4 shows the binding profiles of the two mAbs against the fractionated V8-protease-cleaved *Cry j I*. mAb 026 reacted to fraction V46 and V55, and mAb 027 showed the peaks of the reaction to fractions V31, V37 and V42. The results are consistent with those obtained in the experiments mentioned above.

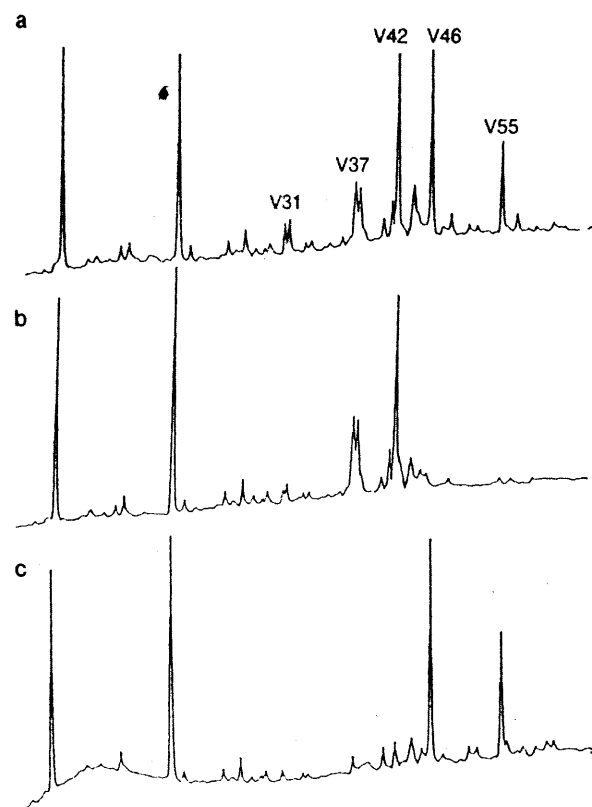


Fig. 3. Detection of mAb 026- or mAb 027-reactive fragments of V8-protease-cleaved *Cry j I* by adsorption on the mAb column. Peptide fragments of V8-protease-cleaved *Cry j I* were mapped out after passing through a control mAb column (a), mAb 026 column (b) or mAb 027 column (c). Comparing the two maps of b and c with the control map, V46 and V55 are missed in b, and V31, V37 and V42 in c.

Pepsin-cleaved *Cry j I* was fractionated, and each fraction was tested by Elisa for the reactivities to mAbs 026 and 027. mAb 026 was found to react with fractions P55 and P59, and mAb 027 could react to P20, P29, P39 and P45 (fig. 5). In the experiment using lysylendopeptidase-cleaved *Cry j I*, fractions L15 and L29 reacted to mAb 027, while no fractions could react to mAb 026 (data not shown).

Consistent with the results mentioned in figure 2, no fragments were found to react to group 1 mAb 046 and group 2 mAbs 029 and 052 by the two methods mentioned above (data not shown).

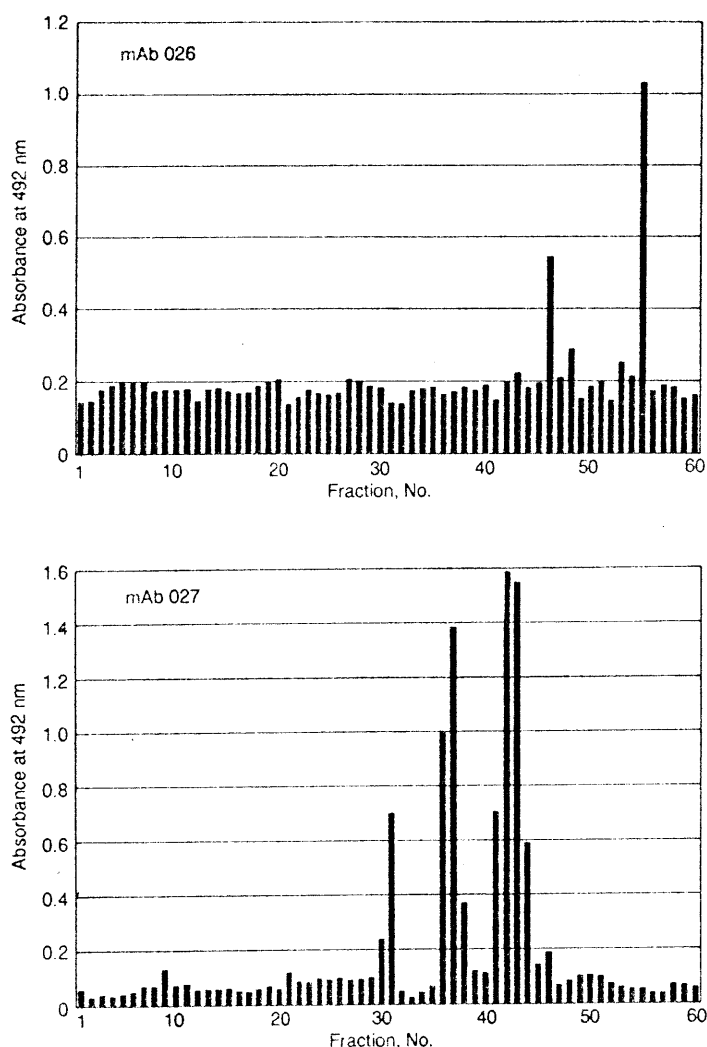


Fig. 4. Binding profiles of mAbs 026 and 027 against fractionated V8-protease-cleaved *Cry j I*. Each fraction of V8-protease-cleaved *Cry j I* obtained by reversed-phase HPLC was covalently coated on microplates. Binding of the mAbs 026 and 027 to the coated Ag was examined by Elisa.

Amino Acid Sequences of the Immunoreactive Peptides Generated by Enzymatic Digestion

Table 1 shows the amino acid sequences of peptides in the immunoreactive fragments. The fragments P39, P45, V42, L15 and L29, reactive to the mAb 027, were found to contain the common amino acid sequence $^{14}\text{Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-}^{24}\text{Lys}$, although in P39 the amino acids after ^{18}Leu could not be read in this experiment. One of the differences between P39 and P45 is that P39 might have sugar at the position of ^{6}Asn , while P45 does not. The results suggest that the epitope recognized by the mAb 027 is located in this common peptide. The amino acid sequences of the peptides in 026-reactive fractions, P55, V46 and V55, were found to begin with Asp-Asn-Pro-Ile-Ser-..., which is consistent with the N-terminal amino acid sequence of *Cry j I*. The epitope for the

mAb 026 is located in a fragment containing the *Cry j I* N-terminal region produced by pepsin or V8 protease digestion, but not in any fragment by lysylendopeptidase digestion.

Binding Activities of mAbs 027 and 026 against the Synthetic Peptide

The peptide Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-Arg, named C1027-1, was synthesized and tested for the binding to mAbs 026 and 027. The results obtained by Elisa are shown in figure 6. The C1027-1 could bind to the mAb 027, but not to mAb 026 (fig. 6a). Moreover, it could inhibit 027 from binding to *Cry j I* and to its immunoreactive fragments (fig. 6b), indicating that this peptide contains an epitope for mAb 027.

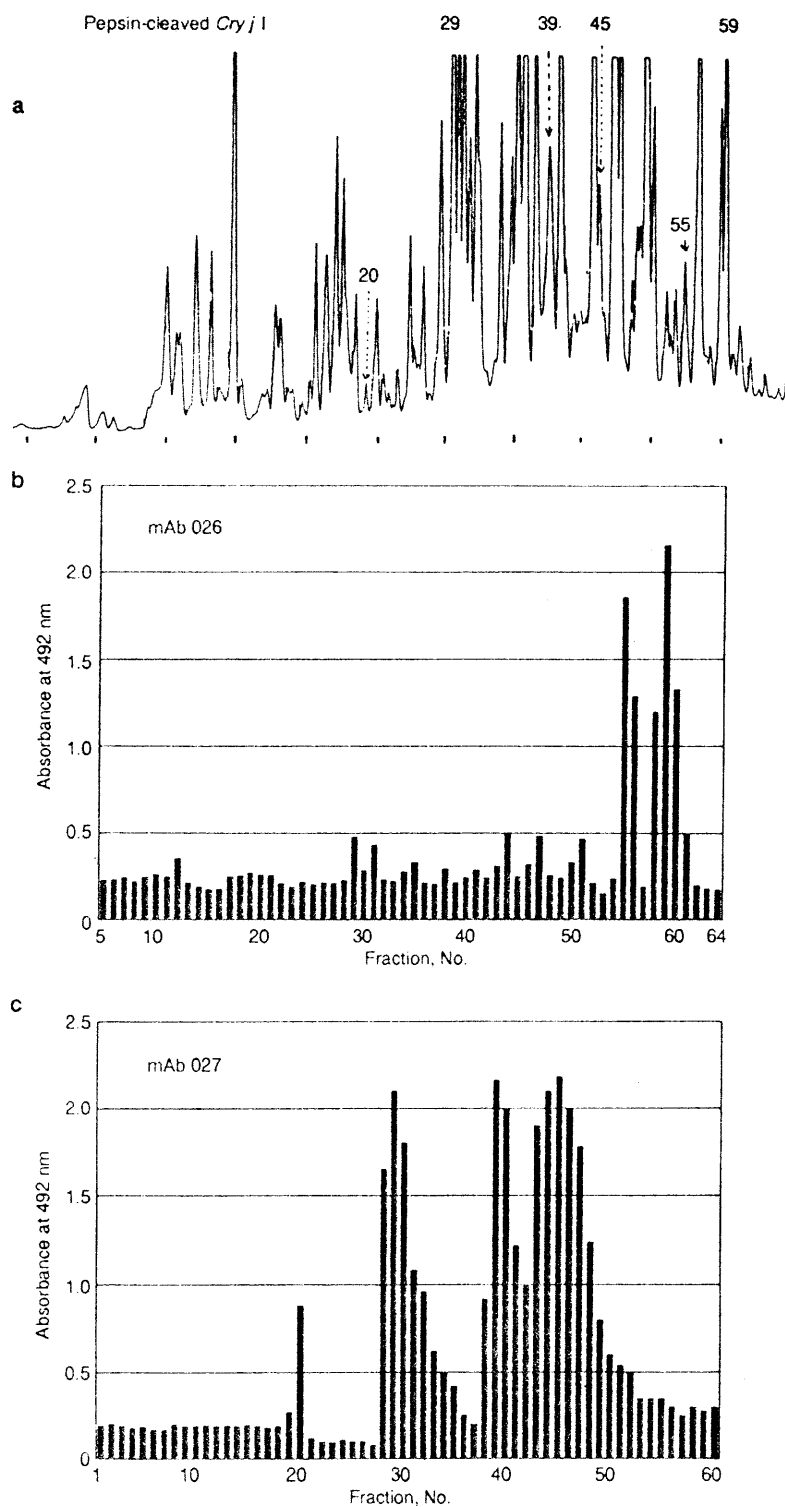


Fig. 5. The chromatogram of reversed-phase HPLC of pepsin-cleaved *Cry j I* (a) and the binding profiles of mAbs 026 (b) and 027 (c) to the fractions. Pepsin-cleaved *Cry j I* was fractionated by reversed-phase HPLC. Each fraction was examined by Elisa for the binding ability against mAbs 026 and 027.

Table 1. Amino acid sequences of the immunoreactive fragments of enzyme-cleaved *Cry j I*

Fragments reactive to the mAb 027

	1	5	10	15	16	20	25
P39	Asn-Val-Glu-Asn-Gly-?	-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-Ala-Gly-Val-Leu-?					
P45	Asn-Val-Glu-Asn-Gly-Asn-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys-Arg						
V42		Asn-Gly-Asn-Ala-Thr-Pro-Gln-Leu-Thr-Lys-Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys					
L15				Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys			
L29				Asn-Ala-Gly-Val-Leu-			

Fragments reactive to the mAb 026

	1	5	10	15	16	20	25
P59	Asp-Asn-Pro-Ile-Asp-Ser-Cys-Trp-Arg-Gly-Asp-Ser-Asn-Trp-Ala-Gln-Asn-Arg-Met-Lys-Leu-Ala-Asp-Lys-Ala-Val-Gly-.....						
V55	Asp-Asn-Pro-Ile-Asp-Ser-.....						
V46	Asp-Asn-Pro-Ile-Asp-Ser-.....						

P45 means fraction No. 45 of pepsin-cleaved *Cry j I*, V and L mean V8-protease- and lysylendopeptidase-cleaved *Cry j I*, respectively.

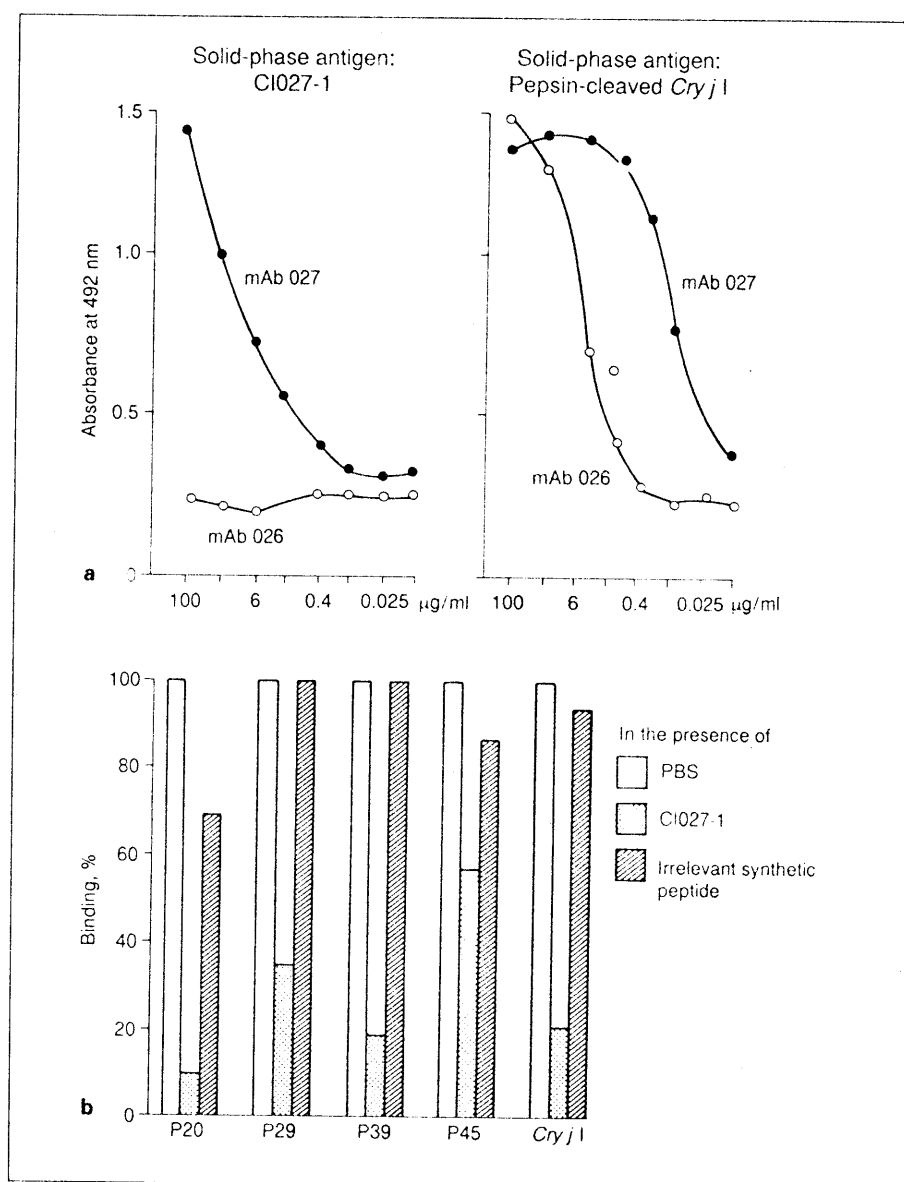


Fig. 6. Specific binding activity of a synthetic peptide, C1027-1, against mAb 027, and its inhibitory ability for the binding of the mAb 027 to *Cry j I* and to the immunoreactive fragments.

Discussion

The structural characterization of an allergenic epitope is not always straightforwardly clarified, because the allergens are usually purified in very small amounts, and the epitopes responsible for allergic reactions are easily destroyed by chemical or physical denaturation [5, 14]. This is true in the case of *Cry j I*. We tried to analyze the four antigenic determinants showing a specificity against our mAbs different from each other [1]. However, the conformationally dependent epitopes, in our case those for group 1 and group 2 mAbs, disappeared by the treatment with guanidine or enzymes, thus they require strategies different from those applied in this study, for instance, limited proteolysis of mAb-bound protein antigen [21–23] or epitope mapping using the mAbs raised against denatured antigen [5].

The epitope for group 4 mAb 027 was determined to be located in the peptide of ten residues, Asn-Ala-Gly-Val-Leu-Thr-Cys-Ser-Leu-Ser-Lys, which was considered to be different from any other known peptides. However, the complete primary structure of *Cry j I* has not been available yet, and we could not know where this peptide is located in the polypeptide chain of the *Cry j I* molecule. The antigenic determinant for mAb 026 was found to be in the fragments containing 27-amino acid residues of the *Cry j I* N terminus, whereas, because of the limited amounts of the allergen, we have not been able to define the precise binding site to mAb 026. In these experiments, the reactivity of a mAb with more than two fragments in an enzyme-digested product, so far tested, indicates that the enzymatic digestion was incomplete, and the resultant products are heterogeneous. Furthermore, reactivity of a mAb to fractions before and behind the peak fractions indicates that the peptides were not separated completely under the condition of this chromatography. Since no fragments could react to both of the 026 and 027 mAbs, and also no fragments could react to 046, 029 and 052, the possibility for the contamination of the reactive fragments with undigested *Cry j I* could be excluded. At any rate, the two mAbs 026 and 027 could be considered to recognize sequential amino acid residues in some parts of the *Cry j I* polypeptide chain. However, the binding strength of the mAbs to the fragments were reduced in some extent, as shown in figure 2, suggesting that some kind of force induced by the conformation of a *Cry j I* molecule enhances the binding ability of the mAbs 027 and 026. The two determinants to mAbs 026 and 027 have been reported in the preceding study [1] to be located in the inner space of *Cry j I* molecules as cryptantigens, then the liquid-phase *Cry j I* could

not bind to the immobilized mAbs 026 or 027, and these determinants were exposed on the surface when the antigen was coated on a plate. The present study revealed that these two determinants are also exposed by the enzymatic cleavage to bind to the corresponding mAb column.

Since the epitopes for the mAbs in the remaining group 1 and group 2 were found to be destroyed by the enzymatic cleavage, structural analysis on these epitopes could not be conducted yet in this study. However, as mentioned in the preceding paper [1], these group mAbs, especially 046, could block effectively the human IgE antibody binding to *Cry j I*. Therefore, the epitopes for these mAbs are considered to have an important role in IgE antibody production and IgE-mediated allergic reactions in humans. To understand the mechanisms of the IgE responses and to make a strategy for the therapy of the pollinosis, we continue to analyze the structures of these epitopes.

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