

Cry j 2, a major allergen of Japanese cedar pollen, shows polymethylgalacturonase activity

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We examined Cry j 2, a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen, for polygalacturonase enzyme activity, since a nucleotide sequence of cDNA of Cry j 2 showed a significant homology with that of tomato polygalacturonase. Polygalacturonase is well known to depolymerize preferentially polygalacturonic acid (PGA) by hydrolysis. However, Cry j 2 did not act on PGA, but was found to depolymerize pectin and methylesterified PGA in a dose-dependent manner. The substrate specificity of Cry j 2 was different from that of polygalacturonase derived from *Aspergillus niger*. The depolymerizing activity of Cry j 2 reached a maximum at 50%–60% of methylesterification of PGA. In contrast, polygalacturonase showed its maximum activity to PGA, and the activity decreased as the degree of methylesterification increased. Interestingly, the pectin-depolymerizing activity of Cry j 2 was due to a hydrolysis, but not a lyase, activity which splits the glycosidic bonds by β -elimination, since no unsaturated uronides were found by measurement of absorbance at 235 nm in the reaction mixture. The enzyme activity was markedly inhibited by anti-Cry j 2 antibodies. These results indicate that Cry j 2 probably has polymethylgalacturonase enzyme activity, as postulated by von Neukom in 1963, although existence of this activity has not yet been proven.

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The number of patients suffering from Japanese cedar pollinosis has rapidly increased since this pollinosis was first discovered in 1964. Two major allergens of Japanese cedar pollen, Cry j 1 and Cry j 2, have already been reported. Cry j 1 was isolated by Yasueda et al. (23), and the cDNA of Cry j 1 was isolated by Griffith et al. (1) and Sone et al. (20). The second major allergen, Cry j 2, was isolated and characterized by Sakaguchi et al. (17), as an allergen completely different from Cry j 1 in immunophyicochemical properties and in N-terminal amino acid sequences. Sawatani et al. (18) reported that most patients suffering from Japanese cedar pollinosis reacted to both Cry j 1 and Cry j 2 *in vivo* (skin test) and *in vitro* (RAST). Very recently, Namba et al. (10) and Komiyama et al. (6) revealed the cDNA nucleotide sequence of Cry j 2.

Recently, we reported that Cry j 1 had a significant amino acid sequence homology to pectate lyase isozymes secreted by pathogenic plant bacteria, and we recognized a pectate lyase enzyme activity in Cry j 1 (22). Analysis of Cry j 2 cDNA revealed that it had a significant sequence homology with that of

polygalacturonase derived from the tomato (19), which is well known to hydrolyze preferentially polygalacturonic acid (PGA). However, it has not yet been shown conclusively that Cry j 2 has polygalacturonase enzyme activity.

In the present study, we found that Cry j 2 did not depolymerize PGA, but did act on methylesterified PGAs, such as pectin, depending on the degree of methylesterification. Interestingly, the depolymerizing activity was caused by hydrolysis. The results presented here indicate that Cry j 2 should probably be categorized as polymethylgalacturonase, as previously proposed by von Neukom (13), but this has not been shown conclusively.

Material and methods

Reagents

Cry j 1 and Cry j 2 were extracted from Japanese cedar pollen with 0.125 M sodium bicarbonate and partially purified as described previously (23). Further purifications of Cry j 1 and Cry j 2 were performed by affinity chromatography with mouse anti-

Cry j 1 monoclonal antibody (mAb) 065- and anti-Cry j 2 mAb N26-immobilized columns, respectively (3, 4). Purities of the Cry j 1 and Cry j 2 preparations used were both greater than 95%, as determined by SDS-PAGE followed by silver staining and Western blotting. PGA derived from orange (P-3889), pectin (70% methylesterification) derived from citrus fruits (P-9135), polygalacturonase derived from *Aspergillus niger* (P-3429), and pectin lyase derived from *Aspergillus japonicus* (P-2679) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

PGA was methylesterified by the method of Manabe (8). Briefly, 0.5 g of PGA was incubated at 70°C in 10 ml of solution containing methanol, distilled water, and hydrochloric acid at a ratio of 90:8:2. After various incubation periods, the reaction mixtures were centrifuged at 3500 rpm for 5 min. The precipitates were washed with 10 ml of 70% methanol, resuspended in 70% methanol, and further incubated overnight at room temperature. Then the precipitates were washed two times with each of the following: 70%, 80%, 90% and 100% methanol and 100% acetone. After washing, the precipitates were dried in a vacuum. The degrees of methylesterification of the PGAs were measured by titration with sodium hydroxide solution and hydrochloric acid. The protein content was determined by the method of Lowry et al. (7) with bovine serum albumin as the standard protein.

Analysis of polygalacturonase and polymethylgalacturonase activities

Reaction mixtures containing 1.0 ml of 1.0% (w/v) substrate solution, 0.2 ml of 1.0 M sodium chloride, 0.2 ml of 0.5 M sodium acetate buffer (pH 4.5), and 0.6 ml of enzyme solution were incubated for 30 min at 30°C, as described by Nasuno & Starr (11). In some experiments, Cry j 2 was preincubated with mouse anti-Cry j 2 polyclonal antibodies, anti-Cry j 2 mAb N26 (3), isotype-matched anti-Cry j 1 mAb 053 (3), or rabbit anti-Cry j 1 polyclonal antibodies for 1 h at room temperature. Enzyme activity was determined by measuring the absorbance of the reaction mixture at 520 nm with a UV-250 spectrophotometer (Shimazu Scientific Instruments and Equipment, Kyoto, Japan), α -D-galacturonic acid (Nacalai Tesque, Inc, Kyoto, Japan) being used as the standard material, according to the Somogyi-Nelson method (12). One unit of polygalacturonase or polymethylgalacturonase activity is the amount of enzyme that releases from the substrate 1 μ M of aldehyde groups per minute at 30°C under the above conditions.

Analysis of pectate lyase activity

Pectate lyase activity was analyzed by the method of Keen et al. (5). Reaction mixtures containing 0.4 ml of 1% (w/v) pectin, 0.2 ml of 0.2 M Tris-HCl buffer (pH 8.5), 0.4 ml of 3 mM CaCl₂, and 0.2 ml of Cry j 1 (25 μ g/ml) or Cry j 2 (100 μ g/ml) were incubated at 37°C. One unit of pectate lyase activity was defined as the amount necessary to form 1 μ M of unsaturated uronide product per minute with an extinction coefficient of 4600 at 235 nm (5).

Analysis of pectin lyase activity

Pectin lyase activity measurement was performed according to the method of Ishii & Yokotsuka (2). Reaction mixtures containing 0.8 ml of 1% (w/v) pectin, 0.8 ml of McIlvaine buffer (pH 5.5), and 0.4 ml of either pectin lyase (5 μ g/ml), Cry j 1 (25 μ g/ml), or Cry j 2 (100 μ g/ml) were incubated at 40°C. One unit of pectin lyase activity was defined as the amount necessary to increase the absorbance at 235 nm by 1.0/min in the reaction mixture.

Results

We investigated whether Cry j 2 had polygalacturonase activity with PGA or pectin as a substrate. As shown in Table 1, Cry j 2 did not depolymerize PGA, but did depolymerize pectin in a dose-dependent manner. In marked contrast, polygalacturonase derived from *A. niger* depolymerized PGA preferentially and acted on pectin only weakly. The optimum pH and temperature for the activity were found to be 4.5 and 30°C, respectively (Figs. 1 and 2).

Table 1. Pectin depolymerization by Cry j 2

Substrate	Enzyme	Enzyme concentration (μ g/ml)	Activity (mU/ml)
PGA	Cry j 2	0	0.0
		25	0.0
		50	0.0
		100	0.0
		200	0.0
	Polygalacturonase	5	240.0
Pectin	Cry j 2	0	0.0
		25	8.4
		50	15.6
		100	39.5
		200	70.1
	Polygalacturonase	5	87.0

Reaction mixtures containing 1.0 ml of 1.0% (w/v) pectin or PGA, 0.2 ml of 1.0 M sodium chloride, 0.2 ml of sodium acetate buffer (pH 4.5), and 0.6 ml of varying concentrations of Cry j 2 or polygalacturonase (5 μ g/ml) were incubated for 30 min at 30°C. Number of reducing groups produced was determined by Somogyi-Nelson method (11), as described in Material and methods.

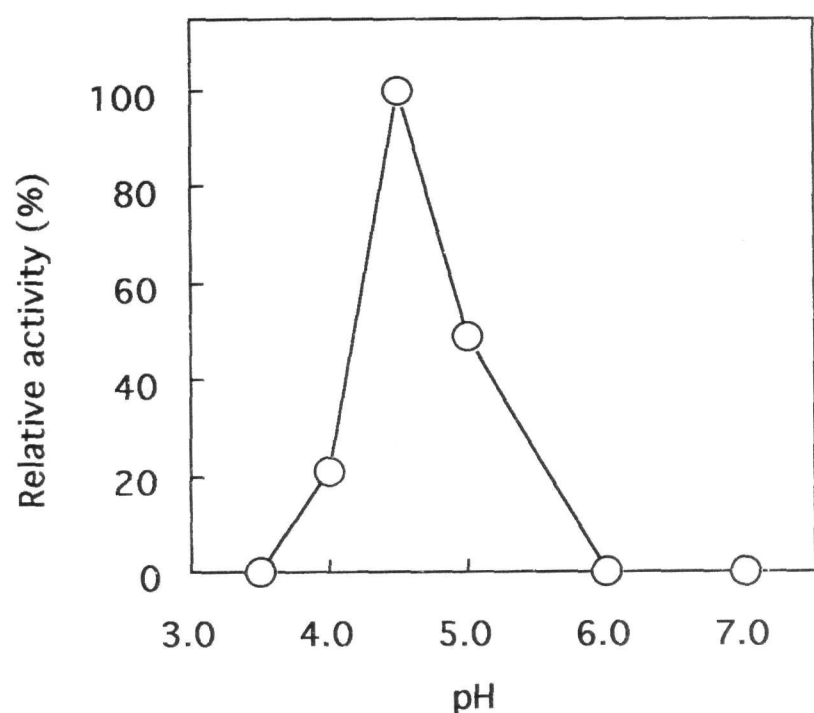


Fig. 1. Effect of pH on pectin-depolymerizing activity of Cry j 2. Reaction mixtures containing 1.0 ml of 1.0% (w/v) pectin, 0.2 ml of 1.0 M sodium chloride, 0.2 ml of various buffers at various pH levels (0.5 M), as described below, and 0.6 ml of Cry j 2 (100 μ g/ml) were incubated at 30°C for 30 min. Two buffers used were sodium acetate buffer at pH 3.5–6.0, and Tris-HCl buffer at pH 6.0–7.0. Number of reducing groups produced was determined by Somogyi-Nelson method, as described in Material and methods. Enzyme activities were expressed as percentage of maximum.

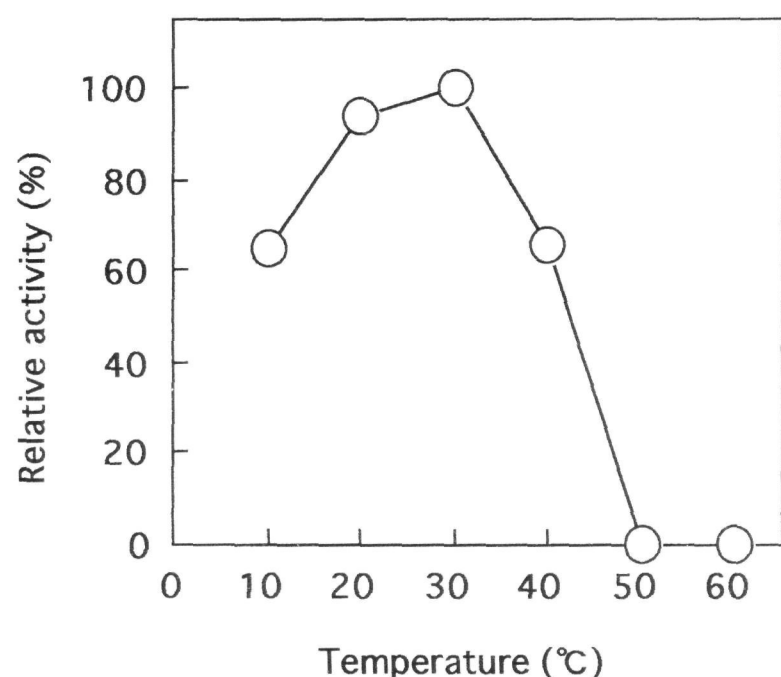


Fig. 2. Effect of temperature on pectin-depolymerizing activity of Cry j 2. Reaction mixtures containing 1.0 ml of 1.0% (w/v) pectin, 0.2 ml of 1.0 M sodium chloride, 0.2 ml of sodium acetate buffer (pH 4.5), and 0.6 ml of Cry j 2 (100 μ g/ml) were incubated for 30 min at various temperatures. Enzyme activities were expressed as percentage of maximum.

For determination of whether the pectin-depolymerizing activity of Cry j 2 is due to hydrolysis or to lyase activity, Cry j 2 was incubated with pectin under several conditions, as described in Table 2, and the reaction products were assessed by measuring the absorbance at 235 nm to detect unsaturated

Table 2. Absence of lyase enzyme activity on pectin in Cry j 2

Experiment	Enzyme	Enzyme concentration (μ g/ml)	pH	Lyase activity (mU/ml)
1	Cry j 2	100	4.5	0
		200		0
2	Cry j 2	100	8.5	0
	Cry j 1	25		172
3	Cry j 2	100	5.5	0
	Cry j 1	25		0
	Pectin lyase	5		229

Lyase activities were determined by monitoring absorbance of reaction mixtures at 235 nm in quartz cuvette. Reactions were carried out under following conditions. Experiment 1 was performed under conditions described in Table 1, which were optimum for Cry j 2 to depolymerize pectin. Experiment 2 was performed under optimum conditions for pectate lyase enzyme activity. Reaction mixtures containing 0.4 ml of 1% (w/v) pectin, 0.2 ml of 0.2 M Tris-HCl buffer (pH 8.5), 0.4 ml of 3 mM CaCl_2 , and 0.2 ml of Cry j 2 (100 μ g/ml), Cry j 1 (25 μ g/ml), or pectin lyase (5 μ g/ml) were incubated at 37°C. Experiment 3 was performed under optimum conditions for pectin lyase (from *A. japonicus*) enzyme activity. Reaction mixtures containing 0.8 ml of 1% (w/v) pectin, 0.8 ml of McIlvaine buffer (pH 5.5), and 0.4 ml of Cry j 2 (100 μ g/ml), Cry j 1 (25 μ g/ml), or pectin lyase (5 μ g/ml) were incubated at 40°C.

uronides. As shown in Experiment 1 of Table 2, no unsaturated uronides were found in the reaction mixtures of Cry j 2 (100 and 200 μ g/ml) under the same conditions optimum for Cry j 2 enzyme activity, as described in Table 1, although at these concentrations Cry j 2 depolymerized pectin to produce significant amounts of reaction products which could be detected by the Somogyi-Nelson method (12) (Table 1).

Experiment 2 was performed to detect pectate lyase activity in the presence of Ca^{2+} . Pectate lyase is known to depolymerize pectin to produce unsaturated uronides (15). Cry j 1, as reported previously (22), exhibited pectate lyase activity. In contrast, Cry j 2 (100 μ g/ml) showed no pectate lyase activity under the same conditions optimum for pectate lyase enzyme activity.

In experiment 3, the pectin lyase activity of Cry j 2 was assessed. Pectin lyase derived from *A. japonicus* depolymerized pectin to produce unsaturated uronides. However, Cry j 2 did not show any activity under the same conditions optimum for pectin lyase enzyme activity. Cry j 1 did not depolymerize pectin under these conditions, probably due to the low pH and the absence of Ca^{2+} . These results indicate that Cry j 2 has no lyase activity.

Therefore, we surmised that the pectin-depolymerizing activity of Cry j 2 is due to hydrolysis. Furthermore, Cry j 2 was found not to require Ca^{2+} to express enzymatic activity, since the Cry j 2 activity was not reduced in the presence of 10 mM EDTA (data not shown).

The major difference between pectin and PGA is the degree of methylesterification. PGA is not

methylesterified entirely. The degree of methylesterification of the pectin used in the present study was 70%. Therefore, we investigated the substrate specificities of Cry j 2 and polygalacturonase, using PGAs as substrates with various degrees of methylesterification. As shown in Fig. 3, the enzyme activity of *A. niger* polygalacturonase was greatest at 0% of methylesterification (PGA itself), and the depolymerizing activity decreased gradually as the degree of methylesterification increased. In contrast, Cry j 2 again showed no activity on PGA. The enzyme activity of Cry j 2 was observed when PGA was methylesterified, and it increased gradually as the degree of methylesterification increased and reached a maximum at 50%–60% of methylesterification. These results clearly indicate that the substrate specificity of Cry j 2 is different from that of polygalacturonase, and that Cry j 2 hydrolyzes methylesterified PGAs, but not PGA itself.

The effects of anti-Cry j 1 and anti-Cry j 2 antibodies on the enzyme activity of Cry j 2 were also studied. As shown in Fig. 4, the pectin-depolymerizing activity of Cry j 2 was inhibited by anti-Cry j 2 mAb N26 and by mouse anti-Cry j 2 polyclonal antibodies in a dose-dependent manner. The isotype-matched anti-Cry j 1 mAb 053 and the rabbit anti-Cry j 1 polyclonal antibodies had no effect. However, the latter has been reported to inhibit Cry j 1 enzyme activity (22). These results indicate that the enzymatic activity observed was due to the Cry j 2 itself, but not to any bacteria-derived polygalacturonase, the lyase proteins, or Cry j 1 contaminating the Cry j 2 preparation.

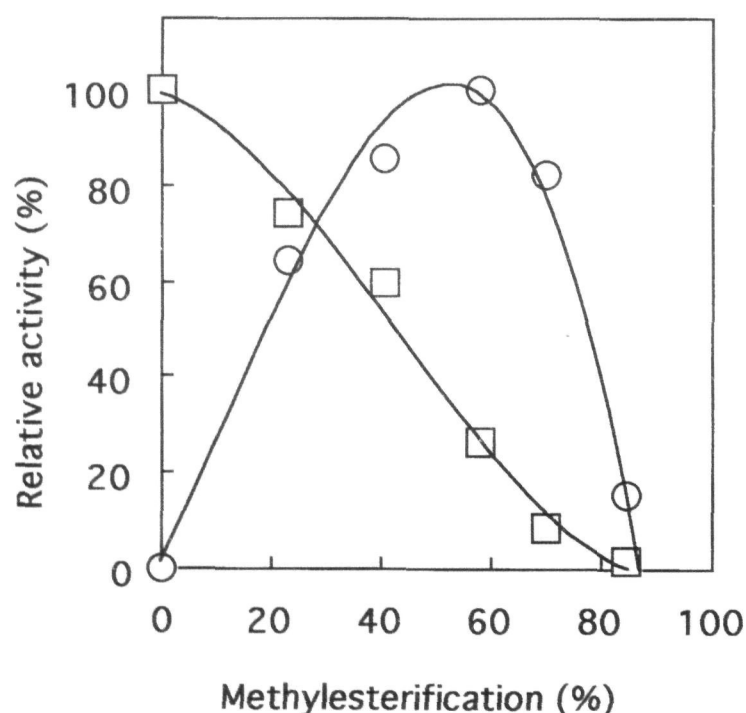


Fig. 3. Substrate specificities of Cry j 2 (○) and polygalacturonase (□) from *A. niger*. Substrate specificities of Cry j 2 and polygalacturonase from *A. niger* were determined with PGAs with various degrees of methylesterification. Enzyme activities were expressed as percentage of maximum.

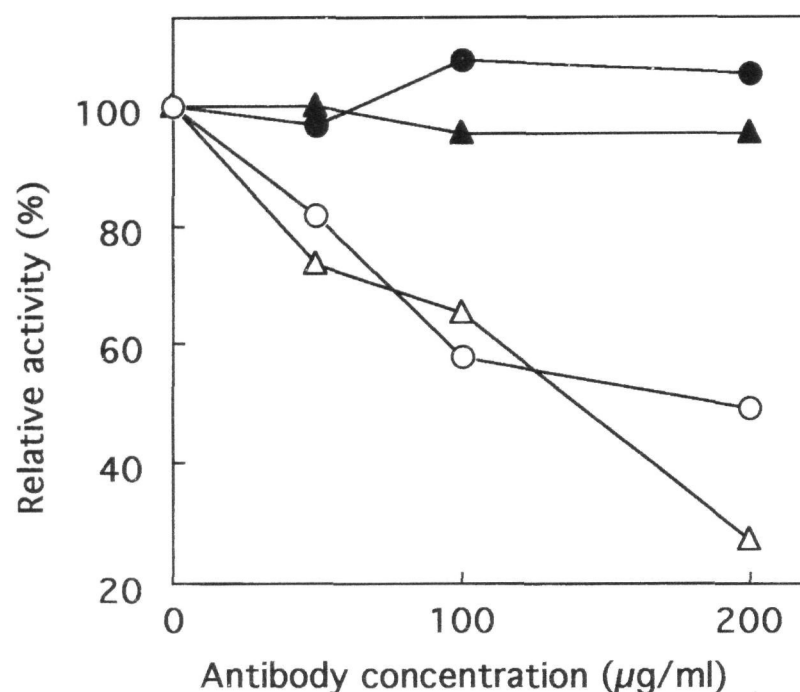


Fig. 4. Effect of anti-Cry j 2 antibodies and anti-Cry j 1 antibodies on pectin-depolymerizing activity of Cry j 2. Cry j 2 was pre-incubated with various concentrations of anti-Cry j 2 mAb N26 (Δ), mouse anti-Cry j 2 polyclonal antibodies (○), rabbit anti-Cry j 1 polyclonal antibodies (●), or anti-Cry j 1 mAb 053 (▲) (isotype-matched to N26) for 60 min at room temperature. Mixtures were then added to sodium acetate buffer (pH 4.5) containing pectin and sodium chloride. Enzyme reaction was carried out for 30 min at 30°C. Number of reducing groups produced was determined as described in Material and methods.

Discussion

In this study, we demonstrated that Cry j 2 had an enzyme activity to depolymerize methylesterified PGAs and pectin, and that the depolymerizing activity was due to a hydrolytic reaction, since no unsaturated uronides produced by β -elimination were found by measuring the absorbance of the reaction mixture at 235 nm under various reaction conditions (Table 2).

Although the nucleotide sequence of Cry j 2 showed significant homology with that of polygalacturonase, the substrate specificities of these two enzymes were different from each other. Polygalacturonase acts preferentially on PGA, while Cry j 2 acts on pectin and methylesterified PGAs, but not on PGA itself. As shown in Fig. 3, our results obtained with polygalacturonase coincided with those of Pilnik et al. (15) showing that polygalacturonase preferentially depolymerizes PGA and that its enzyme activity decreases as the degree of methylesterification of PGA increases. However, Cry j 2 did not act on PGA, but the enzyme activity gradually increased as the degree of methylesterification of PGA increased and reached a maximum at 50%–60% of methylesterification. Nevertheless, both enzymes split glycosidic bonds in the same manner (hydrolysis). The regions showing the sequence homology may be related to a hydrolytic reaction, and the sites responsible for determining the substrate specificities might

be located outside these regions. Alternatively, fine differences between the conformational structures created by the folding of these regions might reflect the substrate specificities. With Taka-amylase, which degrades maltotriose, it was reported that the region which is responsible for degradation is located on the axis of the α/β -barrel in the cleft responsible for substrate recognition (9).

von Neukom (13) classified the pectin depolymerases into eight groups according to the following criteria: the preference for pectin or pectate as the substrate, the hydrolytic or transeliminative splitting of the glycosidic bonds, and the endo- or exomechanism of splitting. According to this classification, the hydrolase which acts mainly on highly methyl-esterified PGAs would be called polymethylgalacturonase. However, the presence of such an enzyme has not yet been demonstrated. Our results suggest that Cry j 2 should be placed in the category of polymethylgalacturonase.

Why the existence of such a polymethylgalacturonase remains undetected is uncertain. Recently, several cDNA sequences having significant homology with that of tomato fruit polygalacturonase were reported to exist in maize pollen (14), although the enzyme activities were not determined. In 1989, Pressey & Reger (16) reported that polygalacturonase activities were found in the pollen of corn, lily, oats, and palm, which all belong to the same category of angiosperms. As far as we know, no depolymerizing pectic enzymes derived from the pollen of gymnosperms have been examined yet except Cry j 1, which is classified as pectate lyase (22). Cry j 2 was classified as polymethylgalacturonase; therefore, it is possible that plant pollens from gymnosperms might contain polymethylgalacturonase.

The fact that both mouse anti-Cry j 2 polyclonal antibodies and mAb N26 inhibited the enzyme activity of Cry j 2, but rabbit anti-Cry j 1 polyclonal antibodies did not, indicates that the enzymatic activity is due to the Cry j 2 protein itself. Furthermore, mAb N26 has been reported to have a significant inhibitory effect on human IgE binding to Cry j 2 (21). Therefore, at least one of the regions with an important role in Cry j 2 enzyme activity is considered to be immunogenic and/or allergenic to mice and man.

The physiologic role of Cry j 2 in pollen remains to be elucidated. Polygalacturonase in pollen has been thought to have a role in the growth of pollen tubes in the pistil (14). It is well known that pectin is located in plant cell walls and that it functions as cement to connect cell walls to each other. Therefore, it is possible that both Cry j 1 and Cry j 2 may also facilitate pollen-tube growth during pollination by degrading female flowers.

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