Antifungal Activity of Rye (Secale cereale) Seed Chitinases: the Different Binding Manner of Class I and Class II Chitinases to the Fungal Cell Walls

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The antifungal activities of rye seed chitinase-a (RSC-a, class I) and -c (RSC-c, class II) were studied in detail using two different bioassays with Trichoderma sp. as well as binding and degradation experiments with the cell walls prepared from its mycelia. RSC-a inhibited more strongly the re-extension of the hyphae, containing mainly mature cells, than RSC-c did. Upon incubation of the fungus with fluorescent chitinases, FITC-labeled RSC-a was found to be located in the hyphal tips, lateral walls, and septa, while FITC-labeled RSC-c was only in the hyphal tip. RSC-a had a greater affinity for the cell walls than RSC-c. RSC-a liberated a larger amount of reducing sugar from the cell walls than RSC-c did. These results inferred that RSC-a first binds to the lateral walls and septa, consisting of the mature cell walls, and degrades mature chitin fiber, while RSC-c binds only to the hyphal tip followed by degradation of only nascent chitin. As a result, RSC-a inhibited fungal growth more effectively than RSC-c. Furthermore, it was suggested that the chitin-binding domain in RSC-a assists the antifungal action of RSC-a by binding to the fungal hypha.

Key words: chitinase; chitin-binding protein; rye seeds; antifungal activity; fungal cell wall

Chitinase (EC 3.2.1.14) is considered to be a defense-related protein in higher plants and to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of many fungi.

Rye seed chitinase-a (RSC-a) is a basic class I chitinase consisting of an N-terminal chitin-binding (CB) domain and a catalytic (Cat) domain connected by a flexible linker. The CB domain is similar in sequence to hevein, wheat germ agglutinin, and pokeweed lectin, which are referred to as chitin-binding proteins. Rye seed chitinase-c (RSC-c) is a basic class II chitinase with 92% sequence similarity to the Cat domain of RSC-a, but lacking the N-terminal CB domain and a linker. RSC-a has 3 times more chitinase activity than that of RSC-c when using colloidal chitin as an insoluble substrate. Yamagami et al. have previously isolated the CB domain and the Cat domain after limited thermolysin hydrolysis of RSC-a. The chitinase activity of the Cat domain, using colloidal chitin, was decreased to the level of RSC-c. We are interested in knowing whether the difference in protein structure is related to an antifungal activity of chitinase.

Our recent work has shown that the actions of RSC-a and RSC-c to hyphae were different, by using novel bioassays. These bioassays showed that the hyphal extension was more consistently inhibited by RSC-a than that affected by RSC-c and the hyphae affected by RSC-a were abnormally extended. We, therefore, presented a hypothesis that RSC-a may have higher affinity to the mature cell walls and may hydrolyze mature chitin fiber better than RSC-c does.

To test the hypothesis, in this study, we used two different bioassays with Trichoderma sp. as well as binding and degradation experiments with the cell walls prepared from its mycelium. In the experiment using FITC-labeled chitinases, a binding site of the proteins on a fungal hypha was disclosed. This is the first report that has clarified the difference of binding manner of class I and class II chitinases to the fungal cell walls. In this paper, the roles of the CB domain and the Cat domain in class I chitinases for antifungal activity are also discussed.

Materials and Methods

Materials. Rye seeds were purchased from Takii Seed Co. (Kyoto, Japan). RSC-a and RSC-c were...
prepared from rye seeds, and the CB domain (Glu1-Pro48) and the Cat domain (Val49-Ala302) were isolated from a thermolysin digest of RSC-a as described by Yamagami et al. The RSC-c mutant (Glu67-Gln), which lost chitinolytic activity, was prepared as described by Ohnuma et al. The following fungal strains were used for antifungal activity: *Trichoderma* sp., *Fusarium oxysporum*, and *Rhizoctonia solani*. All other reagents were of analytical grade.

**Assay for the antifungal activity.**

The *hyphal-extension inhibition assay*. An agar disk (6 mm in diameter) with the hyphae, which was derived from the fungus in an actively growing state previously cultured on a potato dextrose broth with 1.5% (w/v) agar (PDA), was placed in the center of a Petri dish containing PDA. In the assay to examine the effects of pH and ionic strength, the PDA plates contained 20 mM sodium phosphate buffer, pH 6.0, 6.5, 7.0, or 7.5, with or without 0, 0.05, 0.1, 0.15, or 0.2 M NaCl at the final concentration. The plates were incubated at room temperature for 12 h. Wells were subsequently punched into the agar at a distance of 15 mm from the center of the Petri dish. The samples to be tested were placed into the wells in 10 μl of distilled water. The hyphal-extension inhibition was observed with the naked eye or light microscope after incubation at room temperature at 24–72 h.

The *hyphal re-extension inhibition assay*. The hyphal re-extension inhibition assay was done by using *Trichoderma* sp. Agar disks (6 mm in diameter) containing the old hyphae, which were derived from the resting part of the fungus previously cultured on a PDA plate, were put on another PDA plate. The samples were overlaid on the agar disks, then, the plate was incubated at room temperature for 12 h.

**Binding assays.**

An FITC-chitinases binding study. For fluorescein conjugation, 1 mg of chitinases and 0.02 mg of fluorescein isothiocyanate (FITC) were dissolved in 200 μl of 0.25 M sodium carbonate buffer, pH 9.2. The mixture was allowed to react for 3 h at 25°C, in the dark, on a rotary shaker. The conjugated chitinases were separated from unreacted FITC on a Sephadex G-25 column (1.0×10 cm), equilibrated with PBS. By measuring the ratio of absorption at 495 and 280 nm, a labeling degree of 0.7:1 mol (FITC:chitinase) was reached. The conjugated chitinases had an chitinase activity indistinguishable from that of the un conjugated chitinases.

An FITC-chitinases binding study was done as described by Koo et al. The poly-L-lysine-coated cover glasses (12 mm diameter, Asahi Technoglass Corp.) were placed inside of a 24-well microplate and covered with 1 ml of a half-strength potato dextrose broth (PDB) containing 10⁴ spores of *Trichoderma* per ml. After incubation at 25°C overnight, the old medium was replaced with 0.5 ml of a half-strength PDB containing 1 μmol/ml of FITC-conjugated chitinases. After incubation for 15 min at room temperature in the dark, fungal hyphae attached to the cover glasses were washed three times with PDB. The attachment of the labeled chitinase to the fungal material was observed under a fluorescence microscope (fitted with BP 460–490, DM 505, and BA 515F filters).

**Cell-wall column chromatography.** *Trichoderma* sp. was cultured at 30°C for 2 days in a flask containing a liquid medium consisting of 1.0% glucose, 0.75% peptone, 0.5% KH₂PO₄, 0.5% KCl, and 0.5% MgSO₄·7H₂O. The cell-wall fraction was prepared from these mycelia as described by Fukamizo et al. Ion exchange-like cell-wall column chromatography of rye seed chitinases and its domains was done at pH 6.0, 6.5, 7.0, and 7.5. After dialysis against 20 mM sodium phosphate buffer, samples were put on a cell-wall column (0.5×5 cm) previously equilibrated with the same buffer. The protein was eluted by a linear gradient of NaCl from 0 to 0.5 M in the same buffer at a flow rate of 0.5 ml/min. Hydrophobic-like cell-wall column chromatography of rye seed chitinases and its domains was done as follows. After dialysis against 20 mM sodium phosphate buffer, pH 6.0, containing 0.15 M NaCl, these proteins were put on a cell-wall column (0.5×5 cm) previously equilibrated with the same buffer. The protein was eluted by a linear gradient of NaCl from 0.15 to 0 M in the same buffer at a flow rate of 0.5 ml/min.

**Assay for the cell-wall degradation activity.** The cell-wall degradation activity was assayed colorimetrically by using fungal cell wall derived from *Trichoderma* sp. as a substrate. Ten μl of the sample solution was added to 500 μl of a 0.25% (w/v) cell-wall solution in a 50 mM sodium acetate buffer at pH 6.0. After incubating at 37°C for the indicated times, the reducing power of the reaction mixture was measured with the ferriferoxyanide reagent by the method of Imoto and Yagishita.

**Results**

The *hyphal-extension inhibition assay* RSC-a, RSC-c, and the Cat domain inhibited hyphal extensions of *Fusarium oxysporum*, *Rhizoctonia solani*, and *Trichoderma* sp. but the CB domain did not at 30 pmol (Fig. 1). An addition of the CB domain to the Cat domain did not strengthen the inhibitory ability of the Cat domain (Fig. 1, well 6). RSC-a, RSC-c, and the Cat domain had similar inhibition zones with three fungi. Since the inhibition line was clear and hyphal growth is fast, subsequent experiments were done by using *Trichoderma* sp. as a test fungus.
Fig. 1. The Antifungal Activity of Rye Seed Chitinases and Its Domains against Three Fungi.

The antifungal activity assessed by the hyphal-extension inhibition assay was done as described in the "Materials and Methods" section. Inoculated with *F. oxysporum* (A), *R. solani* (B), and *Trichoderma* sp. (C). The wells contained 10 μl of the following solutions: 1, blank (sterile water); 2, 30 pmol of RSC-a; 3, 30 pmol of RSC-c; 4, 30 pmol of the Cat domain; 5, 30 pmol of the CB domain; 6, 30 pmol of the Cat domain with 30 pmol of the CB domain.

Fig. 2. Hyphae Affected by Rye Seed Chitinases and Its Domains.

Observation of hyphae affected by rye seed chitinases and its domains was done as described in the "Materials and Methods" section. Hyphae affected by RSC-a (A), RSC-c (B), Cat domain (C), CB domain (D). Bar = 25 μm.

Observation of hyphae with light microscope

Hyphae affected by rye seed chitinases and its domains were observed with a light microscope. Hyphae exposed to the CB domain were found to grow normally as well as that exposed to sterile water (Fig. 2D). Hyphae affected by RSC-a, RSC-c, and the Cat domain were markedly reduced in size and highly branched. Hyphae affected by RSC-a were more branched and in a higher density than that affected by RSC-c or the Cat domain. In addition, hyphal tips affected by RSC-a made a smooth edge while those by RSC-c and the Cat domain made a rough edge. (compare Fig. 2A with 2B and 2C). These observations suggested that the manners of action of RSC-a and RSC-c on fungal hyphae were different from each other.

The hyphal re-extension inhibition assay

Inhibition of hyphal re-extension from the old hyphae, consisting of mature cell walls, was examined with rye seed chitinases and its domains (Fig. 3). Re-extension from the old hyphae was not observed until about 5 hours of incubation with all samples. The re-extension was observed after 6 hours of incubation with the sterile water (control) and CB domain, and at later hours of incubation with other samples. At 12 hours of incubation, the IC₅₀ values of RSC-a, RSC-c, and the Cat domain were calculat-
ed to be 9, 60, and 18 pmol, respectively. Hyphae affected by RSC-a were extended at a high density and made a smooth edge while those by RSC-c and the Cat domain made rough edges. The CB domain did not inhibit hyphal re-extension. These results indicated that RSC-a inhibited more effectively the hyphal re-extension from old hyphae than RSC-c.

**Binding sites of rye seed chitinases on hyphae**

To investigate the binding sites of rye seed chitinases on hyphae, FITC labeled RSC-a, RSC-c, Cat domain, or CB domain (FITC-RSC-a, FITC-RSC-c, FITC-Cat, or FITC-CB) were used for the binding study (Fig. 4). FITC-RSC-a and FITC-CB were found in hyphal tips, lateral cell walls, and septa even though the signal with FITC-CB was weak (Figs. 4A to 4D and 40 to 4R). On the other hand, FITC-RSC-c was found only in the hyphal tip (Figs. 4E to 4H). FITC-Cat was found located mainly in the hyphal tip and infrequently in spore or lateral cell walls (Figs. 4I to 4L or 4M and 4N). Since lateral walls and septa should contain mainly mature cell walls, these results indicated that RSC-a could bind to mature cell walls.

**Effects of ionic strength and pH on antifungal activity**

By addition of 0.05 M NaCl to PDA, an antifungal activity of RSC-a, RSC-c, or the Cat domain became stronger (compare Fig. 5A with 5B). By addition of over 0.1 M NaCl, the antifungal activity of RSC-c or the Cat domain became weaker with increasing concentrations of NaCl, while that of RSC-a did not change (Fig. 5C to 5E). The antifungal activity of RSC-c or the Cat domain became weaker with increasing pH (Fig. 5F to 5I). The antifungal activity of RSC-a was not altered below pH 6.5, and at over pH 7.0 that of RSC-a became slightly weaker with increasing pH. Besides, the CB domain or the mutant of RSC-c, possessing no chitiniolytic activity, made a very narrow inhibition zone at over 0.1 M NaCl (Figs. 5C to 5E, wells 5 and 6). These results indicated that ionic strength and pH influenced the antifungal activity of rye seed chitinases and the effect was strong in the case of RSC-c and the Cat domain. The results at high ionic strength suggested a contribution of the CB domain in RSC-a to the antifungal activity of RSC-a.

**Cell-wall column chromatography**

To investigate the affinity of rye seed chitinases and its domain for the cell walls of *Trichoderma* sp., chromatography using a column packed with cell
walls prepared from its mycelium was devised. The chromatography was done at several pHs and ionic strengths (Figs. 6A to D and E). When at pH 6.0 and low ionic strength, RSC-a, RSC-c, and the Cat domain were adsorbed to the column. RSC-c and the Cat domain were eluted with about 0.15 M NaCl while RSC-a was eluted with over 0.2 M NaCl (Fig. 6A). On the other hand, the CB domain was not adsorbed to the column at pH 6 to 7.5 under low ionic strength (Fig. 6A to D). Over pH 7.0, RSC-c completely passed through the column, while the Cat domain was partially adsorbed and the retention time of RSC-a was slightly faster than that under pH 6.5 (Fig. 6C). At high ionic strength, RSC-c and the Cat domain rapidly passed through the column without adsorption, while RSC-a and the CB domain were adsorbed to the column and then were gradually eluted (Fig. 6E). These results indicated that ionic and/or hydrophobic interactions contributed to the binding of chitinases and its domains to the fungal cell walls. In addition, the result at high ionic strength strongly supported the contribution of the CB domain to binding ability of RSC-a.

**Cell-wall degradation**

RSC-a, RSC-c, and the Cat domain could degrade the isolated cell walls of *Trichoderma* sp. (Fig. 7).

For the first 2 h, they liberated the reducing sugar at a similar rate, but the release of reducing sugar by RSC-c and the Cat domain stopped as if there was no available substrate. At 24 hours of incubation, the amounts of reducing sugars liberated by RSC-c and the Cat domain were about 60% and 50%, respectively, to that of reducing sugars liberated by RSC-a. A new addition of RSC-a to the reaction mixture, the cell walls and RSC-c after incubating for 12 h (Fig. 7,
Antifungal Activity of Rye Seed Chitinases

Chitinase is considered to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of many fungi. To have antifungal activity, first, a chitinase must bind to fungal cell walls, and second, it must degrade the chitin in it. However, no report of a binding manner of plant chitinase to the fungal cell walls has been found. Plant class I chitinase has at least two binding parts, the N-terminal chitin-binding domain and the catalytic domain, while class II has only one in the catalytic domain. It is not clear whether the CB domain takes part in or reinforces the antifungal activity of class I chitinase.

An antifungal activity of class I and class II chitinases had been demonstrated by using some bioassays. Basic chitinase C (class II) derived from barley seed inhibited the growth of the fungus *Trichoderma viride* as well as basic chitinase T (class I) did. Tobacco basic class I chitinase inhibited growth of *Fusarium solani*, but acidic class II chitinase did not by itself. CHN A (basic class I chitinase derived from tobacco) and its mutant, ΔC CHN (CHN A without chitin-binding domain), were capable of inhibiting growth of *T. viride*, although CHN A had been about five times more effective than ΔC CHN. These results may suggest that a charge of protein and an existence of the CB domain are involved in ability for the antifungal activity of chitinase.

During apical growth in filamentous fungi, chitin and β-glucan fibers are synthesized simultaneously in the tip of the growing hypha. In the fungal mature cell walls, at a distant part from the hyphal tip, the polysaccharides are cross-linked to form mixed chitin-glucan fibers and may be overlaid by other polysaccharides and protein layers. These findings support an idea as follows. At the hyphal tip, the exposed nascent chitin chains are only accessible to hydrolysis by chitinase, whereas the chitin layer in the mature cell walls is inaccessible to degradation by the enzyme.

In our previous paper, we reported that the actions of RSC-a and RSC-c on hyphae were different. In the linear hyphal-extension inhibition assay, the forms of inhibition zone made by RSC-a and RSC-c were different from each other. In the hyphae passing-through slit assay, RSC-a more persistently inhibited the hyphal growth than RSC-c, in addition, the hyphae affected by RSC-a were abnormally extended, while those by RSC-c were normally extended. These results raised a hypothesis as follows. RSC-a may have higher affinity for the mature cell walls and may better hydrolyze mature chitin fiber than RSC-c does. The CB domain of class I chitinase may have affinity for this putative action site in fungal cell walls. Consequently, mature cell walls distant from the hyphal tip are degraded by RSC-a but not by RSC-c and the hyphal growth is persistently inhibited.

In this study, an observation of the hyphae with a light microscope suggested that the actions of RSC-a and RSC-c on the hypha were different. FITC-labeled RSC-a bound to the hyphal tips, lateral walls, and septa but FITC-labeled RSC-c bound only to the hyphal tip. The results in the degradation assay indicated indirectly that RSC-a could degrade nascent chitin and mature chitin fiber, but on the other hand, RSC-c could degrade only nascent chitin. The results in the cell-wall column chromatography indicated that RSC-a had greater affinity for the fungal cell walls than RSC-c. The results in the hyphal re-extension inhibition assay implied that RSC-a acted more effectively on the mature cell walls than RSC-c did, since RSC-a and RSC-c should act on newly re-extended hyphae to similar extent, hence, inhibition of hyphal re-extension by chitinases should be mainly dependent on their action on mature cell wall of old hyphae. All these results strongly supported the

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**Fig. 7.** Hydrolysis of the Cell Wall Prepared from Mycelia of *Trichoderma* sp.

Assay of cell-wall degradation activity was done as described in the "Materials and Methods" section. Five hundred μl of reaction mixtures containing 0.25% of the cell walls and 100 pmol of RSC-a (○), RSC-c (●), or the Cat domain (△) were incubated at 37°C and the amounts of reducing power were measured. △ and ○ indicate reducing powers after the new addition of RSC-a and RSC-c, respectively, at 12 h (an arrow).
hypothesis described above.

The mutant protein of 26-kDa chitinase (basic class II) of barley, with no chitinolytic activity, still had an antifungal activity at a low level.\(^\text{19}\) The mutant protein of basic class I chitinase (derived from chestnut seed) that lost chitinolytic activity had antifungal activity similar to a wild-type chitinase.\(^\text{20}\) These findings may suggest that basic chitinase protein has antifungal activity only by its binding ability of the CB domain and/or the Cat domain to the fungal cell wall. In this study, the CB domain slightly inhibited hyphal extension only under high ionic strength conditions. This observation seems to be related to the binding ability of the CB domain at high ionic strength. The binding of the carbohydrate-binding module (CBM17) from *Clostridium cellulovorans* cellulase 5A to cellobiose and cellobiohexaose was increased by the presence of NaCl and the participation of tryptophan residues in ligand binding was indicated.\(^\text{21}\) For example, in hevein, some hydrophobic amino acids were considered to contribute to a sugar binding. Facts\(^\text{22,23}\) that hydrophobic amino acids, such as Pro13, Leu16, Phe21, Trp23, and Tyr30, were conserved in the CB domain of RSC-a suggest that the binding ability of CB domain may be due to a hydrophobic interaction through hydrophobic amino acids.

Many antimicrobial peptides such as *Ac*-AMP (Antimicrobial peptide derived from *Amaranthus caudatus* seeds), a hevein-like chitin-binding peptide, have high basicity, and basicity of these peptides seem to be important for antifungal activity. An antifungal activity of *Ac*-AMP is reduced at higher ionic strength.\(^\text{24}\) The isoelectric points of RSC-a and RSC-c are 9.7 and over 10, respectively, and that of the Cat domain is higher than that of RSC-c, while the CB domain is acidic (theoretical pH is 4.0).\(^\text{4,5}\) The Cat domain more effectively acted on the old hyphae than RSC-c did and a FITC-labeled Cat domain was partially found in the lateral walls or spores. The Cat domain was partially adsorbed onto the cell-wall column at over pH 7.0, while RSC-c completely passed through and in high ionic strength conditions, the Cat domain behaved similarly to RSC-c (Fig. 5 and Fig. 6). It is speculated that this behavior of the Cat domain might be due to its charge. A high basicity of the Cat domain might cause non-specific binding to the fungal cell walls.

Overall, the antifungal potency of the CB domain by itself was very low, and there is no doubt that the antifungal activity of chitinases is based on their chitinolytic activity even though an RSC-c mutant displaying no catalytic activity had a very low level of antifungal activity. In addition, the result at high ionic strength supported strongly the contribution of the CB domain in RSC-a to binding ability and to antifungal activity of RSC-a.

In summary, our results suggested the following:

First, basic class I chitinase bound to hyphal tips and lateral walls and septa, consisting of mature cell walls, by mainly ionic interaction of the Cat domain and by hydrophobic interaction of CB domain, and second, degraded mature chitin fibers as well as nascent chitin by its hydrolytic action. On the other hand, basic class II chitinase bound only to the hyphal tip by mainly ionic interaction by itself, followed by degradation of only nascent chitin. As a result, the basic class I chitinase more effectively inhibited fungal growth than basic class II chitinase did.

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**References**

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