Molecular Cloning, Functional Expression, and Mutagenesis of cDNA Encoding Rye (Secale cereale) Seed Chitinase-c

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We cloned a complete cDNA encoding rye seed chitinase-c, designated RSC-c, by rapid amplification of cDNA end and PCR procedures. The cDNA of RSC-c consists of 1,018 nucleotides and includes an open reading frame encoding a polypeptide of 266 amino acid residues. A recombinant RSC-c was produced by expression in Escherichia coli Origami(DE3) and purified. rRSC-c had almost the same chitinase activity toward glycochitin and antifungal activity against Trichoderma sp. as the authentic RSC-c did. RSC-c mutants were subsequently constructed and characterized with respect to their chitinase and antifungal activities. Mutation of Glu67 to Gln completely abolished the chitinase activity and diminished the antifungal activity. Considerable decreases in both activities were observed in the mutants of Trp72 and Ser120 to Ala, and Glu89 to Gln. The roles of these residues in the catalytic event of RSC-c are discussed.

Key words: rye seed chitinase; cDNA cloning; expression; mutagenesis; antifungal activity

Plant chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, the β-1,4-linked homopolymer or oligomer of GlcNAc, and have been implicated in the defense reactions of plants against fungal and bacterial infection. A large number of cDNA clones encoding plant chitinases have been isolated and characterized. These have been classified into two different families, families 18 and 19, in the classification system of glycosyl hydrolases, based on the amino acid sequence similarity of their catalytic domains. Family 18 includes plant class III and V chitinases, and family 19 includes plant class I, II, and IV chitinases. In these plant chitinases, the crystal structure of 26-kDa chitinase from barley (Hordeum vulgare L.) seeds, which belongs to family 19, was first solved. After that, the catalytic mechanism and substrate binding mode of barley chitinase were studied using molecular dynamics simulations based on the crystal structure and on splitting mode analyses using GlcNAc and pNP-GlcNAc as the substrate. However, there are few reports of studies that give the experimental evidence for the roles of individual amino acid residues on the chitinase and antifungal activities of family 19 chitinases.

We earlier have reported the purification, characterization, and amino acid sequences of two basic chitinases (RSC-a and -c) from rye seeds, and RSC-a is a class I chitinase consisting of a chitin-binding domain and a catalytic domain connected by a flexible linker, while RSC-c is a class II chitinase with 91.8% sequence identity to the catalytic domain (Gly60-Ala 302) of RSC-a. Furthermore, we suggested that Trp72 was involved in the substrate binding and Asp95 was located at or near the substrate binding site in RSC-c by chemical modification studies.

In the study reported here, in order to elucidate the structural bases for the chitinase and antifungal activities of RSC-c, we cloned the cDNA encoding RSC-c from rye seeds by means of RACE and PCR procedures and expressed it in a functional form in E. coli cells. Subsequently, several RSC-c mutants were constructed and characterized.

Materials and Methods

Materials. Rye (Secale cereale) seeds, which take about 40 days after flowering (DAF) to mature, were harvested 28 DAF in May 1999 in Fukuoka, Japan. The seeds were frozen in liquid nitrogen and stored at −80°C until used. The oligonucleotides used in this

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The nucleotide sequence reported here is available in the DDBJ, EMBL, GenBank Nucleotide Sequences Database under the accession number ABO51579

Abbreviations: RSC-c, rye seed chitinase-c; pNP-GlcNAc, p-nitrophenyl-penta-N-acetylchitopentaoside; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; ORF, open reading frame; IPTG, isopropyl-β-D-thiogalactopyranoside; rRSC-c, recombinant RSC-c; CD, circular dichroism
study were purchased from Amersham Pharmacia Biotech. Restriction endonucleases and DNA modifying enzymes were purchased from either Takara Shuzo or New England Biolabs. The pGEM-T Easy vector was purchased from Promega. Expression vector, pET-22b, and *E. coli* Origami(DE3) were purchased from Novagen. *Pfu Turbo* DNA polymerase was obtained from Stratagene. Nontronit cellulose membranes were from Schleicher & Schull. All other chemicals were of analytical grade for biochemical use. Antiserum against RSC-c was prepared by immunization of a rabbit according to the method described by Taira et al. \(^\text{16}\)

Chitinase activity. Chitinase activity was assayed colorimetrically with glycolchitin as a substrate. Ten microliters of the sample solution was added to 500 μl of 0.2% (w/v) glycolchitin solution in 0.1 M sodium acetate buffer, pH 5.0. After incubation of the reaction mixture at 37°C for 15 min, the reducing power of the mixture was measured with ferri ferrocyanide reagent by the method of Imoto and Yagishita. \(^\text{17}\) One unit of activity was defined as the enzyme activity that produced 1 μmol of GlicNAc per minute at 37°C. Protein concentrations were measured by the bicinchoninic acid method, \(^\text{19}\) using bovine serum albumin as a standard protein.

**SDS-PAGE and Western blotting.** Overproduction of rRSC-c in *E. coli* cells were analyzed by SDS-PAGE \(^\text{19}\) using 15% polyacrylamide gels and Western blotting. \(^\text{20}\) In the Western blotting, the nitrocellulose filter was probed with anti-RSC-c antiserum (1:5,000), developed using goat anti-rabbit IgG conjugated with horseradish peroxidase, and the immune complex was then made visible by addition of a peroxidase substrate solution, 100 mM Tris-HCl, pH 7.5, containing 0.5 mg/ml 3,3′-diaminobenzidine tetra hydrochloride, 0.3% H2O2, and 50 mM imidazole.

**RNA isolation.** Total RNA was prepared from developing rye seeds (28 DAF) by the phenol/SDS method. \(^\text{21}\) Poly(A)\(^+\) RNA was purified from the total RNA using an Oligotex-dT30 Super (Takara Shuzo).

**Cloning of RSC-c cDNA.** The 5′-terminal region of the RSC-c cDNA was amplified by the RACE procedure according to Chen. \(^\text{22}\) The gene specific primers used for the 5′-RACE were designed based on the nucleotide sequence of barley chitinase, in which the amino acid identity between RSC-c and barley chitinase is 91.8%. \(^\text{20}\) The primers, P1~P3, correspond to the nucleotide sequences for the internal amino acid sequences, Gly83-Arg90, Ala79-Gln88, and Glu27-Phe34, in barley chitinase, respectively. Starting from 300 ng poly(A)\(^+\) RNA, isolated from rye seeds (see above), the first-strand cDNA was synthesized with SuperScript II reverse transcriptase using the gene specific primer P1, 5′-GTTCCCTGGTTGAAGCAGTAGCC-3′, followed by hydrolysis of poly(A)\(^+\) RNA with RNase H. To the 1st strand cDNA purified by affinity chromatography on silica matrix, an anchor oligomer, 5′-pGTAGGAATTCCGTTGTTAGGGAGGTGCACATGCC-3′ was attached with T4 RNA ligase in buffer containing 25% PEG 8,000 at 16°C for 24 h. The reaction mixture was used for the PCR template. First PCR was done with an adaptor primer AP1, 5′-GGCAATGTTCGACCTCCCTACAAC-3′ and a gene-specific primer P2, 5′-GCCTCGAGAGCGTACGCCCCGAG-3′, and nested PCR with an adaptor primer AP2, 5′-CTCCCTACAAACCGAATTCC-3′, and a gene-specific primer P3, 5′-AAGCGTGTAGGTAGTGAAG-3′. For the 3′-RACE, the first-strand cDNA was synthesized with an oligo (dT) adapter, 5′-GGCCACCGTGTCGACTAGTACCTTTTTTTTTTTTTTTTTTTT-3′, and used as a template. The 3′-RACE was done with newly synthesized oligonucleotide from the known nucleotide sequence of RSC-c cDNA, P4, 5′-CACCCTAAGCTTACCC-3′, for the forward primer, and an adaptor primer AP3, 5′-GGCCACCGTGTCGACTAGTAC-3′. Finally, a full-length cDNA encoding RSC-c was amplified using the forward primer P4 and reverse primer P5, 5′-GATGCTTTTATTGTAGTGGT-3′, with the sequences of the 3′-RACE products. *Pfu Turbo* DNA polymerase was used for these DNA amplifications. The annealing positions of primers in RSC-c cDNA are indicated in Fig. 1(A).

**Nucleotide sequence analysis.** The nucleotide sequence was analyzed by the double-stranded dideoxy sequencing method \(^\text{24}\) on an ABI 377 DNA sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

**Expression of RSC-c in *E. coli* cells.** To obtain a cDNA fragment encoding mature RSC-c, PCR was done using the RSC-c cDNA as a template with the forward primer, 5′-GCCCATATGAGCGTGTTCC-TCCATCATCT-3′ (underlining indicates the *NdeI* recognition site) and reverse primer, 5′-GGCCGATCCATGAGCGTGTTCC-TCCATCATCT-3′ (underlining indicates the *BamHI* recognition site). The PCR products were ligated into the pGEM-T Easy vector by TA-cloning. After confirmation of the DNA sequence, the DNA fragment encoding the mature RSC-c was excised by digestion with *NdeI* and *BamHI*, and ligated into the expression vector pET-22b, \(^\text{25}\) previously digested with the same enzymes. Introduction of the resulting plasmid, pET-RSC-a, into *E. coli* Origami(DE3) and induction with 1 mM IPTG were done according to the supplier’s instruction.
Purification of rRSC-c. After induction with 1 mM IPTG, the culture was incubated for an additional 24 h at 20°C, then the cells were harvested and disrupted by sonication in 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 1 mM DTT. The sonicated extract was separated into soluble and insoluble fractions by centrifugation at 12,000 × g for 15 min at 4°C.

rRSC-c was purified from the soluble fraction using S-Sepharose column chromatography by one step. The soluble fraction was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and put on a S-Sepharose column previously equilibrated with the same buffer. The elution was done with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The fractions with chitinase activity were pooled. The N-terminal amino acid sequence of the protein was analyzed by a gas phase sequencer PSQ-1 (Shimadzu).

Construction of RSC-c mutants. Site-directed mutagenesis was done using the QuickChange site-directed mutagenesis kit (Stratagene). The nomenclature of RSC-c mutants and the oligonucleotide primers used for mutagenesis are shown in Table 1. A mutation was introduced into the amplified RSC-c cDNA fragment that had been subcloned into plasmid T-vector. After mutagenesis, the RSC-c cDNA fragment was sequenced to verify the presence of the desired mutation. The mutant fragment was then recovered and ligated into the expression vector, pET-22b. Expression and purification of all mutants were done by procedures identical to those described above.

Assay of antifungal activity. The hyphal-extension inhibition assay was done as follows. An agar disk (6 mm in diameter) with the fungus Trichoderma sp. was put in the center of a Petri dish containing potato dextrose agar (PDA) with 1.5% (w/v) agar. After incubation of the plate for 12 h at room temperature, wells were punched into the agar at a distance of 20 mm from the center of the Petri dish. The samples to be tested were placed into the wells in 10 µl of distilled water with 2 µg of purified chitinases. The plate was incubated for 30 h at room temperature and then photographed. In this manner, if the sample being tested was antifungal, a crescent-shaped area of inhibition was observed around the well.

Computer model generation. Swiss-Model, a knowledge-based protein modeling tool was used to predict the tertiary structure of RSC-c from the known X-ray structure of class II barley chitinase (Protein Data Bank entry 2BAA from the Brookhaven Protein Databank). After modeling, the entire structure was shown with the program RasMol.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligonucleotide primer*</th>
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<tbody>
<tr>
<td>E67Q</td>
<td>5'-GCAGACCTCCCAACCAACACCGGGGGG-3'</td>
</tr>
<tr>
<td>W72A</td>
<td>5'-AGACCCGCCCGCGCGCGCGCGCA-3'</td>
</tr>
<tr>
<td>E89Q</td>
<td>5'-TACTGCTTCAAGCAGCACTGGGCGCGCC-3'</td>
</tr>
<tr>
<td>D95A</td>
<td>5'-GGAGCCGCCGCGCATCTGCACCCGG-3'</td>
</tr>
<tr>
<td>S120A</td>
<td>5'-GGGCCCTACGCAGCTCGCCCAACTACAAC-3'</td>
</tr>
</tbody>
</table>

* The mutation sites are underlined.

Results

Cloning and nucleotide sequence of the RSC-c cDNA

The strategy used for the cloning of cDNA encoding RSC-c is given in Fig. 1(A). To obtain the nucleotide sequences of the 5' and 3'-terminal regions of RSC-c cDNA, 5'-RACE and 3'-RACE were done, respectively (see Materials and Methods). The cDNA fragments of about 250 bp were amplified by 5'- RACE reaction. These fragments were cloned into pGEM-T Easy by TA-cloning and sequenced. They were heterogeneous in length and their sequences slightly differed. In these fragments, the longest one encoding an amino acid sequence identical to the published protein sequence in the region encoding the mature protein was used to generate a composite full-length cDNA sequence. The cDNA fragments of about 1,030 bp were amplified by 3'-RACE reaction. They were heterogeneous in length and their sequences slightly differed, too. Therefore, the longest one was selected likewise and used. These cDNA fragments were 203 nucleotides containing the presumed translation initiation codon ATG and 988 nucleotides including a termination codon TAA and poly(A) tail, respectively. Based on the composite nucleotide sequence, a full-length cDNA fragment was then generated by designing the oligonucleotides to the 5'- and 3'-untranslated leader and tailer of the gene, respectively. Overlapping the nucleotide sequence data from the three amplified cDNA fragments revealed the complete nucleotide sequence of a full length of the cDNA encoding RSC-c.

The nucleotide sequence of the cDNA encoding RSC-c and the deduced amino acid sequence are shown in Fig. 1(B). The RSC-c cDNA consists of 1,018 nucleotides and includes an ORF encoding a polypeptide of 266 amino acid residues. Poly(A) additional signal, AATAAA, was found 91 bp upstream of a poly(A) tail. Comparison of the deduced amino acid sequence of RSC-c cDNA with the protein sequence reported previously revealed that the deduced sequence has a putative signal consisting of 23 amino acid residues in the N-terminal region and that the amino acid sequence of the deduced mature protein is identical with that found by the protein sequencing.
Expression and purification of rRSC-c

Expression and purification of rRSC-c were done as described in Materials and Methods. Total proteins from the E. coli cells harboring pET-RSC-c were analyzed by SDS-PAGE and Western blotting. As shown in Fig. 3(A), a protein of 26-kDa was detected by CBB staining after induction by IPTG (lane 3), and it reacted with anti-RSC-c antiserum (lane 6), showing that the 26-kDa protein is rRSC-c. Since the 26-kDa band of rRSC-c was detected in both the soluble and insoluble fractions of the sonicated extract of induced E. coli cells by CBB staining (data not shown), the purification of rRSC-c was done from the soluble fraction using S-Sepharose column chromatography. One symmetrical protein peak with chitinase activity eluting at 0.1 M NaCl concentration was obtained and this peak gave a single band on SDS-PAGE by CBB staining (Figs. 3(B) and 5). The yield of rRSC-c was about 20 mg/liter of induced culture.

The direct sequencing of rRSC-c gave the sequence Ser-Val-Ser-Ile-, indicating that the N-terminal methionine residue was removed. The rRSC-c degraded glycolchitin to the same extent as the authentic RSC-c did (Table 2) and the CD spectrum of rRSC-c was essentially identical to that of authentic RSC-c, suggesting that rRSC-c could be refolded into a conformation similar to that of authentic RSC-c (data not shown).

Construction and characterization of RSC-c mutants

We assumed that Glu67, Glu89, and Ser120 are catalytic residues and Trp72 and Asp95 are involved in substrate binding in RSC-c, based on the structural similarity with barley chitinase20 and our studies concerning inactivation of RSC-c by chemical modification,14,15 respectively. Therefore, five RSC-c mutants, E67Q, W72A, E89Q, D95A, and S120A, were prepared by site-directed mutagenesis, and expressed in E. coli cells. The positions of the mutated amino acid residues were indicated in a 3-D model of RSC-c (Fig. 4). The mutants, purified by the same methods as those of wild type enzyme (Fig. 5), were characterized in terms of the chitinase and antifungal activities. Mutation of Glu67 to Gln (E67Q) completely abolished the chitinase activity. The relative activities of RSC-c mutants, W72A, E89Q, D95A, and S120A, in which the amino acid residues Trp72, Glu89, Asp95, and Ser120 in RSC-c were replaced by Ala, Gln, Ala, and Ala, were 51.2, 0.33, 79.0, and 1.2% of the wild type RSC-c, respectively (Table 2). The secondary structures of each mutant were checked by examining the CD spectrum. The CD spectra of mutants in the short-wavelength region (200–250 nm) were essentially the same as that of wild type RSC-c. Therefore, the backbone conformation of the mutants seemed to be unaltered as that of...
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Fig. 2. Multiple Sequence Alignment of Deduced Amino Acid Sequences for Chitinases from Rye and Barley and Chitinase-antifreeze Protein from Rye.

The deduced amino acid sequence of cDNA encoding RSC-c was aligned with that of cCHI26 encoding barley chitinase (GenBank accession no. AF280438) and CHT46 encoding chitinase-antifreeze protein from rye (GenBank accession no. AF166989). Identical amino acid residues are highlighted on a black background and the dashes indicate gaps. The arrowheads indicate the positions chosen for site-directed mutagenesis.

Fig. 3. SDS-PAGE and Western Blotting of Total Proteins from E. coli Cells Harboring pET-RSC-c (A) and Purification of rRSC-c (B).

(A) The total proteins from E. coli cells harboring pET-RSC-c were separated by SDS-PAGE and stained with Comassie brilliant blue R-250 (lanes 1, 2, and 3). SDS-PAGE gels loaded with the same samples as lanes 1-3, were blotted and made visible in the manner described in Materials and Methods (lanes 4, 5, and 6). M, maker; lanes 1 and 4, RSC-c; 2 and 5, before induction; 3 and 6, after induction by IPTG. Marker proteins used were bovine serum albumin (66,700), egg white albumin (45,000), carbonic anhydrase (29,000), and myoglobin (17,200). (B) The soluble fraction was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and put on a S-Sepharose column previously equilibrated with the same buffer. The elution was done with a linear gradient of NaCl from 0 to 0.3 M in the same buffer.

Wild type RSC-c (data not shown). These results suggested that Glu67, Glu89, and Ser120 are of critical importance to the chitinase activity, but Trp72 and Asp95 are not.

rRSC-c showed the antifungal activity to the same extent as the authentic RSC-c did against Trichoderma sp. as judged from the size of the crescent-shaped inhibition areas. Although the five mutants also had antifungal activities, the inhibitory areas were decreased in proportion to the reduction of chitinase activities (Fig. 6).

Discussion

Our aim in this study was to investigate the roles of individual amino acid residues for the chitinase and
antifungal activities of RSC-c. For this purpose, we first isolated a cDNA encoding RSC-c. The cloned RSC-c cDNA consists of 1,018 nucleotides and includes an ORF encoding a polypeptide of 266 amino acid residues. The N-terminal 23 amino acid sequence is very similar to an eukaryotic signal sequence for endoplasmic reticulum targeting. It is known that tobacco class I chitinase has a C-terminal extra peptide which is necessary and sufficient for the vacuolar localization of this protein. A C-terminal extra peptide was not found in the deduced amino acid sequence of RSC-c cDNA. The localization of RSC-c in its cells is still unclear.

When we used *E. coli* BL21(DE3) cells as a host for expression of rRSC-c, the expressed protein was produced entirely as an insoluble aggregate. Since rRSC-c has three disulfide bonds, as reported previously, rRSC-c might be formed as an insoluble aggregate because of failure of the correct disulfide bonds formation in *E. coli* BL21(DE3) cells. Therefore, *E. coli* Origami(DE3) was chosen as a host for expression of rRSC-c and its mutants. This strain has mutations in both the thioredoxin reductase and glutathione reductase genes, which greatly increases the disulfide bond formation of protein expressed in the cytoplasm. Purified rRSC-c had almost the same chitinase activity as that of authentic RSC-c and there was no difference between the CD spectra of the authentic RSC-c and rRSC-c (data not shown). We succeeded in expressing rRSC-c in a functional form in *E. coli* cells with the yield about 20 mg/liter.

### Table 2. Specific Activities of RSC-c, rRSC-c, and Its Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Specific activity (units/mg)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSC-c</td>
<td>205.0</td>
<td>87.7</td>
</tr>
<tr>
<td>rRSC-c</td>
<td>233.7</td>
<td>100.0</td>
</tr>
<tr>
<td>E67Q</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td>W72A</td>
<td>119.7</td>
<td>51.2</td>
</tr>
<tr>
<td>E89Q</td>
<td>0.78</td>
<td>0.33</td>
</tr>
<tr>
<td>D95A</td>
<td>184.7</td>
<td>79.0</td>
</tr>
<tr>
<td>S120A</td>
<td>2.86</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Chitinase activity was measured as described in Materials and Methods. N.D., no detectable activity.
of induced culture.

The anomer form analyses of hydrolytic products\(^{30}\) and the molecular dynamics simulations of barley chitinase\(^{10}\) suggested that the catalytic reaction of family 19 chitinase is taken place through a single displacement inverting mechanism. According to this mechanism in barley chitinase, Glu67 acts as proton donor for the anomeric oxygen linking sugar residues and Glu89 and Ser120 coordinate with a water molecule that acts as the nucleophile, attacking the C1 atom of the carbonium ion intermediate from the α-site to complete the reaction. Glu89 also assists in the reaction by moving towards the substrate to stabilize the intermediate. Based on barley chitinase as a paradigm, site-directed mutagenesis was done, in which Glu67, Glu89, and Ser120 were possible candidates for amino acid residues directly involved in catalysis of RSC-c. Mutation of Glu67 to Gln completely abolished, and that of Glu89 drastically reduced the chitinase activity (0.33%), compared with that of wild type RSC-c. These results were in good agreement with those of site-directed mutagenesis studies of barley chitinase.\(^{9}\) From these results, it is therefore likely that Glu 67 and Glu89 in RSC-c serve as a general acid catalyst and base catalyst, respectively. Mutation of Ser120 of RSC-c to Ala also greatly reduced the chitinase activity (1.2%), indicating that the hydroxyl group of Ser120 is essential for effective catalysis of RSC-c. Recently, a water molecule held by hydrogen bonds to the carboxyl group of Glu90 and the hydroxyl group of Thr119 (these residues are equivalent to Glu89 and Ser120 in RSC-c) was found in the crystal structure of jack bean chitinase.\(^{29}\)

We earlier examined the effects of chemical modification with N-bromosuccinimide (NBS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-glycine ethyl ester (GEE) on the chitinase activity of RSC-c.\(^{14,15}\) In these experiments, RSC-c became in virtually inactive by the modifications of Trp72 and Asp95, respectively. Therefore, these residues were selected for site-directed mutagenesis. Mutation of Trp72 to Ala reduced the specific activity (51.2%) and increased the $K_m$ (more than 550%) for glycolchitin compared with that of wild type RSC-c (data not shown). These results suggest that Trp72 is involved in the substrate binding in RSC-c. In the case of mutation of Trp72 to Ala, the chitinase activity was not decreased to the extent of that of the Trp72-oxidized RSC-c derivative. Hence, inactivation of the Trp72-oxidized RSC-c derivative might be a consequence of the obstruction on the catalytic cleft by the oxindolealanine residue, preventing RSC-c from productively binding to the substrate. Mutation of Asp95 to Ala had little effect on the chitinase activity. Therefore, it was inferred that the reduction of chitinase activity of RSC-c by the modification of Asp95 with EDC-GEE might be the result of interference of the glycyl residue attached to Asp95 with the binding of substrate to the catalytic cleft.

Yeh et al. suggested that the rye genome contains at least three genes closely related to CHT46 encoding chitinase-antifreeze protein (CHT46-AFP) cloned from cold-acclimated winter rye leaf, by southern blotting.\(^{30}\) In fact, the existence of three kinds of class II chitinases in rye have been so far reported: RSC-c, CHT46-AFP, and a pathogen-induced chitinase lacking an antifreeze activity.\(^{39}\) Although it is unclear whether or not RSC-c has an antifreeze activity, it would be very interesting to investigate the structure-function relationships and physiological roles in rye of these proteins.

This study showed that rRSC-c, which had almost the same chitinase and antifungal activities as the authentic RSC-c did, could be expressed in E. coli cells and purified easily using S-Sepharose column chromatography by one step. Furthermore, we indicated that Glu67, Glu89, and Ser120 of RSC-c are amino acid residues directly involved in the catalysis and Trp72 in the substrate binding and that the antifungal activity of RSC-c depends on mainly its chitinase activity. More valuable information on the roles of individual amino acid residues in the active site of RSC-c should be provided by the investigations of substrate binding subsites based on oligosaccharide digestion experiments and X-ray crystal structures of inactive mutants, such as E67Q RSC-c mutant, complexed with their substrates.

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References

(1993).