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Purification of Two Chitinases from *Rhizopus oligosporus* and Isolation and Sequencing of the Encoding Genes

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Two chitinases were purified from *Rhizopus oligosporus*, a filamentous fungus belonging to the class *Zygomycetes*, and designated chitinase I and chitinase II. Their N-terminal amino acid sequences were determined, and two synthetic oligonucleotide probes corresponding to these amino acid sequences were synthesized. Southern blot analyses of the total genomic DNA from *R. oligosporus* with these oligonucleotides as probes indicated that one of the two genes encoding these two chitinases was contained in a 2.9-kb *Eco*RI fragment and in a 3.6-kb *Hin*dIII fragment and that the other one was contained in a 2.9-kb *Eco*RI fragment and in a 11.5-kb *Hin*dIII fragment. Two DNA fragments were isolated from the phage bank of *R. oligosporus* genomic DNA with the synthetic oligonucleotides as probes. The restriction enzyme analyses of these fragments coincided with the Southern blot analyses described above and the amino acid sequences deduced from their nucleotide sequences contained those identical to the determined N-terminal amino acid sequences of the purified chitinases, indicating that each of these fragments contained a gene encoding chitinase (designated *chi1* and *chi2*, encoding chitinase I and II, respectively). The deduced amino acid sequences of these two genes had domain structures similar to that of the published sequence of chitinase of *Saccharomyces cerevisiae*, except that they had an additional C-terminal domain. Furthermore, there were significant differences between the molecular weights experimentally determined with the two purified enzymes and those deduced from the nucleotide sequences for both genes. Analyses of the N- and C-terminal amino acid sequences of both chitinases and comparison of them with the amino acid sequences deduced from the nucleotide sequences revealed posttranslational processing not only at the N-terminal signal sequences but also at the C-terminal domains. It is concluded that these chitinases are synthesized with pre- and prosequences in addition to the mature enzyme sequences and that the prosequences are located at the C termini.

Chitinases (EC 3.2.1.14), which hydrolyze β-1,4-glucosidic bonds of chitin, are widely distributed in the biological world. Bacteria utilize them for assimilation of chitin as a carbon source (10, 21), and plants have them probably for defense against fungal infections (4, 16, 19, 26). Fungi, in which chitin is often one of the major cell wall components, also have chitinases, and it has long been suspected that chitinase activity is involved in apical growth and branching of fungal hyphae (2, 3).

Recent experiments, in which chitinase activity was suppressed by specific inhibitors or by chitinase gene disruption, showed that chitinase is required for cell separation during growth of the yeast *Saccharomyces cerevisiae* (13, 23). For filamentous fungi, however, there are only a limited number of reports about chitinases, such as a cell wall-bound chitinase of *Aspergillus nidulans* (20) and a membrane-bound chitinase of *Mucor mucedo* (9). Their roles in hyphal growth and regulation to which they are subject are almost totally unknown. Here we describe primary structures of two chitinases from *Rhizopus oligosporus*, which supposedly contains large amounts of chitinases, as the cells are autolyzed during the late stage of growth on a solid medium or on the surface of a liquid medium. This is the first report describing the structures of filamentous fungal chitinases which may have morphogenetic roles in hyphal growth.

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MATERIALS AND METHODS

Strains and culture conditions. Chitinases were purified from *R. oligosporus* Saito IFO 8631. A genomic DNA library of *R. oligosporus* was constructed in λ2001 vector (11) and propagated in *Escherichia coli* PLK-17 (*lac mcrA mcrB hadR gal supE*). *E. coli* MV1190 [Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*) *306*::Tn*10* Tet^r (F′ *traD36 proAB lacI^{q}ZΔM15*)] was used as a host for plasmid construction and CJ236 {*dut ung thi relA* (pCJ105 [Cm^r])} was used as a host for the preparation of uracil-containing single-stranded DNA. *R. oligosporus* was grown and maintained in SIV medium (28).

Enzyme and protein assay. Chitinase activity was assayed as follows. The reaction mixture that contained 250 μl of colloidal chitin (25), 250 μl of McIlvaine's buffer (pH 4.0), and 500 μl of enzyme solution was incubated for 2 h at 37°C. After centrifugation, 500 μl from the supernatant fluid was mixed with 100 μl of 0.8 M boric acid, the pH of which had been adjusted to 10.2 with KOH. The test tube was heated for 3 min in boiling water. After the mixture had cooled, 3 ml of *p*-dimethylaminobenzaldehyde (DMAB) solution (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% [vol/vol] hydrochloric acid) was added, and the mixture was incubated for 20 min at 37°C. A_{585} was measured against water. One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 μmol of *N*-acetylglucosamine per min under the above condition.

Protein concentration was determined by using bicinchoninic acid protein assay reagent (Pierce Chemical Co.) with bovine serum albumin as a standard.

Gel electrophoresis. Sodium dodecyl sulfate-polyacryl-

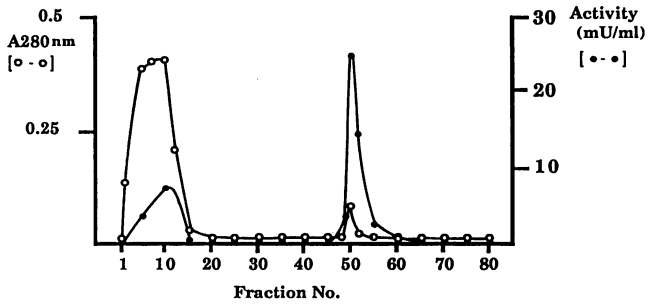

FIG. 1. Elution profile of chitinolytic activity in a column of regenerated chitin. Fractions of 10 ml were collected, A_{280} was determined (left), and chitinolytic activity was assayed (right).

amide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (14). Proteins were stained with Coomassie brilliant blue R250 or silver salts (18).

Purification of chitinases. Unless otherwise stated, the following steps were done at 4°C.

(i) **Step 1.** *R. oligosporus* IFO8631 was cultivated in six 5-liter Erlenmeyer flasks, each containing 80 ml of SIV medium, without shaking at 30°C. After 8 days, culture medium and surface-grown mycelial cells were mixed and filtered through Toyo no. 2 paper (Toyo Roshi Co., Tokyo, Japan) on a Büchner funnel. Residual materials were washed with 150 ml of ice-cold water.

(ii) **Step 2.** To the culture filtrate from step 1, solid ammonium sulfate (603 g/liter) was added with stirring to give 90% saturation. After the mixture was allowed to stand overnight, precipitate was collected by centrifugation (10,000 × *g*, 30 min) and dissolved in 40 ml of 20 mM sodium bicarbonate buffer (pH 8.4). The solution was dialyzed against the same buffer for a day and then centrifuged at 15,000 × *g* for 20 min. The precipitates were discarded.

(iii) **Step 3.** The supernatant was put on a regenerated chitin column (2.6 by 5 cm) which had been equilibrated with 20 mM sodium bicarbonate buffer (pH 8.4) (17). After being washed with the same buffer, the column was eluted with 200 ml of pH gradient (pH 5.4 to 3.2) in 20 mM sodium acetate buffer and then with 200 ml of 20 mM acetic acid (pH 3.2) at a flow rate of 52 ml/h. Fractions of 10 ml were collected. The enzyme activity appeared at flow-through fractions and the fractions that were eluted with 20 mM acetic acid (Fig. 1). The latter fractions (no. 50 to 69) were pooled, adjusted to pH 7.5 with a mixture containing 1 M Tris and 2 M NaOH, and then concentrated by using an ultrafiltration membrane (Amicon YM-10).

(iv) **Step 4.** The concentrated enzyme solution was put on a Sephadex G-75 column (2.6 by 66 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with the same buffer, at a flow rate of 15 ml/h. Fractions of 10 ml were collected. Fractions (no. 14 to 18) were pooled (Fig. 2).

(v) **Step 5.** The enzyme solution was put on a DEAE-Toyopearl 650M column (1.6 by 16 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer and then with a 600-ml linear salt gradient (0 to 30 mM NaCl) in the starting buffer, at a flow rate of 15 ml/h. Fractions of 10 ml were collected.

N-terminal amino acid sequences. Purified chitinase (25 μg), after being dialyzed against water, was subjected to automated Edman degradation with a model 477A protein sequencer equipped with a model 120A phenylthiohydantoin analyzer (Applied Biosystems).

C-terminal amino acid analysis. The C-terminal amino acid sequence was determined by carboxypeptidase digestion. Purified chitinase (250 μg) dissolved in 120 μl of 50 mM trimethylamine acetate buffer (pH 8.0) was incubated with carboxypeptidase A and carboxypeptidase B under the conditions described by Watanabe et al. (31). Aliquots (20 μl) withdrawn at intervals were dried in a Pico-Tag work station, and the released amino acids were analyzed after being converted to phenylthiocarbamoyl-amino acid derivatives.

Deglycosylation of chitinase with endoglycosidase H and trifluoromethane-sulfonic acid (TFMS). Purified chitinase I (5 μg) denatured by being boiled in SDS solution was treated with 20 mU of endoglycosidase U (Seikagaku Kogyo Co. Ltd) per ml, according to the manufacturer's instructions. Treatment with TFMS and recovery of protein from the reaction mixture were done according to the method of Edge

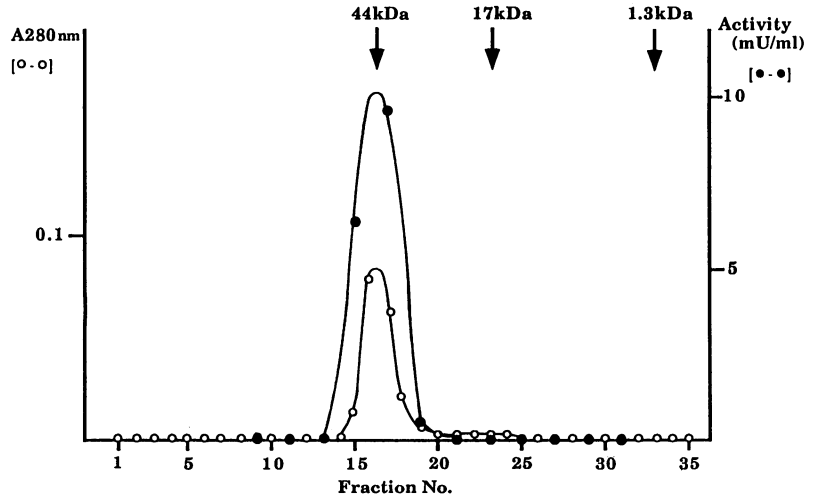

FIG. 2. Elution profile of chitinolytic activity in a column of Sephadex G-75. Ovalbumin, myoglobulin, and vitamin B-12 were used as molecular weight markers. See the legend to Fig. 1 for fractionation and assay descriptions.

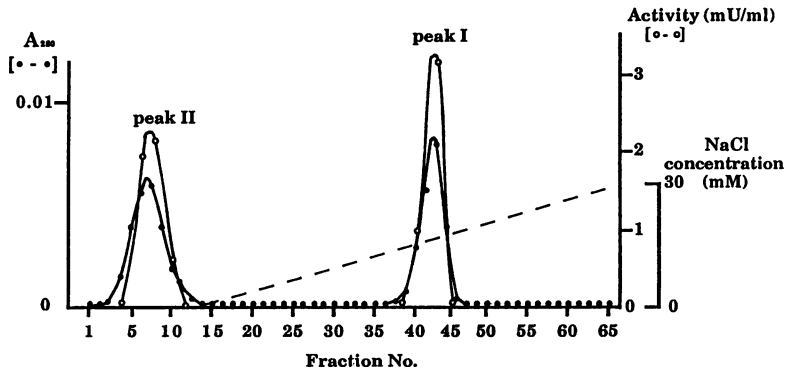

FIG. 3. Separation of chitinases by DEAE-Toyopearl 650M column chromatography. See the legend to Fig. 1 for fractionation and assay descriptions, except that the concentration of NaCl in the gradient was determined (right).

et al. (7). Chitinase (10 μg) was dissolved in 15 μl of TFMS and incubated at 0°C for 60 min. A 50-fold excess of diethyl ether containing 10% (vol/vol) of *n*-hexane was added, and the mixture was allowed to stand at −40°C. After 20 min, a drop of pyridine was added and the incubation was continued for 40 min. Protein was then pelleted by centrifugation, followed by an ether wash and recentrifugation. The resultant pellet was resuspended in ice-cold 95% ethanol, recentrifuged, and freed of remaining solvents by vacuum drying.

Construction of a genomic DNA library. *R. oligosporus* total DNA, isolated as described previously (8), was partially digested with *Sau*3AI and fractionated on an 0.8% agarose gel. Fragments of 10 to 20 kb were recovered from the gel, treated with alkaline phosphatase, and ligated to λ2001 phage DNA which had been doubly digested with *Bam*HI and *Eco*RI. The ligated DNA was packaged in vitro, and the phage particles were transfected to *E. coli* PLK-17.

DNA manipulations. Lambda phage DNA was isolated by the method of Davis et al. (6). Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (15). *E. coli* was transformed by using standard procedures (15). DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (24), with plasmid pUC119. Oligonucleotides were synthesized by the automated phosphoramidite method in a model 391 DNA synthesizer (Applied Biosystems). Recombinant DNA manipulations were done by standard methods (15).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL, and GenBank data bases under accession numbers D10157 and D10158.

TABLE 1. Purification of chitinases from *R. oligosporus*

Step	Protein (mg)	Total mU	Sp act (mU/mg of protein)	Yield (%)	Purification (fold)
Culture filtrate	378.6	4,080	10.78		
Ammonium sulfate precipitation	68.68	2,410	35.09	59.1	3.3
Chitin column	8.75	619	70.74	15.2	6.6
Sephadex G-75	2.02	268	132.67	5.8	12.3
DEAE-Toyopearl					
Peak I	0.51	73	143.13	1.8	13.3
Peak II	1.06	68	64.15	1.7	6.0

RESULTS

Purification of chitinases and their characterization. Chitinolytic activity was detected in the culture supernatant when *R. oligosporus* was grown to the late stage of growth on the surface of SIV liquid medium, and chitinases were purified from the culture medium. The first step, after ammonium sulfate precipitation, was affinity chromatography on a chitin column. Most proteins were not absorbed, but most of the activity was absorbed on the chitin column at pH 8.4 and eluted when the pH of the buffer was lowered to 3.2 (Fig. 1). The fraction with the activity was concentrated and subjected to gel chromatography in a column of Sephadex G-75. The activity was eluted as a single peak at the same fraction as the major protein peak with an estimated molecular mass of 45 kDa (Fig. 2). The activity was further purified by ion-exchange chromatography on a column of DEAE-Toyopearl 650M and it was separated into two peaks; one was eluted by washing the column with the charging buffer (peak II), and the other was eluted by a linear salt gradient in the vicinity of 15 mM NaCl (peak I) (Fig. 3). We designated the latter enzyme chitinase I and the former enzyme chitinase II. The results of purification are summarized in Table 1.

Both preparations of chitinase I and II were almost homogeneous, as judged by SDS-PAGE, with estimated molecular masses of 50 and 52 kDa, respectively (Fig. 4, lanes 2 and 3). Comparison with the results of gel filtration

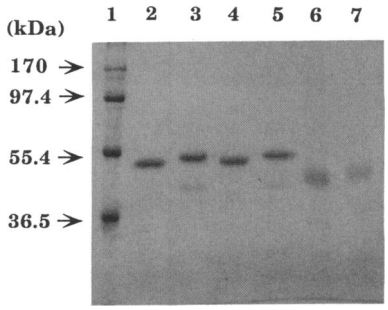

FIG. 4. SDS-PAGE of chitinase I and II before and after endoglycosidase H or TFMS treatment. Protein was stained with Coomassie brilliant blue R250. Lanes: 1, molecular weight markers indicated on the left; 2, chitinase I; 3, chitinase II; 4, endoglycosidase H-treated chitinase I; 5, endolgycosidase H-treated chitinase II; 6, TFMS-treated chitinase I; 7, TFMS-treated chitinase II.

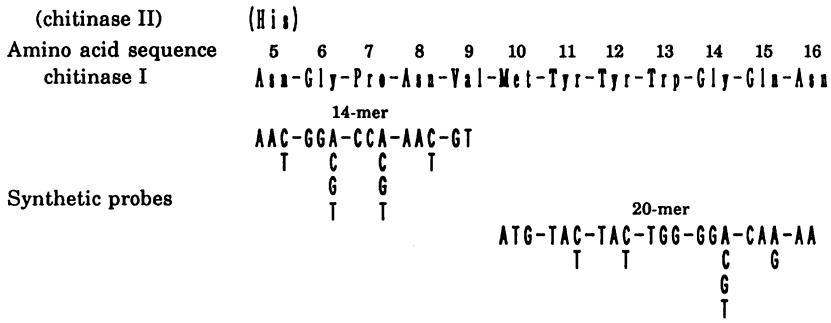

FIG. 5. Synthetic oligonucleotide mixture probes. Two kinds of oligonucleotide mixtures were synthesized; one was 14-mer which is hybridizable to a gene encoding only chitinase I, and the other was 20-mer which is hybridizable to both genes encoding chitinase I and chitinase II.

suggested that the native forms of these chitinases were monomeric.

Two chitinases were subjected to SDS-PAGE after enzymatical and chemical deglycosylation (Fig. 4, lanes 4 to 7). The treatment with endoglycosidase H did not change the mobility of either chitinase on the gel, whereas the treatment with TFMS increased the mobility of both proteins. The molecular masses of the deglycosylated chitinase I and II with TFMS were approximately 44.5 and 46.5 kDa, respectively. These results strongly suggested that the two chitinases were *O* glycosylated to similar extents and that the difference in their molecular masses was caused by the difference in the lengths of the polypeptides.

The pH optima for the activities of chitinase I and II in McIlvaine buffer were 4.0 and 3.5, respectively. Heating of chitinase I and II for 10 min at 60°C resulted in losses of 25 and 45%, respectively, in the activity. Thin-layer chromatography revealed *N,N'*-diacetylchitobiose as a major product and *N*-acetylglucosamine and *N,N',N''*-triacetylchitotriose as minor products of hydrolysis of colloidal chitin by both chitinase I and II. In the assay system for chitinase used in the present experiment, the sensitivity of detection was more than 300-fold lower for the disaccharide than for the monosaccharide, so the activity detected by using the purified enzymes was supposed to be dependent on the presence of the monosaccharide produced as minor product by the enzyme reaction. We observed that β-*N*-acetylglucosaminidase in the cell extract which cleaves the disaccharide to the monosaccharide was eluted in the nonabsorbed fraction in the chitin column chromatography and was separated from the main activity peak of chitinase (Fig. 1). This result explains why the yield and the purification fold were so low after the step of chitin column during purification (Table 1).

N-terminal amino acid sequences of chitinase I and II were determined as A-W-S-S-N-G-P-N-V-M-Y-Y-W-G-Q-N-S-A-G-G-S-N-T-Q-A-S-L-G-T-Y and A-W-S-S-H-G-P-N-V-M-Y-Y-W-G-Q-N, respectively. Two chitinases had very similar N-terminal amino acid sequences, with a difference to the 16th N-terminal residue in only the 5th amino acid residue, which was Asn in chitinase I and His in chitinase II.

Cloning of two chitinase genes, *chi1* and *chi2*. Mixed oligonucleotides of 20- and 14-mer (Fig. 5) were synthesized. The 20-mer probe was expected to hybridize to both chitinase genes, and the 14-mer probe was expected to hybridize to only the chitinase gene *chi1*, encoding chitinase I, but not to the other chitinase gene, *chi2*, encoding chitinase II.

Southern blot analysis of total DNA of *R. oligosporus* digested with either *Eco*RI or *Hin*dIII was carried out with the ^{32}P-labelled 20-mer probe under the conditions at which the hybridization temperature was 42°C and the washing temperature was 55°C. A single band at 2.9 kb in the total DNA digested with *Eco*RI and two bands at 3.6 and 11.5 kb in the total DNA digested with *Hin*dIII were detected. Then, the *R. oligosporus* genomic DNA library was screened with the ^{32}P-labelled 20-mer probe under this hybridization condition. All positive clones obtained had a 2.9-kb *Eco*RI fragment hybridizable with the 20-mer probe. Some of them had a 3.6-kb *Hin*dIII fragment and the others had a 11.5-kb *Hin*dIII fragment hybridizable with the 20-mer probe. When probed with the ^{32}P-labelled 14-mer probe, those clones containing only the 3.6-kb *Hin*dIII fragment were hybridizable.

The 3.6- and 11.5-kb *Hin*dIII fragments were subcloned into the *Hin*dIII site of plasmid pUC119, yielding plasmids pCHI6 and pCHI9, respectively, and the nucleotide sequences of the regions which were hybridizable with the probes were determined by using the strategy shown in Fig. 6. The results are shown in Fig. 7 and 8.

In the nucleotide sequence of the insert of pCHI6, there are sequences identical to those contained in both 20- and 14-mer probes. Furthermore, a sequence of 30 amino acid

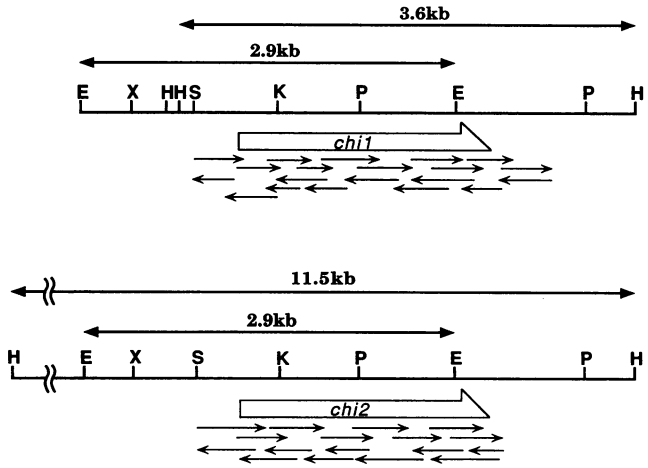

FIG. 6. Restriction endonuclease maps and sequencing strategies for *chi1* and *chi2* of *R. oligosporus*. Chitinase-encoding regions and the directions of transcription are indicated by open arrows. The abbreviations for restriction enzymes are E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sph*I; and X, *Xba*I.

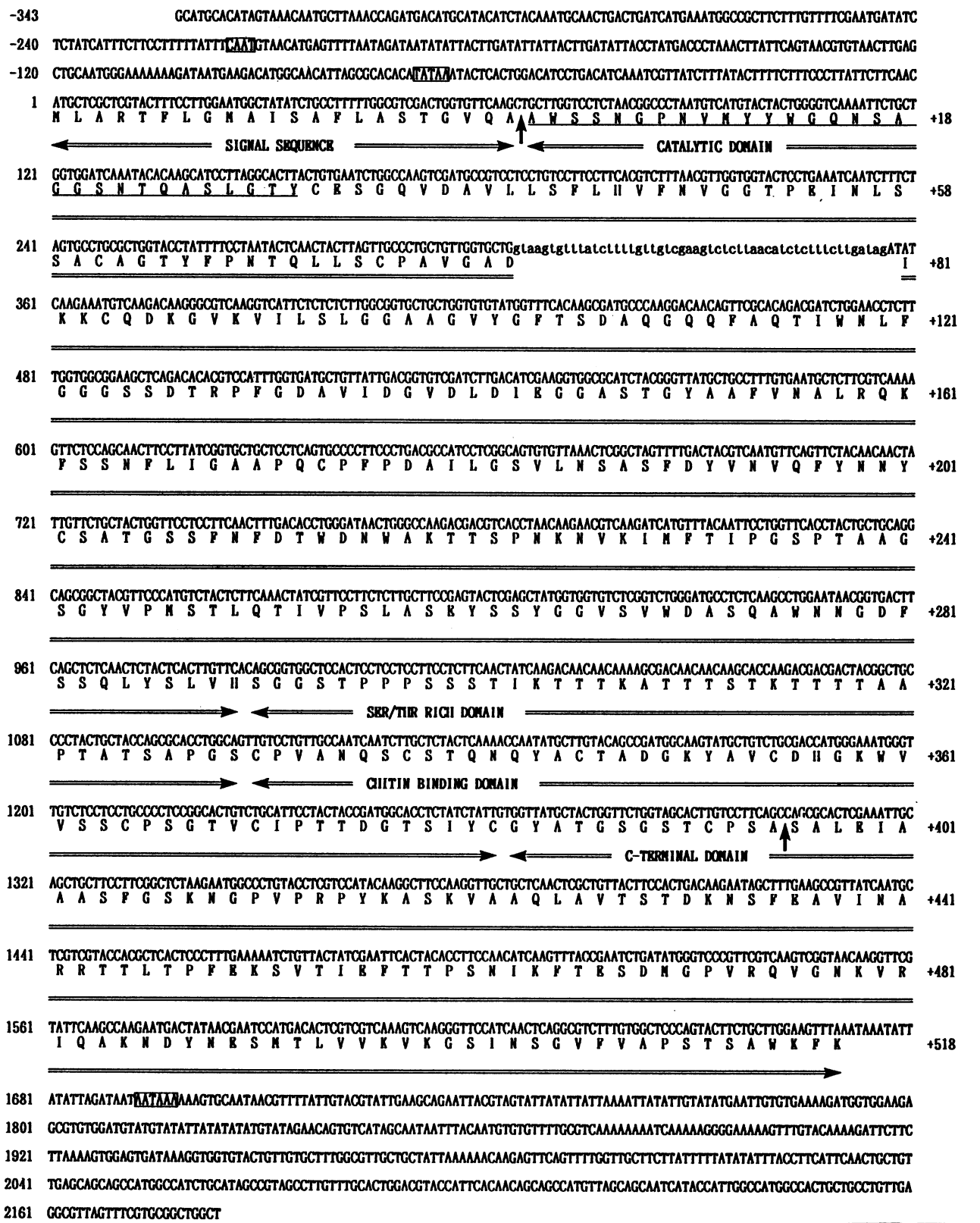

FIG. 7. Nucleotide and deduced amino acid sequences of *chi1* of *R. oligosporus*. The entire nucleotide sequence of *chi1* is shown, with the corresponding amino acid sequence printed below it. The nucleotide and amino acid sequences are numbered on the left and right, respectively. The A nucleotide of the presumed ATG initiation codon and the N-terminal alanine of the chitinase I are designated +1 in the nucleotide and amino acid sequences, respectively. The sequences CAAT, TATAA, and AATAAA are boxed, and the N-terminal amino acid sequence determined by sequencing of the purified chitinase I is underlined. Lowercase letters indicate the nucleotides in the presumed intron sequence. The N- and C-terminal processing sites suggested in this experiment are indicated by arrows. In the case of the C-terminal processing site, there are two alternative sites (see text). Each of the proposed five domains is indicated by a double line with arrowheads.

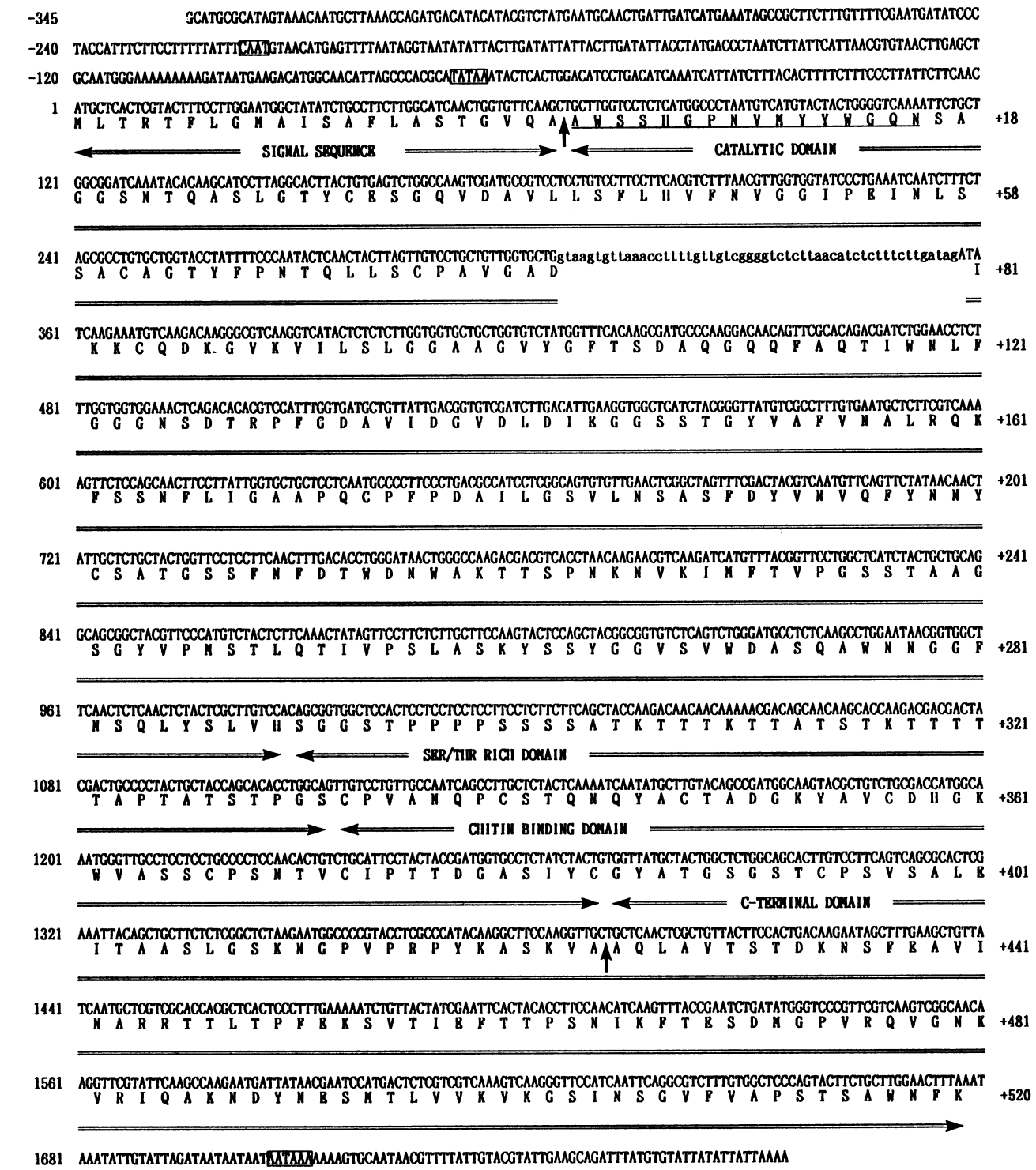

FIG. 8. Nucleotide and deduced amino acid sequences of *chi2* of *R. oligosporus*. For explanation of symbols and abbreviations, see the legend to Fig. 7.

residues identical to the N-terminal amino acid sequence of the purified chitinase I is present in the deduced amino acid sequence. In the case of pCHI9, there is a sequence identical to that contained in the 20-mer probe but there is not a sequence identical to that contained in the 14-mer probe. In the deduced amino acid sequence, there is a sequence consisting of 16 amino acid residues identical to the N-terminal amino acid sequence of the purified chitinase II. From these results, it is concluded that these regions of pCHI6 and pCHI9 encode chitinase I and II, respectively.

Several features indicate that the first ATG codons of the determined nucleotide sequences are the most probable

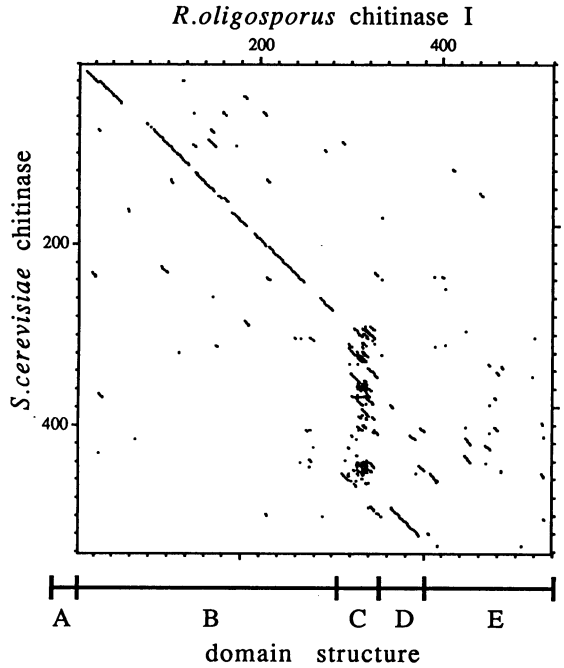

FIG. 9. Harr plot analysis of the amino acid sequences deduced from the nucleotide sequences between the *chi1* gene of *R. oligosporus* and the chitinase gene of *S. cerevisiae* (13). Points represent identity in 5 of 12 amino acids. See Fig. 10 for the domain structures shown at the lower part.

translational start sites. Putative TATA elements are present at 68 and 70 nucleotides upstream of these ATG codons for the *chi1* and *chi2* genes, respectively.

Stop codons in-frame are present at 224 and 200 nucleotides downstream of the 20-mer-probe-hybridizable sequence in *chi1* and *chi2*, respectively, indicating the possible presence of introns. It is suggested that *chi1* and *chi2* contain introns of 52 and 53 nucleotides, respectively, at the sequence encoding +80 Asp in Fig. 7 and 8 for two reasons. First, so far, we have cloned and sequenced several genes from *Rhizopus* spp. and found the following intron consensus sequence: 5'-GTAa · · · PuPyTNAPy · · · PyAG-3'. Both predicted introns in *chi1* and *chi2* have this consensus sequence. Secondly, there is a significant homology between the published amino acid sequence of *S. cerevisiae* chitinase (13) and those of chitinase I and II deduced from the intron-deleted DNA sequences of *chi1* and *chi2*. In Fig. 9, the results of Harr plot analysis for chitinases of *S. cerevisiae* and *R. oligosporus* (I) are shown.

It is suggested that the TAA codons after the sequences encoding +518 Lys of *chi1* and +520 Lys of *chi2*, respectively, are true termination codons. A-T-rich regions, which are characteristic in 3'-noncoding regions of *Rhizopus* genes, and poly(A) addition signal sequences are present downstream of these TAA codons.

The DNA sequences of *chi1* and *chi2*, when these putative introns are deleted, contain open reading frames of 1,620 and 1,626 nucleotides, respectively, corresponding to proteins of 540 amino acids for chitinase I and 542 amino acids for chitinase II. Their structures are very similar, with respect to the restriction enzyme maps, nucleotide sequences including noncoding regions (94.6% identity), and deduced amino acid sequences (94.8% identity). Although the ploidy of *R. oligosporus* is unknown, it is possible that *chi1* and *chi2* are allelic genes.

The molecular masses of the purified chitinase I and II were different by 2 kDa, judging from the analysis by SDS-PAGE. But the calculated molecular masses of the polypeptides from which the signal sequence had been removed of *chi1* and *chi2* were almost the same (53.987 kDa for the *chi1* product and 54.258 kDa for the *chi2* product), and they were larger than those of deglycosylated purified chitinase I and II (Fig. 4). It was then speculated that the signal sequence-removed products of these genes were processed also at their C termini, to produce chitinase I and II. To examine this possibility, the C-terminal amino acid sequences of chitinase I and II were determined. The proteins were digested with carboxypeptidase A and B, and the released amino acids were analyzed. The C-terminal amino acid sequences of chitinase I and II were found to be -Ser-Ala and -Val-Ala, respectively, which are different from those deduced from the nucleotide sequences of the genes. Therefore, C-terminal parts of the products of *chi1* and *chi2* are certainly processed.

In the deduced amino acid sequence of *chi1*, there are seven -Ser-Ala sequences at the Ala positions +60, +168, +184, +327, +395, +397, and +514. Considering the molecular mass of the deglycosylated chitinase I (44.5 kDa), the Ala's at +395 and +397 are candidates for the cleavage site. For the same reason, the Ala at +424 is most likely the processing site of the *chi2* product among five -Val-Ala sequences at the Ala positions +153, +336, +364, +424, and +511 in the deduced amino acid sequence of chitinase II. This suggestion of the processing site of the *chi2* product is supported by the result of the kinetic analysis of released amino acids from purified chitinase II by digestion with carboxypeptidases, which indicated that the C-terminal region contained lysine, serine, and tyrosine residues in addition to alanine and valine. Considering these results, we conclude that chitinase I and II are synthesized with pre- and prosequences and that the prosequences, which are processed to produce the mature forms reducing the molecular masses by more than 10 kDa, are located at the C termini.

DISCUSSION

In this study, we purified two chitinases and isolated their genes from *R. oligosporus*. *R. oligosporus chi1* and *chi2* encoded the proteins consisting of 540 and 542 amino acid residues, respectively. In comparison with the amino acid sequence of *S. cerevisiae* chitinase (13), it is suggested that the structures of the products of the *chi1* and *chi2* genes are divided into five domains: the signal sequence, the catalytic domain, the Ser- and Thr-rich domain, the chitin-binding domain, and the C-terminal domain (Fig. 9 and 10).

The first 22 amino acid residues (from −22 Met to −1 Ala) have characteristics of a cleavable signal sequence (27), that is, a short, positively charged N terminus (−19 Arg), a long central hydrophobic core (from −17 Phe to −7 Ala), and a polar C terminus (from −6 Ser to −1 Ala). In fact, the N-terminal amino acid sequences predicted from polypeptides from which the signal sequence had been removed are identical to those determined for the purified chitinases I and II. The existence of signal sequences in *Rhizopus* chitinase precursors indicates that they are transported through the usual cellular secretion pathway. As they do not have a potential membrane-spanning region as judged from the hydropathy profiles, they may be secreted out into periplasmic space and bind to chitin there or in the cell wall.

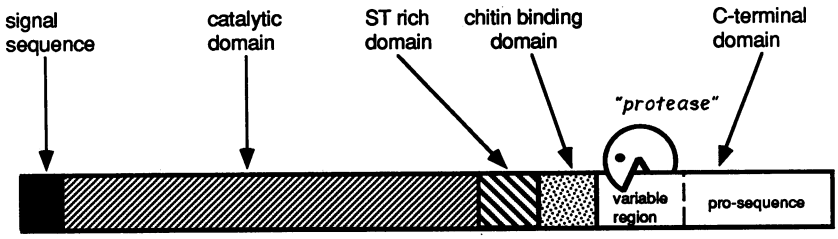

FIG. 10. Schematic representation of *R. oligosporus* chitinases. Five domains, two regions in the C-terminal domain, and the "protease" processing site are described in the text. Five domains described here (the signal sequence, the catalytic domain, the Ser/Thr-rich domain, the chitin-binding domain, and the C-terminal domain) correspond to A, B, C, D, and E, respectively, in the lower part of Fig. 9.

The sequence consisting of the following 290 amino acid residues (from +1 Ala to +290 His) seems to be the catalytic domain, because it is homologous with the proposed catalytic domain of the *S. cerevisiae* chitinase (13) and because there are two small regions in it that are conserved in several enzymes which cleave the β-1,4-glycosidic bonds between adjacent *N*-acetylglucosamine residues (13); region I is located from +90 Lys to +104 Phe, and region II is located from +137 Asp to +150 Gly. Subsequent to the catalytic domain, the third domain, consisting of about 40 residues (from +291 Ser to +330 Ser in the *chi1* product and from +291 Ser to +332 Ser in the *chi2* product), contains serine and threonine in more than 50% of the total amino acid residues in this domain. Carbohydrate residues (Fig. 4) may be attached to this domain by *O*-glycosidic linkage as in the cases of some other fungal carbohydrolases (12). The fourth domain, consisting of 41 amino acid residues in the *chi1* gene product (from +331 Cys to +371 Cys) and the *chi2* gene product (from +333 Cys to +373 Cys), has approximately 50% homology with the chitin-binding domain of the *S. cerevisiae* chitinase. The fifth domain, which is absent in the *S. cerevisiae* chitinase (Fig. 9), is a long C terminus. Comparison of the amino acid sequences deduced from the nucleotide sequences with the C-terminal amino acid sequences of the purified chitinases indicated the processing of precursors at the C termini. It is suggested that the fifth domain consists of two regions; one is a variable region whose sequence is different for the two chitinases and where the C-terminal precessing event occurs, and the other region is the prosequence which is removed during maturation of these chitinases of *R. oligosporus*. So, the general structure of the two chitinases may be summarized as shown in Fig. 10. The structures of these chitinases are very similar to those of fungal carbohydrolases (12), but the existence of the C-terminal domain is specific for the chitinases.

There are limited examples for C-terminal processing. For a bacterial serine protease of *Serratia marcescens*, the processing of the C-terminal portion of the proenzyme appears to be involved in secretion of the mature protein, even though it also has the N-terminal signal sequence (32). The C-terminal part of endo-β-1,4-glucanase of *Bacillus subtilis* is postulated to be processed. Although the function of this region is unknown, it does not inhibit the enzymatic activity of the mature protein since the precursor is fully active (22).

Fungal extracellular carbohydrolases are in multiple forms (12, 29). Generally, the larger molecules can be absorbed on an insoluble substrate and can digest it easily. On the other hand, the smaller molecules cannot digest insoluble substrates but are fully active against soluble substrates (30). Proteolytic processing has been proposed to explain the diversity of fungal carbohydrolases; proteolytic removal of the terminal substrate-binding domains results in low affinity to insoluble substrates. These processings are dissimilar to that of *Rhizopus* chitinases, because the processed mature-form *Rhizopus* chitinases purified from the culture supernatant contain the chitin-binding domains and are active against insoluble substrates even after the processing events.

In *M. mucedo*, at least one of membrane-bound chitinases is activated with trypsin treatment, and therefore, it appears to be a zymogen (9). The preliminary analyses of the full and truncated *chi1* products that were secreted from *S. cerevisiae* suggested that the pro-enzyme with the C-terminal domain did not have chitinase activity (data not shown).

Interestingly, the chitinase of *S. cerevisiae* is synthesized as an activated form. Chitin represents only approximately 1% of the cell wall in *S. cerevisiae* but more than 9% in filamentous fungi (1). This suggests that chitin plays more important roles in cell wall assembly in filamentous fungi than in *S. cerevisiae*. Therefore, chitin synthesis and degradation may be coordinated more delicately in filamentous fungi, and chitinase as well as chitin synthase (5) may be regulated posttranslationally. Whether the *Rhizopus* chitinases play morphogenetic roles as described above requires further experiments such as disruption of their structural genes or inhibition of their gene expression with antisense RNAs. We have constructed a host-vector system of *Rhizopus niveus* (33, 34). The chitinase genes of *Rhizopus* isolated in this study may enable us to carry out such experiments.

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