Intracellular chitinase gene from *Rhizopus oligosporus*: molecular cloning and characterization

Naoki Takaya,† Daisuke Yamazaki, Hiroyuki Horiuchi, Akinori Ohta and Masamichi Takagi

Author for correspondence: Masamichi Takagi. Tel: +81 3 3812 2111 ext. 5169. Fax: +81 3 3812 9246. e-mail: amtakag@hongo.ecc.u-tokyo.ac.jp

Multiple chitinases have been found in hyphae of filamentous fungi, which are presumed to have various functions during hyphal growth. Here it is reported, for the first time, the primary structure of one such intracellular chitinase, named chitinase III, from *Rhizopus oligosporus*, a zygomycete filamentous fungus. Chitinase III was purified to homogeneity from actively growing mycelia of *R. oligosporus* using three steps of column chromatography. Its molecular mass was 43.5 kDa and the pH optimum was 6.0 when p-nitrophenyl N,N',N"-β-D-triacetylchitotrioside was used as a substrate. Chitinase III also hydrolysed chromogenic derivatives of chitobiose, but had no N-acetylglucosaminidase activity. The gene encoding chitinase III (chi3) was cloned using PCR with degenerate oligonucleotide primers from the partial amino acid sequence of the enzyme. The deduced amino acid sequence of chi3 was similar to that of bacterial chitinases and chitinases from mycoparasitic fungi, such as *Aphanocladium album* and *Trichoderma harzianum*, but it had no potential secretory signal sequence in its amino terminus. Northern blot analysis showed that chi3 was transcribed during hyphal growth. These results suggest that chitinase III may function during morphogenesis in *R. oligosporus*.

Keywords: *Rhizopus oligosporus*, zygomycetes, chitinase

INTRODUCTION

Chitin, a homopolymer of 1,4-β-linked N-acetyl-D-glucosamine, is widely distributed in invertebrates, fungi and algae and confers strength against mechanical forces from the environment as external skeletons or cell walls. In filamentous fungi, chitin is one of the major components of the cell wall and is thought to be important for maintaining cell wall integrity. Proper chitin metabolism may be essential for hyphal growth and morphology.

Chitinase (EC 3.2.1.14) is an enzyme which hydrolyses chitin at 1,4-β-bonds between N-acetylglucosamine units. The enzyme is found in bacteria, plants, insects, humans and fungi (Flach et al., 1992; Collinge et al., 1993; Boot et al., 1995) and is thought to function in assimilation of chitin, defence against fungal pathogens and separation of dividing cells, although the actual roles of most chitinases remain to be elucidated. In filamentous fungi, chitinases are thought to be involved in processes requiring cell wall digestion (Gooday, 1990), viz. germination of spores, tip growth of hyphae (Bartnicki-Garcia, 1973), branching of hyphae, hyphal autolysis and differentiation into spores, as well as in assimilation of chitin and mycoparasitism. There have been few reports of chitinases of filamentous fungi. In *Mucor rouxii*, multiple chitinase activities have been observed in mycelial extracts (Rast et al., 1991) and germinating spores (Pedraza-Reyes & Lopez-Romeo, 1991). Their roles in fungal growth and mechanisms of chitinase regulation are almost totally unknown.

*Rhizopus oligosporus* is a filamentous fungus that belongs to the phylum Zygomycota, whose cell walls are mainly composed of chitin and its deacetylated de-
rivative, chitosan (Davis & Bartnicki-Garcia, 1984). Because of the abundance of chitin and chitosan, R.
oligosporus could be a good organism for the analysis of cell wall chitin metabolism. We have already cloned two
chitin synthase genes (Motoyama et al., 1994) and also two chitinase genes, chi1 and its allelic gene chi2, encoding chitinase I and chitinase II, respectively, from this organism (Yanai et al., 1992). In the course of our
study, it was revealed that chitinase I and chitinase II are induced in the late stage of culture, when the hyphae
autolyse, so that we could purify the enzymes from the culture medium. It was also found that they are
synthesized with N-terminal signal sequences for secretion. These results suggest that chitinase I and II are
not involved in hyphal growth.

With the aim of revealing the function of chitinases in growing hyphae, we report here the purification and
characterization of chitinase III from mycelial extracts of R. oligosporus and the cloning of the gene encoding
this enzyme. Our results suggest that chitinase III has a role during hyphal growth and that filamentous fungi
have evolutionarily divergent chitinases with markedly differing roles in chitin metabolism.

METHODS

Strains and media. R. oligosporus IFO8631 was used throughout this work. R. oligosporus was grown in SIV (Takaya et al., 1994) or potato dextrose medium. Escherichia coli MV1190 (Δlac-proAB thr supE Δm17 recA) 306::Tn10 Tet' (F tetD36 proAB lacIqZAM15) was used for the propagation of plasmids. E. coli was grown in LB medium and 50 μg ampicillin ml⁻¹ was added if necessary.

Culture conditions. Surface culture was done as described previously (Yanai et al., 1992). Submerged culture was done as follows: spores (approx. 8 x 10⁶) of R. oligosporus were inoculated into a 5 litre Erlenmeyer flask containing 1 litre SIV medium and cultivated in a rotary shaker at 120 r.p.m., 30 °C.

Enzyme activity assay. Chitinase assay was performed with the chromogenic substrate p-nitrophenyl N₃N₅N₇N₉-p-tri-acetylchitotrioside (PNP chitotrioside; Seikagaku-Kogyo). Reactions contained 0.1 M potassium citrate (pH 6 5), Triton X-100 (0.1 %, v/v), 0.5 mM PNP chitotrioside at 37 °C in a total volume of 100 μl. The reaction was stopped with 500 μl 1 M Na₂CO₃ and the absorption of p-nitrophenol was spectrophotometrically determined at 415 nm (Hitachi U-2000 spectrophotometer). One unit of enzymic activity was defined as that which released 1 nmol p-nitrophenol min⁻¹.

Enzyme purification. Chitinase I was purified as described previously (Yanai et al., 1992). For purification of chitinase III, 12 litres SIV medium was inoculated with 10⁶ spores of R. oligosporus and incubated with shaking at 30 °C for 18–20 h. Mycelia (130 g wet wt) were harvested by filtration, washed with 20 mM NaHCO₃ (pH 8.4) and suspended in 600 ml of the same buffer containing 1 mM EDTA, 1 mM PMSF and 10 mM β-mercaptoethanol. Mycelia were broken with a Waring blender at top speed. Triton X-100 was added to a final concentration of 1 % and the broken mycelium mixture stirred for 3 h at 4 °C. After centrifugation at 7000 g for 30 min, the supernatant was loaded onto a regenerated-chitin column (16 x 70 mm) and washed with 600 ml 20 mM NaHCO₃ (pH 8.4) containing Triton X-100 (0.1 %) at a flow rate of 30 ml h⁻¹. Fractions containing chitinase activity that were eluted by 20 mM sodium acetate (pH 3.2) containing Triton X-100 (0.1 %) were immediately neutralized with 1 M Tris. They were combined (105 ml), dialysed against 20 mM Tris/HCl (pH 8.0) containing Triton X-100 (0.1 %) and applied to a DEAE-Sephadex A50 column (Pharmacia) (10 x 100 mm). The column was washed with 100 ml of this buffer at a flow rate of 50 ml h⁻¹ and chitinase activity eluted with a linear gradient from 0 to 500 mM NaCl in 300 ml of the same buffer. Fractions (84 ml) containing chitinase activity were pooled, dialysed against 1 mM sodium phosphate, 0.1 % Triton X-100 (pH 6.8) and loaded onto a hydroxyapatite (Wako Chemicals) column (10 x 60 mm). After washing with 100 ml 1 mM NaCl, 40 mM octyl D-glucoside (Dojindo), chitinase activity was eluted with 1 mM sodium phosphate (pH 6.8), 40 mM octyl D-glucoside. Fractions containing chitinase activity were pooled, dialysed against 40 mM octyl-
D-glucoside and used as the purified chitinase III preparation.

Subcellular fractionation. Subcellular fractionation of R. oligosporus was done as follows: 0.5 g (wet wt) of mycelia of R. oligosporus cultured in SIV medium containing glucose as carbon source was suspended in 5 ml 50 mM sodium phosphate (pH 7.2) and broken with a Potter-Elvehjem homogenizer. Cell extracts were centrifuged at 1000 g for 15 min and the supernatant was centrifuged at 10000 g for 15 min at 4 °C to prepare the 100000 g pellet. The supernatant was centrifuged at 100000 g for 1 h at 4 °C to obtain the 100000 g pellet and 100000 g supernatant.

Solubilization of proteins. Solubilization of the 10000 g and 100000 g pellets was carried out as follows: 10000 g or 100000 g pellets containing approximately 20 mg protein were suspended in 50 mM sodium phosphate (pH 7.2) to a final volume of 4 ml. Triton X-100 was added to a final concentration of 1 % and the resuspension incubated for 1 h at 4 °C. It was then centrifuged at 10000 g for 15 min at 4 °C. The supernatants were loaded directly onto the chitin column.

SDS-PAGE and Western blot analysis. SDS-PAGE was done as described by Laemmli (1970). Western blot analysis was performed with ECL-Western blotting detection reagents (Amersham) according to the manufacturer’s instructions.

Protein sequencing. In situ digestion of chitinase III was done essentially according to the method of Aebersold et al. (1987). Chitinase III (about 60 μg) was subjected to SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The protein bands were detected by staining with Ponceau S (Sigma) and a protein of 43.5 kDa excised and digested with trypsin. The tryptic fragments were separated by C18 reverse-phase HPLC and the amino terminal sequences of three of these fragments (P15, P32 and P34) determined using an automated amino acid sequencer (Applied Biosystems model 373A).

Isolation of the chi3 gene. R. oligosporus total DNA, prepared as described previously (Horiuchi et al., 1988), was used as a template for PCR with the following degenerate oligonucleotide primers: 31, 5′ GGGATCNCCNACCACN 3′ (corresponding to the sequence of tryptic peptide P32); and 32, 5′ TTYGCNAAAYTNCARCC 3′ and 33, 5′ ATRTCNCCR- TANGCRT 3′ (both corresponding to the sequence of tryptic peptide P34). N, Y and R indicate any of the four nucleotides, a pyrimidine and a purine, respectively). The first PCR was done with primer 31 (100 pmol), primer 32
(100 pmol) and 0.5 μg total DNA as a template. Cycling conditions were: 10 min at 94 °C for the first denaturation; 30 cycles of 0.5 min at 94 °C, 1 min at 42 °C and 2 min at 72 °C for amplification; then 10 min at 72 °C for extension. Products of about 700 bp were purified by agarose gel electrophoresis and used as a template for the second PCR. The second PCR was performed using the same conditions except for the use of primers 32 and 33. An amplified DNA fragment of the expected size was obtained and purified. Labelled probes were prepared using [α-32P]dCTP by the random primer labelling method (Feinberg & Vogelstein, 1983) and used for screening of a genomic library constructed in vector λ2001 (Yanai et al., 1992) by plaque hybridization (Sambrook et al., 1989).

**Nucleotide sequencing.** A 3.8 kb Sall–PstI fragment bearing the chi3 gene was inserted into pUC119 and a series of deletions generated using exonuclease III and mung bean nuclease. The truncated chi3 DNAs were sequenced by the dideoxy chain-termination method (Sanger et al., 1977). In some cases, restriction fragments of the chi3 DNA were cloned into pUC119 and sequenced. Primer extension analysis was done as described by Rothmel & LeClerc (1989) using the oligonucleotide 5' CAGGTCCTTGGTGTGCTGAGGTG-GTCC 3' as a primer.

**RNA analysis.** Spores of R. oligosporus (10^6 ml^-1) were cultured in SIV medium containing glucose (2%, w/v) at 30 °C for 24 h. Mycelia were then transferred to SIV medium containing no carbon source, 0.1% glucose, 2% glucose, or 10% (w/v) colloidal chitin (Yanai et al., 1992) and incubated at 30 °C for 24 h. Total RNA was prepared from the mycelia using the RNeasy Total RNA kit (Qiagen) according to the manufacturer's instructions. Thirty micrograms of each RNA preparation were separated by agarose gel electrophoresis, blotted to Hybond-N (Amersham) and hybridized with the [α-32P]-labelled chi3 cDNA fragment as a probe. Signals were detected using a BioImaging Analyzer BAS2000 (Fuji Photo Film).

**RESULTS**

**Purification of chitinase III**

To purify intracellular chitinase in the present study, we used actively growing hyphae of R. oligosporus as starting material, in contrast to our previous work (Yanai et al., 1992). Mycelial homogenates were extracted with buffer containing Triton X-100 (1%) and the soluble fraction was subjected to a series of column chromatography procedures. During the first regenerating-chitin column chromatography step, significant activity was eluted as a single peak with 20 mM acetate (pH 3.2) containing Triton X-100 (0.1%) and was subjected to further purification. As no activity was eluted with the buffer lacking Triton X-100, the detergent was included in the buffer in the following steps. During DEAE-Sephadex chromatography, the enzyme activity was eluted as a broad peak from 100 mM to 400 mM NaCl concentration. This step had little effect on purification, but was helpful for concentrating the enzyme activity in the following final step. By the procedure described, we purified the chitinase activity by 2400-fold with a yield of 1.3% from the detergent-soluble fraction of mycelia. SDS-PAGE of this fraction showed a single band of molecular mass of 43.5 kDa (Fig. 1, lane 4) and we designated this protein chitinase III. A summary of the representative purification steps is shown in Table I.

**Characterization of the purified enzyme**

The isoelectric point of chitinase III was approximately 7.0 (data not shown). No positive signal was obtained by periodic acid/Schiff reagent staining (Poehling & Neuhoff, 1981) after SDS-PAGE (data not shown). This was consistent with the lack of consensus sequences for asparagine-linked glycosylation in the deduced amino acid sequence (see below), suggesting that chitinase III has no glycosyl chain. The optimum pH for enzyme activity with PNP chitotrioside as substrate was approximately 6.0, which is much higher than that for chitinase I (optimum pH 3.0), another chitinase purified from this fungus (Yanai et al., 1992) (data not shown). The specific activity for PNP chitotrioside was 198 U (mg protein)^{-1}, which was 8.9-fold lower than that of chitinase I. The specificity of each chitinase for chromogenic chitooligoside substrates was determined. The activity of chitinase III with PNP chitobioside or PNP chitotetraoside as substrate was approximately one-fifth and sevenfold that for PNP chitotrioside, respectively, whilst the activity of chitinase I with PNP chitobiode or PNP chitotetraoside as substrate was below one-fiftieth and 10-fold that for PNP chitotrioside, respectively (experiments were undertaken three times with the same results, data not shown). K_m values of chitinase III and chitinase I for PNP chitotrioside were 7.8 x 10^{-5} M and 1.3 x 10^{-4} M, respectively. The K_i of chitinase III for allosamidin, a competitive inhibitor of chitinases, was 3.0 x 10^{-8} M, which was one-sixth that of chitinase I (1.8 x 10^{-7} M). MnCl_2 (1 mM) increased the activity of...
Table 1. Purification of chitinase III from mycelial extract of R. oligosporus

Data from a typical purification procedure are presented. Three independent isolation experiments gave comparable results.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity [U (mg protein)^-1]</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>7728</td>
<td>64000.0</td>
<td>8.28</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Regenerated chitin</td>
<td>1.56</td>
<td>5950.0</td>
<td>3814.1</td>
<td>9.3</td>
<td>460.6</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>0.80</td>
<td>1182.1</td>
<td>1477.5</td>
<td>2.0</td>
<td>178.4</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.043</td>
<td>853.3</td>
<td>19844.2</td>
<td>1.3</td>
<td>2396.6</td>
</tr>
</tbody>
</table>

chitinase III 2.7-fold, but had no effect on chitinase I. The metal ion chelator (10 mM EDTA) had no effect on chitinase III activity, but inhibited chitinase I activity by approximately 50%. The activities of these chitinases were not affected significantly by DTT (10 mM), β-mercaptoethanol (10 mM), MgCl₂ (1 mM), CaCl₂ (1 mM), ZnCl₂ (1 mM), NaMoO₄ (1 mM) or CoCl₂ (1 mM) (data not shown).

A preliminary result suggested that the activity of chitinase III increased in the presence of Triton X-100 (0.1%) and this may be indicative of association with other macromolecules or membranes. Therefore, we examined the effects of the detergent, a phospholipid and serum albumin on enzyme activity. The results showed that activity of the purified enzyme increased approximately fourfold in the presence of each of Triton X-100 (0.1%), serum albumin (1 μg ml⁻¹) or phosphatidylcholine (1 μg ml⁻¹) (data not shown).

Intracellular localization of chitinase III

We investigated the distribution of chitinase III in R. oligosporus by Western blot analysis of a few subcellular fractions using antibody, raised against Bacillus circulans chitinase A1, which did not react with chitinase I or II. Using this antibody, only a single protein band of 43-5 kDa was detected following Western blot analysis of the fraction, eluted from the chitin column during enzyme purification, that contained chitinase III activity. We added Triton X-100 to each of the 10000 g and 100000 g pellet fractions (final concentration, 1%) to solubilize the pellet (see Methods section), purified chitinase activity from these and the 100000 g supernatant fraction using the regenerated-chitin column, and carried out Western blot analysis with anti-chitinase A1 antiserum (Fig. 2). Chitinase III was detected in both 100000 g supernatant and 10000 g pellet fractions. These results suggest that some portion of chitinase III is associated with membrane- or cell-wall structures in R. oligosporus.

Amino acid sequencing of chitinase III

Direct sequencing of the amino-terminal amino acids of chitinase III was unsuccessful. Therefore, three tryptic peptides of chitinase III (P15, P32 and P34) were sequenced. The amino acid sequences of P15, P32 and P34 were YNVVDVATQANK, LVVGMPLYGR and AFANVQPDGQVVLGDAYADI, respectively. Searching the protein databases (SWISS-PROT and PIR), we found that peptide P32 had similarity to chitinase 1 of Aphanocladium album (7 of 10 amino acid positions were identical) (Blaiseau & Lafay, 1992) and chitinase A1 of B. circulans (8 of 10 amino acid positions were identical) (Watanabe et al., 1990). Peptide P34 also had similarity to the A. album chitinase (10 of 20 amino acid positions were identical), whilst peptide P15 showed no significant homology to known sequences.

Cloning of the chitinase-III-encoding gene

To clone the gene encoding chitinase III, nested PCR (McPherson et al., 1995) was performed with three degenerate oligonucleotide primers based on the sequences of peptides P32 and P34. The nucleotide sequence of a specifically amplified 0-11 kb fragment was determined and its deduced amino acid sequence completely matched the sequence of P34, if we assumed
Intracellular chitinase from *Rhizopus oligosporus*.

Fig. 4. Northern blot analysis using *chi3* cDNA as hybridization probe. (a) *R. oligosporus* was cultured in SIV medium containing glucose as carbon source for 24 h at 30°C, then transferred to SIV medium containing one of the following carbon sources: none (lane 1); glucose (0.1%) (lane 2); glucose (2%) (lane 3); colloidal chitin (10%) (lane 4) and incubated for 24 h at 30°C (Yanai et al., 1992). RNA was isolated as described in the text and 30 µg RNA electrophoresed per lane. (b) Total RNA was prepared from submerged culture in SIV medium containing glucose (2%) as sole carbon source after 24 h (lane 1), surface culture on the same medium after 24 h (lane 2) and surface culture after 48 h (lane 3). RNA was isolated and electrophoresed as described above. Positions of rRNAs are shown on the left in kb.

Fig. 3. Primer extension analysis of *R. oligosporus* *chi3*. (a) Lanes A, C, G and T indicate profiles of DNA sequences assayed by the dideoxy method. Lane P, products of primer extension reaction. Transcription start sites are shown by dots on the left. (b) Transcription start sites and the translation initiation site are indicated by dots and box, respectively.

an intron between the fourteenth amino acid (glycine)- and fifteenth amino acid (aspartic acid)-encoding sequences. A genomic DNA library was screened using this DNA fragment as a hybridization probe and four positive clones were obtained. They were divided into two classes by their restriction maps. A 3.8 kb *SalI−PstI* fragment from one of the positive clones was inserted into *pUC119* and its nucleotide sequence was determined. One ORF containing three putative intron sequences was present and its deduced amino acid sequence contained sequences that matched peptides P15, P32 and P34.

Since the transcription start site was at the twenty-seventh T, as determined by the primer extension method (Fig. 3a), the translation initiation site was assigned as shown in Fig. 3(b). The presence of the introns was confirmed by the sequence of the cDNA of *chi3* (data not shown). These results indicated that this DNA fragment contained the chitinase-Ill-encoding gene, which was designated *chi3*. The predicted amino acid sequence was comprised of 400 amino acids and its calculated molecular mass was 44.9 kDa, which corresponds well to that of chitinase II. There is neither a potential secretory signal sequence in its amino terminus nor a long hydrophobic stretch in the internal sequence. The partial nucleotide sequence of another clone with a different restriction map was also determined. Its deduced amino acid sequence was very similar to that of chitinase III (data not shown), suggesting that this clone could contain an allele of *chi3*, which is consistent with the observation that *R. oligosporus* is diploid or aneuploid (Motoyama et al., 1994; Yanai et al., 1992).

### Homology to other chitinases

Although there is no potential secretory signal sequence, the amino acid sequence of chitinase III is similar to a number of sequences reported previously for chitinases, demonstrating 42, 42, 42, 42, 40 and 25% similarity to *A. album* chitinase 1 (Blaiseau & Lafay, 1992), *Trichoderma harzianum* ThEn-42 chitinase (Hayes et al., 1994), *T. harzianum* ECH-42 chitinase (Carsolio et al., 1994), *T. harzianum* CHIT42 chitinase (Garcia et al., 1994),
Expression of chi3 mRNA in R. oligosporus

Northern blot analysis was performed using chi3 cDNA as a hybridization probe. chi3 mRNA was abundant in actively growing cells and was not increased by addition of colloidal chitin (Fig. 4a). It was reduced when the mycelia were transferred to a medium which contained only a low concentration of carbon or no carbon source. Furthermore, chi3 mRNA was not detected during the late stage of surface culture (Fig. 4b, lane 3), under conditions where chitinase I was produced in abundance (data not shown), indicating that the expression of chi3 and of chi1 is regulated differently during growth.

DISCUSSION

In this study, we have purified and characterized intracellular chitinase III from the mycelial extract of R. oligosporus, cloned its encoding gene and analysed the structure of the gene. This is the first report of the isolation of the gene encoding an intracellular chitinase from a filamentous fungus. The optimum pH for enzyme activity was nearly neutral and higher than that of extracellular chitinase I, suggesting that chitinase III may function in the cytosol. The deduced amino acid sequence of chitinase III had no potential secretory signal sequence and no possible sequence for modification by carbohydrate chains commonly found in secretory proteins. Interestingly, its deduced amino acid sequence indicates that it belongs to the bacterial-type chitinase subgroup rather than to the fungal-type subclass of yeasts and filamentous fungi.

Chitinase III exhibited a higher affinity than chitinase I for small chitosan oligosides. Chitinase I has a serine/threonine-rich domain and a chitin-binding domain on the carboxy terminus, which have been suggested to act as acceptor sites for O-glycosylation and high-affinity chitin binding, respectively (Kuranda & Robbins, 1991). In contrast, chitinase III had no such domains. This difference may be related to the varying substrate specificity of the two enzymes and to the different chitinase subgroups to which they belong, since the bacterial-type chitinase (Chit42) from T. harzianum cleaves smaller oligomers of chitin than the fungal-type chitinase (Chit33) (De la Cruz et al., 1992).

In the subcellular fractionation experiment, significant amounts of chitinase III were detected in the 10000 g pellet, which could be solubilized in the presence of Triton X-100, and the activity of this enzyme was stimulated by Triton X-100, serum albumin and phospholipid. These results suggest that chitinase III could be most active in the cells in contact with membrane- or cell-wall structures. In M. rouxii and Candida albicans cell extracts the presence of membrane-bound chitinases is suggested and the activities of these enzymes are inhibited by treatment with phospholipases and stimulated by detergents (Humphreys & Gooday, 1984; Dickinson et al., 1991). A 45 kDa chitinase was purified from the cytosolic fraction of C. albicans. The enzyme has an optimum pH of 5.0 and chitinase activity was stimulated 1.5-fold when phosphatidyicholine was present in the reaction mixtures (Mellor et al., 1994). These characteristics are similar to those of chitinase III studied during the present investigation. We are interested in the possibility that the 45 kDa chitinase of C. albicans is a bacterial-type chitinase, similar to those detected in filamentous fungi, which has not so far been detected in yeasts. In this regard it is important to note that C. albicans is a dimorphic fungus capable of filamentous growth.

The expression pattern of chi3 suggests that the enzyme is produced during hyphal growth. Chitinases are thought to be involved in hyphal tip growth and branching. It is possible that chitinase III may function in loosening the cell wall at the hyphal tip to enable turgor pressure to extend the hypha at the apex (Bartnicki-Garcia, 1973). Rast et al. (1991) proposed a model for functional relationships between chitinases and chitin synthases. According to this model, there are two types of chitinases in mycelia of M. rouxii. The type A chitinases, which hydrolyse smaller chitosan oligosides efficiently, are proposed to provide N-acetylglucosamine to activate chitin synthase(s) allosterically. The other, type B chitinases, are speculated to provide substrates for type A chitinases by hydrolysing long-chain cell wall chitin. Our results suggest that chitinase III hydrolyses well small chitosan oligosides rather than large ones and could interact with membrane- or cell-wall structures in the cell, proposals which are compatible with the ideas of Rast et al. (1991) described above. Stimulation of chitinase activity by Mn2+, as observed during the present study, has also been reported for some chitin synthases (Sudoh et al., 1995).

Recently, both fungal- and bacterial-type chitinase genes were cloned from each of two fungi: T. harzianum (Carsolio et al., 1994; García et al., 1994; Hayes et al.,
ACKNOWLEDGEMENTS

We are grateful to Dr S. Sakuda and Dr T. Watanabe for providing allosamidin and anti-chitinase A Ig antisera, respectively. We are also grateful to Dr S. Nakaju and Dr K. Nakaya for the amino acid sequencing analysis of chitinase III.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. This work was performed using the facilities of the Biotechnology Research Center, The University of Tokyo.

REFERENCES


canCHS1A, a variant gene of Candida albicans chitin synthase. Microbiology 141, 2673–2679.


Received 24 March 1998; revised 7 May 1998; accepted 14 May 1998.