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# Improved Production of *Trichoderma harzianum* Endochitinase by Expression in *Trichoderma reesei*

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**The chromosomal endochitinase gene (*ThEn-42*) of the mycoparasite fungus *Trichoderma harzianum* P1 was isolated and overexpressed in the filamentous fungus *Trichoderma reesei* under the promoter of the major cellulase gene *cbh1*. The host strain RutC-30 did not produce any endogenous endochitinase activity. The prepro region of the *T. harzianum* endochitinase was correctly processed in *T. reesei*. No differences in expression were observed when the prepro region was replaced with the CBHI signal sequence. Shake flask cultivation yielded 130 mg of active enzyme per liter, which in terms of activity represents about a 20-fold increase over the endochitinase activity produced by *T. harzianum*. The presence of multiple copies of the expression cassette in the transformant resulted in limitation in transcription and/or regulation factors needed for full activity of the *cbh1* promoter, although this was not the major limiting factor for higher expression of endochitinase. The endochitinase was very sensitive to an acidic protease at the late stages of *T. reesei* cultivation. *T. reesei* RutC-30 appeared to be tolerant of the endochitinase and can be used as a production host for this enzyme, which has antifungal activity toward plant pathogens.**

The filamentous fungus *Trichoderma harzianum* is a mycoparasite of plant pathogens and has been accepted as one of the most potent agents for biocontrol of plant diseases. Although the mechanism of mycoparasitism is not completely understood, expression of extracellular cell wall-degrading enzymes is assumed to be involved in this process. Together with other hydrolases, chitinolytic enzymes and glucanases are considered to have a major role in biocontrol through their action on cell wall constituents of the target fungi (4).

In plants, a great number of endochitinases and β1-3 glucanases have also been identified as pathogenesis-related proteins involved in plant defense, since they are present after infection and increase induced resistance against all types of pathogens. Combinations of plant endochitinases and β1-3 glucanases have been shown to inhibit the growth of several ascomycetes in in vitro experiments (6).

Of the extracellular proteins produced by strain P1 of *T. harzianum*, three different enzymes with chitinolytic activity have been purified and partially characterized: *N*-acetylglucosamidase, chitobiosidase, and endochitinase (13). In vitro experiments performed to test the antifungal activity of the pure enzymes showed that the endochitinase strongly inhibited the growth and development of the chitin-containing fungi tested, while the chitobiosidase had a similar effect but at higher concentrations (19). Combining the two enzymes had a significant synergistic effect, resulting in greater inhibition of spore germination of pathogens than with either enzyme tested alone (19). In addition, combinations of chitinolytic and glucanolytic enzymes showed a high level of synergy, especially when the endochitinase and a β1-3 glucanase were present in the mixtures (20). In comparison to other reports, the chitinolytic enzymes of *T. harzianum* appeared to be more effective than plant and bacterial chitinases against a wide range of plant pathogens (19).

*T. harzianum* produces only moderate levels of chitinolytic enzymes. The recent isolation of the gene encoding the endochitinase, *ThEn-42*, from strain P1 of *T. harzianum* (14) makes possible production studies of the endochitinase in efficient expression hosts. This opens up new possibilities for application of chitinases. The availability of enzyme preparations with high chitinase activity could be very useful not only in biological control but also in bioconversion of chitin-containing waste materials, such as shellfish (7), and in production of chitooligosaccharides for various applications (34).

The cellulolytic filamentous fungus *Trichoderma reesei* is not suitable for biocontrol of plant pathogens but has been used extensively for industrial production of hydrolases because of its high secretion capacity. Hypersecretory mutant strains of *T. reesei* produce more than 35 g of extracellular protein per liter (8), approximately half consisting of the main cellulase cellobiohydrolase I (CBHI). *T. reesei* has also been used successfully to produce heterologous proteins, such as calf chymosin and antibody fragments, using the strong *cbh1* promoter (12, 24). In this report, we describe the construction of *T. reesei* strains capable of overproducing the endochitinase from *T. harzianum* under the control of the *cbh1* promoter.

## MATERIALS AND METHODS

**Strains, media, and growth conditions.** *T. reesei* RutC-30 (ATCC 56765) (22) was used as the host for production of the endochitinase. *T. harzianum* P1 (ATCC 74058) (33) was used in isolation of the chromosomal endochitinase gene. The strains were grown for 5 to 6 days at 28°C on plates containing PD medium (Difco) to produce conidiospores. Liquid cultivations to screen for endochitinase-producing transformants were carried out in 50 ml of cellulase-inducing medium, which was *Trichoderma* MM (26)-based medium in which glucose was substituted with whey (24). The flasks were inoculated with $5 \times 10^7$ spores and incubated in a rotatory shaker for 3 to 4 days at 28°C. The transformant VTT-D-95434 and RutC-30 were grown for 3 days in shake flasks with 50 ml of *Trichoderma* MM containing 2% (wt/vol) glucose and then transferred to shake flasks containing 500 ml of cellulase-inducing medium. Samples were taken at different time points of cultivation. Culture media were separated from mycelia by filtration through GF/B glass microfiber filters (Whatman). The mycelia were stored at −70°C, and culture supernatants were kept at 4°C until

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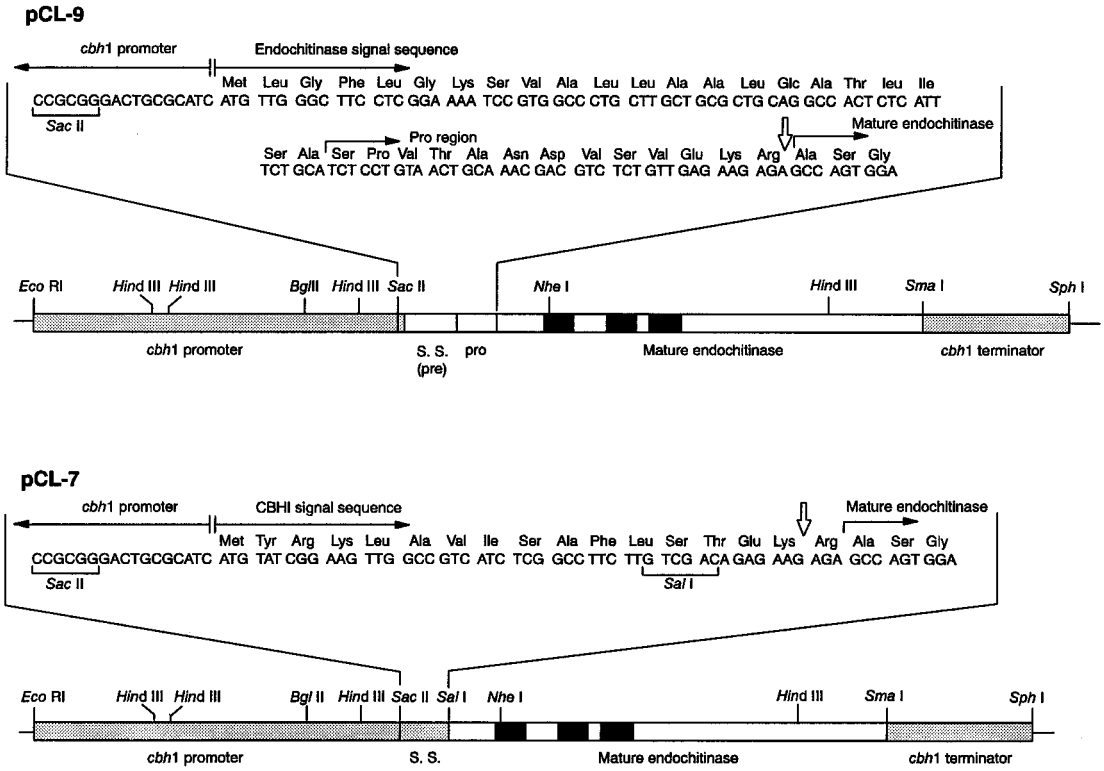


FIG. 1. Genetic constructions for endochitinase expression in *T. reesei*. Plasmid pCL-9 contains the fusion between the *cbh1* promoter and the prepro region of the endochitinase gene, and pCL-7 contains the fusion of the *cbh1* promoter and CBHI signal sequence (S. S.) with the mature endochitinase. Shaded area, *cbh1* sequences; solid area, endochitinase introns; open area, endochitinase coding region. The rest of the plasmid is vector pUC18. Open arrows indicate the processing sites of the secreted proteins, determined by N-terminal sequencing.

used. Cultivations to obtain mycelia for DNA isolation were done on *Trichoderma* MM containing 2% glucose.

**Genetic constructions.** The exact coding region of the endochitinase gene was amplified by PCR from the genomic DNA of *T. harzianum* P1 by using primers designed from the published endochitinase cDNA sequence (14). The first 21 nucleotides of the primer, 5′-AACCGCGGACTGCGCATCATGTTGGGCTT CCTCGGAAAATCCGTG-3′ (sense), correspond to the nucleotides from the *Sac*II site in the *cbh1* promoter up to the first ATG of the *cbh1* gene. A *Sma*I site was included in the antisense primer, 5′-GGCCCGGGCTAGTTGAGACCGC TTCGGATGTT-3′. Plasmid pCL-9 (Fig. 1) was generated by cloning the amplified *ThEn-42* gene (1,485 bp) between the *Sac*II and *Sma*I sites present in plasmid pEM-F5. Plasmid pEM-F5 was previously constructed to express the *cbh1* gene in *T. reesei* under the control of the *cbh1* promoter and terminator. This plasmid was obtained when the *Sac*II-*Sma*I fragment from plasmid pPLE-3 containing the *egl1* cDNA was replaced by the *cbh1* cDNA isolated with *Sac*II and *Sma*I from plasmid pTTc01 (25, 31).

A fragment of 405 bp containing part of the *cbh1* promoter and a region coding for the first 14 amino acids of the CBHI signal sequence was amplified from plasmid pEM-F5 with the primer 5′-GCCTAAGATCTCGGGCCCTCG GGC-3′ (sense), which amplifies from the *Bgl*II site located in the *cbh1* promoter, and the primer 5′-CCGGTCGACAAGAAGGCCGAGATGACGGCC-3′ (antisense), which includes nucleotide changes to generate a *Sal*I site. The *Sal*I site changes an Ala in the CBHI signal sequence to a Ser (Fig. 1). Further, a fragment of 325 bp containing the sequence coding for part of the mature endochitinase was amplified from plasmid pCL-9 with the primer 5′-CCGTCGACA GAGAAGAGAGCCAGTGGATACGCA-3′ (sense), which contains a *Sal*I site and the sequence coding for the last three amino acids of the endochitinase pro region, and with the primer 5′-GGGCTAGCATACAATCGTCGTCAT-3′ (antisense), which amplifies from the *Nhe*I site found in the first intron of the endochitinase gene. Plasmid pCL-7, encoding a fusion between the CBHI signal sequence and the mature endochitinase, was obtained when the amplified sequences were linked to plasmid pCL-9 digested with *Bgl*II and *Nhe*I (Fig. 1). DynaZyme DNA polymerase was used in all the PCRs described above, following the reaction conditions recommended by the manufacturer (Finnzymes Oy, Espoo, Finland). The restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim and used as recommended.

**Transformation of *T. reesei* RutC-30.** Fungal transformations were performed by the standard transformation method for *T. reesei*, selecting for $AmdS^+$ transformants (26). Plasmid p3SR2, which contains the *amdS* gene of *Aspergillus nidulans* (15), was used for selection.

**Western (immunoblot) analysis.** Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and blotted to nitrocellulose filters (32). Endochitinase was detected with antiendochitinase polyclonal antibodies (14), which were incubated before use with the culture supernatant of RutC-30 grown in cellulase-inducing medium to avoid a cross-reaction. Antibody reaction was detected by commercial alkaline phosphatase-labelled anti-rabbit immunoglobulin G (IgG; Sigma). CBHI was detected with the anti-CBHI-specific monoclonal antibody CI-89 (1) and afterwards recognized by commercial alkaline phosphatase-labelled anti-mouse IgG (Sigma). The staining was done with the Protoblot kit (Promega).

**Manipulation of nucleic acids.** Chromosomal DNA was isolated by phenol-chloroform extraction as described by Raeder and Broda (27). Total RNA was extracted by cesium chloride gradient centrifugation as described by Chirgwin et al. (5). Southern and Northern (RNA) blotting was performed by standard methods (29). Nylon membranes (Hybond; Amersham) were used in all nucleic acid hybridizations. The probes were labelled with [$\alpha$-$^{32}$P]dCTP (Amersham) with a random primer kit (Boehringer Mannheim). The relative amounts of mRNA were determined with a PhosphorImager SI (Molecular Dynamics), and the values given in the text were calculated as averages among all time points.

**Protein purification and enzyme assays.** Transformants were grown in inducing conditions as described above, and endochitinase was purified from the culture filtrate by gel filtration and electrofocusing as described before (13). The endochitinase activity was measured by reduction of turbidity of a suspension of colloidal chitin (13). Protease activity was assayed at pHs 3.7 and 5.5 with the artificial substrate azocasein (A2765; Sigma) and the procedure described previously (9).

**Protein determinations.** The protein concentration in the culture filtrate was determined by using Coomassie brilliant blue G-250 (Bio-Rad protein assay), with IgG as the standard. The protein concentration of the pure endochitinase was measured as the $A_{280}$, considering the theoretical extinction coefficient of 95,300 $M^{-1}$ $cm^{-1}$.

**Analysis of the N-terminal amino acid sequences.** The N-terminal amino acid sequence of the purified protein was determined at the University of Kuopio, Kuopio, Finland, with a model 477A protein sequencer equipped with a 120A analyzer (Applied Biosystems Inc., Foster City, Calif.).

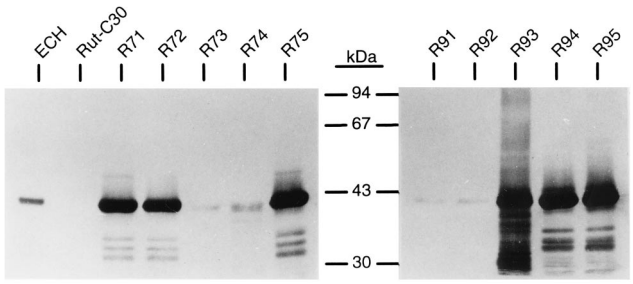


FIG. 2. Analysis of transformants producing endochitinase. Western blotting of the culture media of strains transformed with plasmids pCL-7 (R71 to R75) and pCL-9 (R91 to R95) and of the nontransformed host strain RutC-30 cultivated on cellulase-inducing medium for 4 days was done with antiendochitinase antibodies. Total extracellular protein (1 μg) from the cultivations and 20 ng of pure endochitinase from *T. harzianum* P1 (ECH) were loaded. The positions of molecular size markers are shown in the middle.

## RESULTS

**Construction and analysis of *T. reesei* strains producing endochitinase.** In order to produce the *T. harzianum* endochitinase in *T. reesei* under the control of the *cbh1* promoter, the genomic copy of the endochitinase gene *ThEn-42* was first amplified by PCR from the genomic DNA of *T. harzianum* P1 with primers designed on the basis of the published cDNA sequence (14). Sequencing of the gene revealed three introns of 56, 69, and 71 bp, starting at positions 216, 315, and 365, respectively, in the published cDNA sequence. The sizes and positions of the introns were similar to those found in a genomic endochitinase gene (*ech-42*) isolated from another *T. harzianum* strain (3).

In order to express the endochitinase with its own signal sequence, plasmid pCL-9 was made by linking the coding region of the endochitinase gene to 2.3 kb of the *cbh1* promoter and 0.7 kb of the *cbh1* 3′-flanking region comprising the transcription terminator (Fig. 1). In addition to a signal sequence, the *T. harzianum* endochitinase appears to contain a pro region of 13 amino acids, ending with two basic residues, Lys and Arg, which has been suggested to be a processing site for a KEX2-like protease (14). In order to test the possible need for the pro region in endochitinase production, plasmid pCL-7 was constructed, in which the mature part of the endochitinase was linked to the CBHI signal peptide (Fig. 1). In this construct, the KEX2-like processing site was maintained (Fig. 1).

The constructed plasmids pCL-7 and pCL-9 were separately cotransformed into *T. reesei* RutC-30 with plasmid p3SR2, which contains the selective marker *amdS*. Transformants were selected and spore purified on plates containing acetamide as the only nitrogen source. Twenty putative transformants from both constructions, pCL-7 and pCL-9, were grown in shake flasks on cellulase-inducing medium. The culture filtrates obtained after 4 days of cultivation were analyzed by Western blotting. Approximately 80% of the transformants produced endochitinase.

Five of the producing strains for each construct were chosen for further analysis, and cultivations were repeated as before. The endochitinase produced by the transformants comigrated with the enzyme purified from *T. harzianum* P1 in Western blots (Fig. 2). Comparison between the best producers from both constructions showed no significant differences in the amount of secreted enzyme. This indicates that the endochitinase can be secreted by *T. reesei* equally well with its own secretion sequence or with the CBHI signal peptide and that the pro region present in the native endochitinase seems to be dispensable.

The genomic DNAs of the transformants were analyzed by Southern blotting (Fig. 3). Signals of different sizes were observed in most of the transformants, suggesting that integration of more than one copy of the expression cassette had occurred at different places in the genome. Integration in a tandem fashion seems to have taken place, because intense signals of approximately the size of the plasmids (6.8 kb) can be seen in the *Bgl*II digestion, which linearizes plasmids pCL-7 and pCL-9. *Hin*dIII digestion is expected to give a single band of approximately 1.54 kb. In addition, other signals of different sizes appeared, indicating that integration also took place throughout the endochitinase gene, probably giving rise to inactive forms of the expression unit. The endogenous *cbh1* gene was intact in all the transformants analyzed (data not shown), showing that no integration had occurred at this locus.

By comparing the intensities of the signals obtained in Western blotting, the transformants can be grouped into low and high producers. The amount of endochitinase secreted seems to correlate with the copy number of the expression cassette,

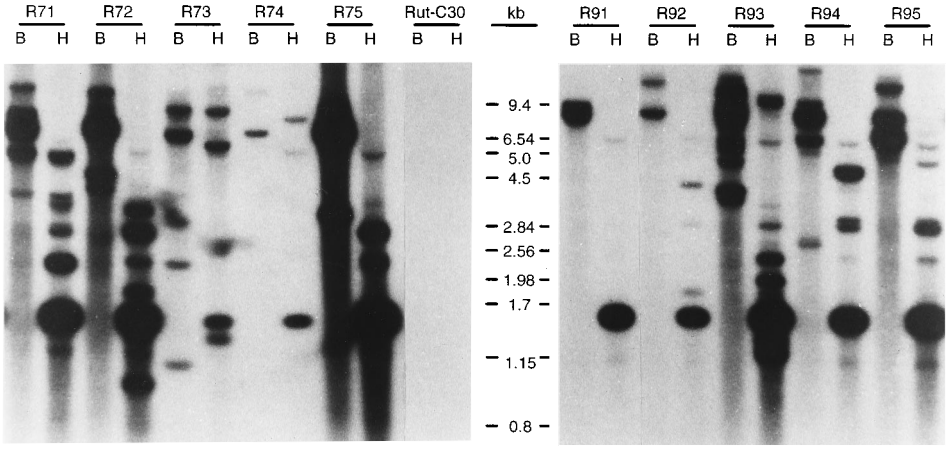


FIG. 3. Analysis of transformants producing endochitinase. Southern analysis of 2 μg of genomic DNA isolated from transformants R71 to R75 and R91 to R95 and the host RutC-30, digested with *Bgl*II (B) and *Hin*dIII (H), was performed. The *Sal*I-*Hin*dIII fragment of the endochitinase gene from plasmid pCL-7 was used as the probe (see Fig. 1). The positions of molecular size markers are shown in the middle.

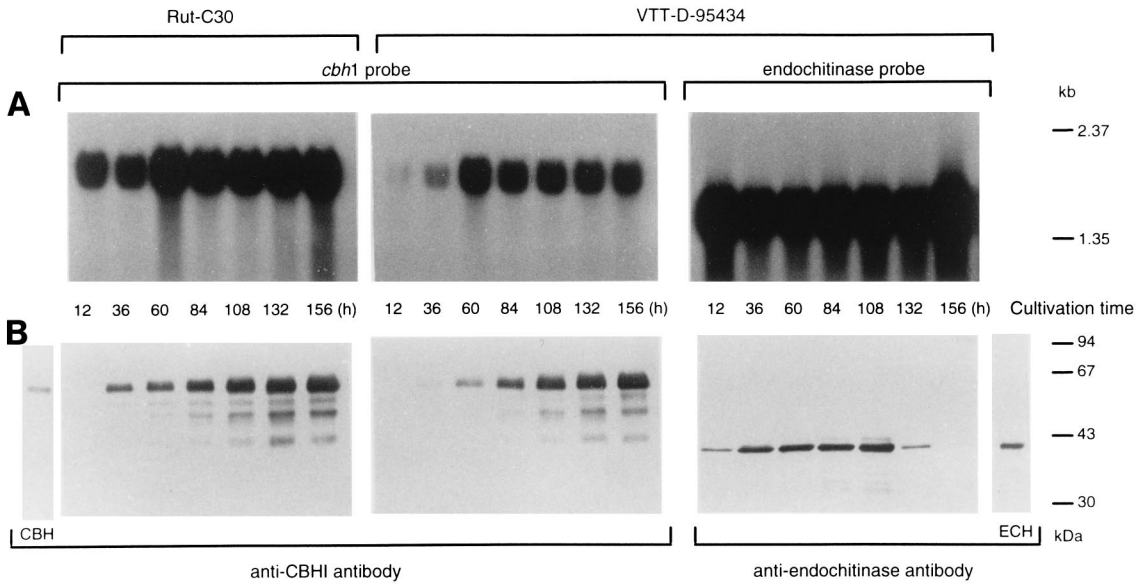


FIG. 4. Analysis of mRNA and secreted protein from the cultivation of strains VTT-D-95434 and RutC-30 for 156 h on cellulase-inducing medium. (A) Northern analysis of 2 μg of total RNA isolated from samples collected at different time points (12 to 156 h) of cultivation of RutC-30 and VTT-D-95434. RNA concentrations were carefully determined spectrophotometrically, and equal loading was further confirmed by acridine orange staining of the gel before blotting. The *Nco*I-*Bgl*II fragment (954 bp) from the *cbh1* gene and the *Pvu*II-*Sma*I fragment (942 bp) from the endochitinase gene were used as probes. The positions of molecular size markers are indicated. (B) Western blots of culture medium (10 μl of a 1:10 dilution) analyzed with anti-CBHI and antiendochitinase antibodies. As a control, 40 ng of pure CBHI (CBH) of *T. reesei* and 20 ng of pure endochitinase (ECH) from *T. harzianum* P1 were loaded. The positions of molecular size markers are indicated.

since transformants producing larger amounts of protein have more copies integrated into the genome.

**N-terminal processing of the endochitinase.** The endochitinase produced by one of the best producers transformed with each construct was purified. Strain R75 (VTT-D-95435), transformed with plasmid pCL-7, and strain R93 (VTT-D-95434), transformed with plasmid pCL-9, were grown in shake flasks, and the enzyme was purified from the culture filtrate (unpublished data). The N termini of the purified endochitinases were sequenced. The enzyme produced by strain VTT-D-95434 started with the same Ala (see Fig. 1) which has been shown to be the N-terminal amino acid in the mature enzyme secreted by *T. harzianum* (14). The endochitinase produced by strain VTT-D-95435 had been cleaved between, and not after as above, the two basic residues forming the KEX2-like processing site, leaving Arg at the N terminus. The reason for this is not known. The specific activity of both enzymes was determined, but no differences were observed (results not shown). Apparently the extra Arg at the N terminus has no effect on the endochitinase activity.

**Endochitinase expression in strain VTT-D-95434.** Endochitinase expression in strain VTT-D-95434, expressing the prepro form of the endochitinase, was studied in more detail. This strain and the nontransformed *T. reesei* RutC-30 were grown in shake flasks in 500 ml of cellulase-inducing medium for 156 h. Samples were collected at different time points for analyses of mRNA, protein, and growth characteristics.

No differences in growth rate between the strains were observed. Compared with the nontransformed strain, no changes in morphology of the hyphae or growing tips were apparent under the optical microscope in the endochitinase-producing strain (results not shown), not even when expression was maximal (see below). Thus, although *T. reesei* contains chitin in its cell wall, the production of endochitinase does not seem to be harmful to the host.

An aspect of interest in this work was how the expression of the endochitinase from the *cbh1* promoter compares with that of the endogenous *cbh1* and whether the presence of extra copies of the *cbh1* promoter in the transformant affects expression of the endogenous *cbh1* gene. Northern analysis showed that the endogenous *cbh1* mRNA levels appeared to be lower in the transformant than in RutC-30 at all time points analyzed (Fig. 4A), being approximately 2.5-fold lower, as quantified with the PhosphorImager (data not shown). The Northern results were confirmed by analysis of CBHI secreted into the culture medium (Fig. 4B). There was a good correlation in both strains between the level of *cbh1* mRNA and the amount of CBHI secreted.

Endochitinase expression was analyzed with a probe of approximately the same length and specific activity as the *cbh1* probe (Fig. 4A). The endochitinase gene was strongly transcribed in the early stages of growth, and its expression was high throughout cultivation. As anticipated, the intensity of the endochitinase-specific signals was stronger than that of the *cbh1* mRNA produced in the same strain, reflecting the fact that endochitinase was produced from several copies expressed under the *cbh1* promoter, while the endogenous *cbh1* is a single-copy gene. However, endochitinase mRNA levels were only approximately threefold higher than those of *cbh1* (data not shown), which is not in direct correlation with the copy number, remaining lower than expected, considering that the transformed strain contains at least 10 copies of the expression cassette.

Extracellular endochitinase production was monitored by Western blotting and activity measurements (Figs. 4B and 5A). The secreted endochitinase accumulated in the growth medium reaching a maximum at 108 h. On the basis of activity measurements, the calculated amount of endochitinase produced by the transformant at this time point was 130 mg/liter (considering the specific activity of 2.3 U/μg for purified *T. reesei*-produced endochitinase). Although the amount of endochitinase produced was large, it was not in accordance with

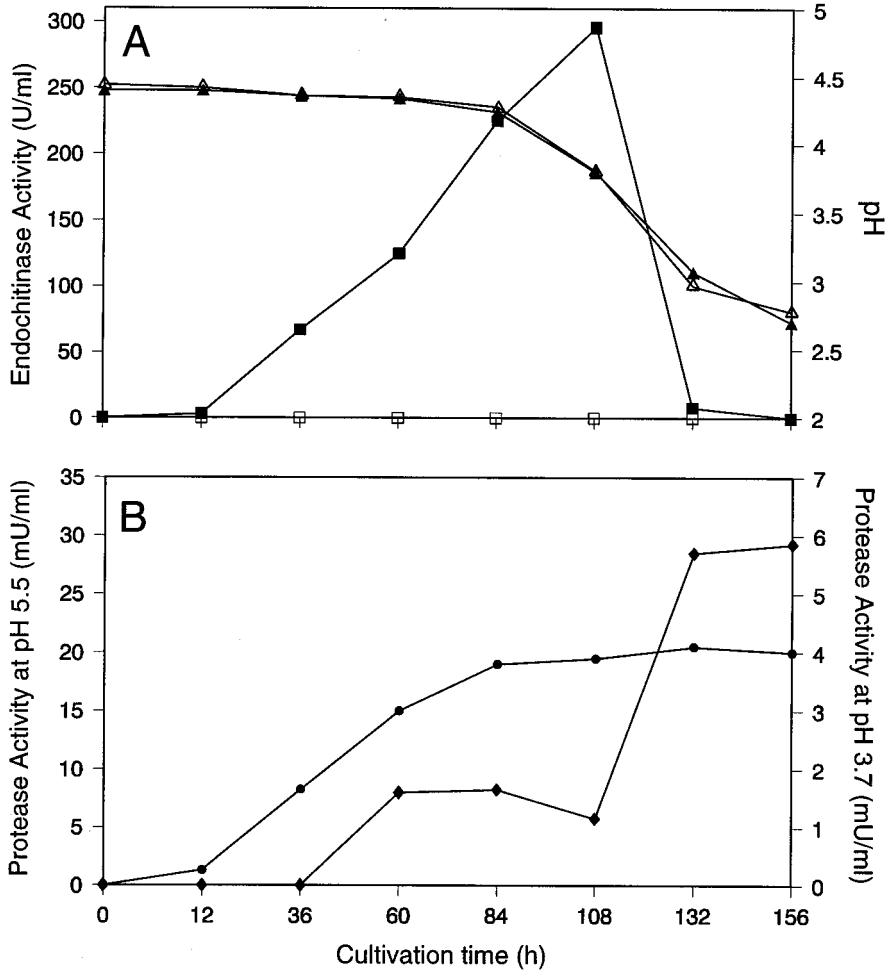


FIG. 5. (A) Production of endochitinase and change in culture pH as a function of cultivation time. Endochitinase activity was determined in the culture medium of VTT-D-95434 (■) and RutC-30 (□), and pH was determined in the culture medium of VTT-D-95434 (▲) and RutC-30 (△). (B) Protease activity at pH 3.7 (◆) and pH 5.5 (●) produced during cultivation of strain VTT-D-95434.

the steady-state mRNA levels if the ratio of endochitinase mRNA to protein is compared with that of CBHI. Although there was less *cbh1* mRNA in the transformant, and even in the nontransformed RutC-30 (approximately 30% lower; data not shown), than endochitinase mRNA, there was more extracellular CBHI protein than endochitinase. The absolute amounts of secreted protein cannot be properly estimated by Western blotting. However, it is known that RutC-30 produces more than 1 g of CBHI per liter in conditions similar to those used in this experiment (2). If comparable, the amount of endochitinase mRNA in the transformant should have yielded more than 1 g of secreted enzyme per liter.

**Stability of the endochitinase in *T. reesei* growth medium.** As mentioned earlier, the amount of endochitinase in the growth medium reached a maximum at 108 h of cultivation. After this time point, a remarkable decrease in endochitinase amount was observed at the last two time points of cultivation, 132 and 156 h (Fig. 4B and 5A), although at these time points, endochitinase mRNA levels were high (Fig. 4A). The decrease in activity coincided with a decrease in the culture pH (Fig. 5A). A similar change in pH occurred in the cultivation of the nontransformed strain RutC-30, which did not produce any detectable endochitinase activity (Fig. 5A). This indicates that the decrease in activity could be associated with a change in the physiological stage of the fungus not related to expression of endochitinase.

Extracellular proteolytic activity from cultivation of strain VTT-D-95434 was measured at pHs 5.5 and 3.7 (Fig. 5B). The activity determined at pH 5.5 increased gradually until 84 h of cultivation, after which it stayed more or less stable. On the other hand, the activity measured at pH 3.7 increased sharply at 108 h and remained high thereafter. This suggests that acidic protease activity occurred concomitantly with the change in the culture pH. Similar results were obtained when the proteolytic activity of the culture medium from the RutC-30 cultivation was analyzed (data not shown).

Although the most significant degradation of endochitinase was detected at the last two analysis points, measurable proteolytic activity was present throughout the cultivation (Fig. 5B). In order to test the stability of the endochitinase in the culture supernatant of *T. reesei*, purified endochitinase produced by strain VTT-D-95434 was incubated in filtered supernatants collected at different time points from the RutC-30 cultivation and analyzed by Western blotting (Fig. 6). The protein appeared to be very stable in the growth medium from 12 to 108 h, but no protein was detected in growth medium between 132 and 156 h. This confirms that from 108 h onwards,

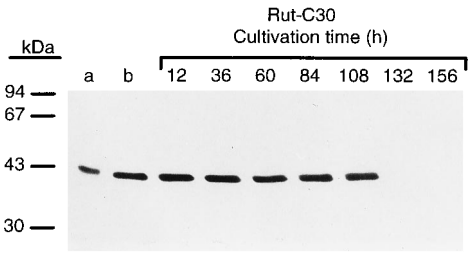


FIG. 6. Stability of endochitinase in the growth medium of *T. reesei* RutC-30. Endochitinase purified from *T. reesei* VTT-D-95434 was incubated at 100 μg/ml for 24 h at 28°C in 50 μl of filtered growth medium obtained at different time points (12 to 156 h) of RutC-30 cultivation on cellulase-inducing medium. $NaN_3$ (0.03%) was added to avoid microbial growth. After incubation, the samples were analyzed by Western blotting. Lane a, endochitinase not incubated (20 ng); lane b, endochitinase incubated for 24 h at 28°C in 50 mM potassium phosphate buffer (pH 6.7). Reaction volumes corresponding to 40 ng of endochitinase were loaded on the gel.

a pronounced protease activity and, from the data, an acidic protease lead to degradation of the endochitinase.

## DISCUSSION

Considerable amounts of *T. harzianum* endochitinase could be produced in active form by *T. reesei*. The endochitinase levels secreted by the best-producing strains analyzed in this work represent activities which are more than 20-fold higher than the endochitinase activities normally produced by *T. harzianum* P1 (data not shown).

The pro region of the endochitinase was processed similarly in *T. reesei* and *T. harzianum*, most probably by a Kex2-like protease. On the other hand, the presence of this pro region seems not to be essential for the production of the enzyme in *T. reesei*, which indicates that the region might be dispensable for production and secretion and seems to have no role, for instance, in reducing intracellular activity of the enzyme, which might be harmful for the producing host.

Strains containing multiple copies of the expression cassettes gave higher production levels. None of the best producers had the expression cassette integrated into the *cbh1* locus, which has previously been observed to give higher expression levels with the *cbh1* promoter (11, 12, 24, 28). It is possible that higher expression can be obtained by integrating the expression cassette into the *cbh1* locus and especially by simultaneous inactivation of expression of the endogenous *cbh1* gene. *cbh1* is one of the most highly expressed genes in *T. reesei* and gives rise to an abundance of secreted protein, which may set a significant load on the expression and secretion machinery of the cell.

A more detailed analysis of strain VTT-D-95434, which contains at least 10 copies of the expression cassette, gave indications of a partial inhibition of expression from the endogenous *cbh1* gene. Although the levels of endochitinase mRNA in this strain were higher than those of the endogenous *cbh1* mRNA produced by RutC-30, the endochitinase mRNA levels remained lower than expected on the basis of the copy number of the expression cassette. These results suggest that transcription and/or regulatory factors needed for full activity of the *cbh1* promoter might be limiting. This suggestion has also been reached earlier, proposing that three copies of this strong promoter could be sufficient to titrate out regulatory proteins or essential transcription factors (17). This kind of effect of promoter competition has also been observed, for instance, in the yeast *Saccharomyces cerevisiae*, in expression from the *GAL* promoter (23), and in *Aspergillus niger*, with the glucoamylase promoter (35). Overexpression of *trans* activator factors could overcome this limitation, as shown with overexpression of the transcriptional activator Gal4p in the *GAL* promoter-based expression system of *S. cerevisiae* (18, 30). On the other hand, the amount of secreted endochitinase produced by strain VTT-D-95434 was lower than that of CBHI, although the amount of endochitinase mRNA was higher. This indicates that transcription is not the major limiting factor for high expression of endochitinase.

At the later stages of cultivation, *T. reesei* produced an acidic protease associated with a decrease in pH, which may have degraded the endochitinase. The proteases produced by *T. reesei* and their mode of regulation are not well known, but there is some evidence that the availability of organic nitrogen and pH in the medium influences protease production, as is the case for *A. niger* (9, 16, 21). It is to be expected that removal of the proteolytic activity could lead to even higher amounts of endochitinase accumulation in the culture medium, because endochitinase mRNA is still abundant at the later stages of cultivation and significant extracellular accumulation of, for instance, CBHI still occurs at this production phase. Optimized fermentor cultivations usually increase production yields. It is noteworthy that by using the strains constructed in this work, production of endochitinase can be raised in fermentor cultivations to levels of grams per liter (10).

The antifungal activity of the chitinolytic enzymes of *T. harzianum* P1 has been tested against a number of fungal strains. Endochitinase caused morphological effects, including lysis of germ tubes, spores, and mycelia. Inhibition of growth accompanied by hyphal distortion and swelling was also observed when mixtures of endochitinase and chitobiosidase were used (19, 20). Perhaps surprisingly, no changes in growth or morphology of *T. reesei* were observed in the cultivations of the endochitinase-producing strains, although strain RutC-30 does not naturally produce endochitinase activity, as noticed in this work. The levels of active enzyme in the growth medium in the shake flask cultivations of the transformants (up to 130 mg/liter) are comparable to the inhibitory effect values obtained for most of the chitin-containing fungi tested previously (50% effective doses of 30 to 135 mg/liter) (19), and the levels obtained in the fermentor cultivation are even higher. Also, *T. harzianum* P1 appears to be very resistant to its own chitinases, as analyzed in in vitro assays. The production of a chitinase inhibitor in liquid medium and biochemical modifications of the cell wall composition have been suggested as possible reasons for this resistance (19). It is possible that during the process of transformation, we selected *T. reesei* strains with tolerance to endochitinase. This is, however, unlikely, because no difference was observed in the number of transformants obtained when RutC-30 was transformed with the selection plasmid only or when it was cotransformed together with the endochitinase constructions. In vitro experiments to determine the resistance of *T. reesei* strains to endochitinase are in progress.

In conclusion, *T. reesei*, for which there are well-developed molecular tools for protein production and established conditions for industrial-scale enzyme production, seems to provide an efficient host for overproduction of a chitinolytic enzyme that is potentially harmful to fungal producing hosts. Production in *T. reesei* allows easier purification of large quantities of the enzyme for various application trials. The fact that *T. reesei* also produces a great array of glucanases and cellulases in the inducing conditions used here to express endochitinase could have special advantages, for instance, in biocontrol applications, since the synergistic activity of chitinolytic and glucanolytic enzymes plays a major role in the degradation of cell walls

of plant pathogens (13, 20). Because of the large amounts of cellulases produced by the fungus, the enzyme mixture now produced might prove useful against a broad spectrum of plant-pathogenic fungi, including oomycetes, which have cellulose as a cell wall component and lack appreciable amounts of chitin.

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