

Identification and Characterization of the *Trichoderma harzianum* Gene Encoding α -1,3-Glucanase Involved in Streptococcal Mutan Degradation

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Abstract

α -1,3-Glucanases (mutanases) are currently of great interest due to their potential use in the field of dental care. These enzymes have been reported in several bacteria, yeasts and fungi, but up to now, characterization of this family of proteins has been relatively poor. In this study, we identify and characterize a mutanase gene from *Trichoderma harzianum* CCM F-340. Sequence analysis, on the nucleotide and amino acid levels reveals that this α -1,3-glucanase is highly homologous to α -1,3-glucanases from *T. harzianum* isolate CBS 243.71 and *T. asperellum* CECT 20539. *T. harzianum* CCM F-340 mutanase is a 634-aa residue protein with a calculated molecular mass of 67.65 kDa, composed of two distinct, highly conserved domains (a long N-terminal catalytic domain and a short C-terminal polysaccharide-binding domain) separated by a less conserved Pro-Ser-Thr-rich linker region. The mutanase gene was expressed in an *E. coli* BL21(DE3) host, under the transcriptional control of T7 promoter. The purified enzyme migrated as a band of about 68 kDa after SDS-polyacrylamide gel electrophoresis, which coincided with the predicted size based on the amino acid sequence. Our data indicate that this enzyme is highly conserved in *Trichoderma* and can be produced in active form in such heterologous expression system.

Key words: *Trichoderma harzianum*, α -1,3-glucanase, cloning, expression, mutanase

Introduction

Fungal species of the *Trichoderma* genus are soil-borne, green-spored ascomycetes, found all over the world (Schuster and Schmoll, 2010). *Trichoderma* spp. have been used as biological control agents against a wide range of phytopathogenic fungi for many years (Papavizas, 1985), *Trichoderma harzianum* being especially effective. After host recognition, *Trichoderma* spp. attach themselves to the host hyphae *via* coiling, and penetrate the cell wall by secreting cell wall-degrading enzymes (CWDEs) (Viterbo *et al.*, 2002). One of these enzymes, α -1,3-glucanase, a so-called mutanase, is also a useful tool in dentistry.

Mutanases (exo- α -1,3-glucanase: E.C. 3.2.1.84; endo- α -1,3-glucanase: E.C. 3.2.1.59), both fungal and bacterial, have the ability to hydrolyse α -1,3-glucosidic bonds in streptococcal mutans and to remove dental as well as denture plaques (Budtz-Jørgensen and Kelstrup, 1977; Inoue *et al.*, 1990). Therefore, mutanases could be applied as active ingredients in mouthwash,

toothpaste, dental gel or chewing gum to prevent the accumulation of glucose biopolymers. Mutanases might be useful in preventive oral hygiene, but their molecular characterization is limited. Previously, mutanases have been reported in bacteria, such as *Bacillus circulans* (Matsuda *et al.*, 1997), *Paenibacillus* spp. (Pleszczyńska *et al.*, 2007), *Flavobacterium* spp. (Ebisu *et al.*, 1975) and *Microbispora rosea* (Chung *et al.*, 2001); yeasts, such as *Schizosaccharomyces pombe* (García *et al.*, 2005), *Endomyces tetrasperma* (Meyer and Phaff, 1977), *Cryptococcus albidus* (Meyer and Phaff, 1977) and *Rhodotorula minuta* (Meyer and Phaff, 1977); and filamentous fungi, such as *Aspergillus nidulans* (Wei *et al.*, 2001), *Cladosporium resinae* (Walker and Hare, 1977), *Penicillium purpurogenum* (Fuglsang *et al.*, 2000; Shalom *et al.*, 2008), *Penicillium funiculosum* (Reese *et al.*, 1972), *Trichoderma asperellum* (Sanz *et al.*, 2005), *Trichoderma viride* (Hasegawa *et al.*, 1969), *Trichoderma harzianum* (Wiater and Szczodrak, 2002) and *Verticillium malt-housei* (Reese *et al.*, 1972). Some microbial mutanase genes have been cloned in the past few years. Several

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of these genes are from fungi, including *A. nidulans* (Wei et al., 2001), *P. purpurogenum* (Fuglsang et al., 2000; Shalom et al., 2008), *T. asperellum* (Sanz et al., 2005) and *T. harzianum* (Fuglsang et al., 2000; Ait-Lahsen et al., 2001).

The aim of this study was cloning of the α -1,3-glucanase gene from *T. harzianum* CCM F-340 and its expression in a heterologous *Escherichia coli* expression system using a T7 promoter. Certain properties of the heterologous expressed enzyme were also investigated.

Experimental

Materials and Methods

Strain cultivation. *Trichoderma harzianum* CCM F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) isolate was cultivated in a liquid medium containing 10 g/l peptone and 15 g/l malt extract (pH 6.0). The culture was maintained in this medium at 30°C for 14 days without shaking. An *E. coli* strain TOP10F⁺ (*F*⁺ *mcrA* Δ (*mrr*-*hsdRMS*-*mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^R) *enda1* *nupG*) (Invitrogen, USA) was used as a host for plasmid construction and cultivated in LB medium (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0). For *E. coli* derivatives harboring plasmids pJET1.2 and pET15b, the LB medium was supplemented with ampicillin (100 μ g/ml). The expression strain *E. coli* BL21(DE3) *plysS* (*F*⁻, *ompT*, *hsdS_B* (*r_B*⁻, *m_B*⁻), *dcm*, *gal*, λ (DE3), *plysS*, Cam^R) (Novagen, USA) was cultivated as described in the "Expression of the recombinant *T. harzianum* α -1,3-glucanase" section (see below).

DNA methods and sequence analysis. Standard techniques were used for genomic and plasmid DNA isolation, agarose gel electrophoresis, restriction enzyme digestion, cloning and transformation (Sambrook et al., 1989). For standard PCR amplifications, DNA polymerase (Hypernova, Poland) was used. Sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, UK) and the ABI Prism 310 sequencer. Database searches were conducted with the BLAST and FASTA programs available at the National Center for Biotechnology Information (Bethesda, USA) and the European Bioinformatic Institute (Hinxton, UK). For phylogenetic tree construction, sequences of mutanases from *T. harzianum* and other related organisms were aligned using ClustalX software. Phylogenetic analysis was performed using the PhyML 3.0 program for maximum likelihood analysis.

Gene isolation. After 14 days of *T. harzianum* growth, total RNA from the microorganism pellet was isolated using the Total RNA isolation kit (A&A Bio-

technology, Poland) according to the manufacturer's instructions. The isolated RNA was used in reverse transcription reaction where oligo(dT)₁₅ served to prime the synthesis of the first cDNA strand. On the basis of the N-terminal amino acid sequence of the native mutanase from *T. harzianum*, degenerated primer TrihN1 (5'-GTIIIICAYTTYTTYATGATHATHGTIGGIGGIGAY-3') was designed. RT-PCR reaction was performed on the template of 1 μ g total RNA using the set of primers TrihN1-terminal and oligo(dT)₁₅ and a SMART cDNA library construction kit (Clontech, USA). PCR reaction was set in the mixture containing: 0.2 μ M of TrihN1 and oligo(dT)₁₅ primers, 0.2 μ g of cDNA, 250 μ M of each dNTP, 1 U of DNA polymerase (Hypernova, Poland) in 1 \times PCR buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 3.4 mM MgCl₂, 0.15% Triton X-100). The reaction mixture was incubated for 3 min at 95°C, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final incubation for 5 min at 72°C using a Mastercycler Gradient (Eppendorf, Germany). The resulting PCR product was subcloned into pJET1.2 vector (CloneJet kit, Fermentas, Lithuania) giving plasmid pJET1.2T1, and the insert of this plasmid was sequenced.

On the basis of the obtained DNA sequence for mutanase gene, more specific primers Trih2 (5'-CTGG GCAGGGCGGTGGCGTAC-3') and Trih3 (5'-GGA GCCTGCAGCAGA GCGCAACGC-3') were designed to synthesize the first cDNA strand with the leader sequence of the gene. These primers were used instead of the oligo(dT)₁₅ primer. All further manipulations were performed with a SMART cDNA library construction kit (Clontech, USA) according to manufacturer's instruction. The first cDNA strand was synthesized using the SmartIV oligonucleotide (5'-AAGCAGTG GTATCAACGCAGAGTGGCCATTACGGCCGGG-3') and the Trih2 primer. Then, dsDNA synthesis was performed using the primer pair: 5'PCR (5'-AAGCAGTG GTATCAACGCAGAGT-3') and the Trih2 in the PCR mixture containing 0.2 μ M of each primer, 0.2 μ g of the first strand cDNA, 250 μ M of each dNTP, 1 U of DNA polymerase in 1 \times PCR buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 3.4 mM MgCl₂, 0.15% Triton X-100). The reaction mixture was incubated for 3 min at 95°C, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final incubation for 5 min at 72°C. The PCR product (447 bp) containing cDNA with the signal sequence for mutanase was subcloned into pJET1.2, resulting in pJET1.2T2A plasmid, and sequenced. In addition, in order to determine the possible presence of introns in the native mutanase gene, genomic DNA fragment encoding the discontinuous gene was isolated. The genomic DNA was isolated from cell pellet by use of Yeast Genomic DNA isolation kit (A&A Biotechnology, Poland). PCR reaction

was carried out using *T. harzianum* genomic DNA as a template and oligonucleotide primers Trih3B (5'-GCTTCTTCTGCTGACCGTCTCGTATTC-3') and Trih4H (5'-TTTTAAGCTTCTAGCAGTATTGACATGCCGTTGGCGGG-3'). The cycle conditions for this reaction mixture were the same as for the primer pair: TrihN1 and oligo(dT)₁₅. The resulting PCR product (2200 bp) was subcloned into pJET1.2 vector giving pJET1.2T2 plasmid, and sequenced. The presence of introns in the mutanase gene was determined after a comparison of the full-length gene sequence with its cDNA sequence.

Expression plasmid construction. The *T. harzianum* mutanase gene without the leader sequence was PCR amplified by using as a template the pJET1.2T1 plasmid and a set of primers: Trih4H (5'-TTTTAAGCTTCTAGCAGTATTGACATGCCGTTGGCGGG-3') and Trih5 (5'-ATGGCTTCTTCTGCTGACCGTCTCGTATTC-3'). PCR amplification was set in mixture containing 0.2 μ M of each primer, 0.2 μ g pJET1.2T1, 250 μ M of each dNTP, 1 U of DNA polymerase in 1 \times PCR buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 3.4 mM MgCl₂, 0.15% Triton X-100). The reaction mixture was incubated at the same temperature conditions as mentioned above for the primer pair: Trih3B and Trih4H. The 1849-bp-long amplicon was digested with *Hind*III endonuclease and cloned into *Hind*III site and previously filled with Klenow polymerase *Nco*I site of pET15b vector (Novagen, USA). The resulting pET15bTM recombinant vector was transformed into competent cells of *E. coli* BL21(DE3) plysS strain (Novagen, USA) using a standard procedure (Sambrook *et al.*, 1989).

Expression of the recombinant *T. harzianum* α -1,3-glucanase. An *E. coli* BL21(DE3) plysS strain harboring the pET15bTM recombinant plasmid was grown overnight in LB broth containing 1% of glucose supplemented with ampicillin (100 μ g/ml) at 37°C in an air shaker at 220 rpm. Expression broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing the same antibiotic was inoculated with the preculture (1%, v/v). Cultivation was continued at 37°C to OD₆₀₀ of 0.3. Then, the culture was supplemented with isopropyl- β -D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, USA) to 1 mM final concentration. The expression from *E. coli* BL21(DE3) plysS containing pET15bTM was performed at 16°C for 16 h on a rotary shaker at 220 rpm. The expression of the α -1,3-glucanase gene at higher temperatures did not result in the synthesis of the target protein (data not shown).

Purification of the recombinant *T. harzianum* α -1,3-glucanase. After α -1,3-glucanase expression in an *E. coli* host, cells were harvested by centrifugation at 5 000 \times g for 20 min, the cell pellet was resuspended in 50 ml of buffer A (20 mM K₂HPO₄-KH₂PO₄, pH 7.5, 50 mM KCl) and frozen at -20°C for 15 min. After

thawing at room temperature, the samples were sonicated (6 \times 30 s with 59 s intervals on ice, Sonics Vibra-Cell VCX 130) and centrifuged at 10 000 \times g for 30 min. The supernatant containing the desired protein was applied onto anion exchange resin DEAE-Sepharose (GE Life Science, Sweden) equilibrated with four volumes of buffer A. The protein was collected as the flow-through fraction. The mutanase present in this fraction was under pressure concentrated 10-times by membrane filters MWCO 30 000 (Vivaflow2, Sartorius GmbH, Germany). The protein was then purified by gel filtration (Size Exclusion Chromatography) (Sephadex 200 26/200 on a column equilibrated with buffer D (20 mM KH₂PO₄-K₂HPO₄, 150 mM KCl, pH 7.5) (GE Life Science, Sweden). The fraction containing the purified protein was collected and lyophilized.

Enzyme activity assay and determination of protein concentration. The recombinant enzyme was analysed for its activity in mutan hydrolysis by a modified Wiater *et al.* (2008) method as described previously, where 0.1% (w/v) mutan was used as a substrate in the hydrolysis reaction carried out in 50 mM acetate buffer solution (pH 5.5) at 37°C for 1 hour. The protein concentration was assessed by the Bradford method (Bradford, 1976), using bovine serum albumin as standard, with absorbance measured at 595 nm.

Results and Discussion

Isolation of *T. harzianum* α -1,3-glucanase gene and construction of the expression plasmid. In order to isolate the α -1,3-glucanase gene from the *Trichoderma harzianum* CCM F-340 genome, degenerate primer TrihN1 was designed, based on the N-terminal amino acid sequence of the enzyme produced by the isolate. The corresponding 5'-end of the mutanase gene primer and oligo(dT)₁₅ primer were used in RT-PCR reaction with total RNA of *T. harzianum* to obtain cDNA of the mutanase gene without introns or leader sequence. The obtained PCR product was cloned into pJET1.2 vector resulting in pJET1.2T1 plasmid, and was then sequenced. Based on the sequence of this DNA fragment, additional reverse Trih2 and forward Trih22 primers were designed. A DNA fragment containing the leader sequence of the gene was obtained using these primers and SMART technology from Clontech. A full-length mutanase gene termed *mutAW* was obtained by combining sequences of these two PCR fragments.

To investigate whether the mutanase gene from *T. harzianum* CCM F-340 isolate contained introns, DNA fragment containing the full-length gene was amplified by PCR from the organism's genomic DNA. The comparison of this sequence with the sequence of the mutanase cDNA revealed the presence of

two introns. They were 52-bp and 43-bp in length, at 570 bp (intron 1), sequence: 5'-TAAGTCGCAACTT-TAGAGTCGAGAGCAGGGGAGCAATACTGAT-GTCTTTATA-3'; and at 1778 bp (intron 2), sequence: 5'-GCCCCCATAACCCTTACTTGCTTCCTTAAC-TAATCCTTCATAG-3', respectively. The nucleotide sequence of *mutAW* and the deduced amino acid sequence of the mutanase from *T. harzianum* CCM F-340 are presented in Figure 1.

The entire mutanase-encoding gene is 2000-bp long while the cDNA lacking both introns contains a 1905-bp open reading frame initiating with an ATG codon and terminating with TAA stop codon at nucleotide position 1903. It encodes a protein of 634 amino acids. The first 111 nucleotides at 5'-end of the gene are predicted to encode a 37-aa-long signal peptide. The sequence of the *T. harzianum* CCM F-340 mutanase gene *mutAW* has been deposited in the GenBank database (accession no HQ871941). The molecular mass for the proenzyme calculated from the amino acid sequence and of the mature enzyme was 67.65 kDa and 63.86 kDa, respectively. Based on the amino acid sequence, the isoelectric point of the mutanase was estimated to be 5.68.

The nucleotide sequence of the *T. harzianum* CCM F-340 *mutAW* was analyzed and aligned with sequences of homologous genes available at GeneBank (Fig. 2). The gene displayed 97.6% identity to the deposited sequence of a mutanase gene from another *T. harzianum* isolate CBS 243.71 (accession no AJ243799), 96.2% identity to a homologous gene from *Hypocrea lixii* (accession no HC050999), 82.8% identity to *T. asperellum* homolog (accession no AJ784420) and 72.7% identity to *P. purpurogeum mutA* gene (accession no EU341820). The amino acid sequence of the protein encoded by the *T. harzianum mutAW* gene shared significant homology with proteins belonging to the glycohydrolase-71 superfamily, corresponding to the family of α -1,3-glucanases. MutAW protein showed the highest sequence homology (98% identity and 99% similarity, respectively) to the mutanase of *T. harzianum* isolate CBS 243.71 (accession no. CAC80493) (Fig. 2). A lower homology of this protein to α -1,3-glucanases from *T. asperellum* (88% identity and 94% similarity) (accession no CAH04820), *Penicillium marneffei* ATCC 18224 (60% identity and 71% similarity) (accession no EEA20869) and *P. purpurogenum* (56% identity and 70% similarity) (accession no AAF27912) was observed. The sequence analysis revealed that *T. harzianum* CCM F-340 mutanase contains two distinct domains: C-terminal domain responsible for binding of the polysaccharide substrate (mutan) and a long N-terminal catalytic domain. This finding concurs with the data described by Fuglsang *et al.* (2000), where authors demonstrated that mutanases from *T. harzianum* CBS 243.71 and *P. purpurogenum* CBS 238.95 are composed

of two distinct domains: a N-terminal catalytic domain and C-terminal polysaccharide-binding domain, separated by an O-glycosylated Pro-Ser-Thr-rich linker peptide. Similarly to the *T. harzianum* CCM F-340 mutanase gene identified by us, α -1,3-glucanase cDNA from *T. harzianum* CBS 243.71 encoded a 634-aa-residues protein with 37-aa-long signal peptide. In contrast, *P. purpurogenum* CBS 238.95 mutanase gene contained four introns and its compiled coding sequence encoded a protein of 630 aa, with 30-aa signal peptide (Fuglsang *et al.*, 2000). On the other hand, cDNA for mutanase from another isolate of *P. purpurogenum* CBS 257.37 was only 1803-bp long and encoded a protein of 600 aa (63.5 kDa) (Shalom *et al.*, 2008), demonstrating a significant diversity among mutanase genes on both the genomic and protein levels. The N-terminal catalytic domain of *T. harzianum* mutanase encompasses most of the protein length (region 1-453 aa). However, the mutan-binding C-terminal domains of the enzymes from these two species are relatively short and contain two regions of internal similarity (Fig. 2) (Fuglsang *et al.*, 2000). In *T. asperellum* genome *agn13.2* gene was identified and shown to encode α -1,3-glucanase of 635 aa (Sanz *et al.*, 2005). The coding sequence of *A. nidulans mutA* gene homologous to *T. harzianum* CCM F-340 *mutAW* was interrupted by three introns and encoded a protein of 642 aa with predicted N-terminal 22-aa-long secretion signal. It was established that the *mutA* gene was expressed in this organism during sexual development (Wei *et al.*, 2001).

Using the amino acid sequences of several mutanases available from Genbank, we performed a phylogenetic analysis of *Trichoderma* species and other closely related genera (Fig. 3). In general, the compared α -1,3-glucanases from different *Trichoderma* species and the other closely related organisms demonstrate significant diversity with respect to the amino acid sequence length and the level of homology. The constructed tree shows that the microorganisms from *T. harzianum* form a tight cluster and are most closely related to *T. asperellum* species (Fig. 3) (Sanz *et al.*, 2005). Among other analyzed organisms, *T. harzianum* mutanase was the most similar to those of *Neurospora crassa*, *P. chrysogenum* and *Aspergillus flavus*, but more different from those of *A. nidulans*, *P. purpurogenum*, *P. marneffei* and *Talaromyces stipitatus* (Fig. 3) (Fuglsang *et al.*, 2000; Wei *et al.*, 2001).

Expression and purification of the recombinant *T. harzianum* α -1,3-glucanase. In nature, *T. harzianum* produces and secretes the mutanase to the environment upon induction by α -1,3-glucan. To produce this enzyme at a constitutive level, independent of such inductors, a recombinant mutanase was generated in the *E. coli* BL21(DE3) *plyS* expression system. For this purpose, the DNA fragment containing the

M L G V F R R L R L G A L A A A A L S S L G S A
 1 ATGTTGGGCG TTTTCCGCGC CCTCAGGCTC GGCGCCCTTG CCGCCGCAGC TCTGTCTTCT CTCGGCAGTG
 A P A N V A I R S L E E R A S S A D R L V F C
 71 CCGCTCCCGC CAATGTGCT ATTCGGTCTC TCGAGGAACG TGC TTCTTCT GCTGACCGTC TCGTATTCTG
 H F M I G I V G D R G S S A D Y D D D M Q R A
 141 TCACTTCATG ATTGGTATCG TTGGTGATCG TGGCAGCTCG GCAGACTATG ATGACGATAT GCAACGTGCC
 K A A G I D A F A L N I G V D G Y T D Q Q L G Y
 211 AAAGCCGCTG GCATTGACGC CTTGCGCCCTG AACATCGGCG TTGACGGCTA TACCACCAG CAGCTCGGAT
 A Y D S A D R N G M K V F I S F D F N W W S P
 281 ATGCTTATGA CTCGCGGAT CGTAATGGCA TGAAAGTCTT CATCTCATTC GATTCAATT GGTGGAGCCC
 G N A V G V G Q K I A Q Y A N R P A Q L Y V D
 351 CGGCAATGCA GTTGGTGTG GCCAGAAGAT TGCGCAGTAC GCCAACCGCC CTGCCAGCT GTATGTCGAC
 N R P F A S S F A G D G L D V N A L R S A A G S
 421 AACCGACCAT TCGCCTCTTC CTTGCGCCGT GACGGTCTAG ATGTAAATGC GTTGCCTCT GCTGCAGGCT
 N V Y F V P N F H P G Q S S P S N I D G A L N
 491 CCAACGTTTA CTTTGTTCCT AACTTCCACC CTGGCCAATC TTCTCCCTCA AACATTGATG GTGCCCTTAA
 W M A
Intron 1
 561 CTGGATGGCG TAGTCGCAAC TTAGAGTCGA GAGCAGGGGA GCAATACTGA TGCTTTTATA G
 W D N D G N N K A P K P G Q T V T V A D
 622 C TGGGATAATG ATGGAAACAA CAAGGCACCC AAGCCGGGCC AGACTGTCAC GGTGGCAGAC
 G D N A Y K N W L G G K P Y L A P V S P W F F T
 683 GGTGACAACG CTTACAAGAA TTGGTGGGT GGAAGCCTT ACCTAGCGCC TGCTCACCT TGGTTTTTTA
 H F G P E V S Y S K N W V F P G G P L I Y N R
 753 CCCATTTCCG CCCGAAGTT TCATACTCCA AGAACTGGGT CTTCCAGGT GGTCTCTGA TCTACAACCG
 W Q Q V L Q Q G F P M V E I V T W N D Y G E S
 823 GTGGCAACAG GTC TTGCAGC AGGGCTTCCC CATGGTTGAG ATCGT TACCT GGAATGACTA CCGCGAGTCT
 H Y V G P L K S K H F D D G N S K W V N D M P H
 893 CACTACGTCG GTCCTACTAAA GTCTAAGCAT TTTCGATGAT GCAACTCCAA ATGGTCAAT GATATGCCCC
 D G F L D L S K P F I A A Y K N R D T D I S K
 963 ATGATGGATT CCTGGATCTT TCAAAGCCGT TTATTGCTGC GTATAAGAAC AGGGATACCG ACATCTCCAA
 Y V Q N E Q L V Y W Y R R N L K A L D C D A T
 1033 GTATGTTCAA AATGAGCAGC TTGTTTACTG GTACCGCCGC AAC TTGAAGG CATTGGACTG CGAGCCACC
 D T N S N R P A N N G S G N Y F M G R P D G W Q
 1103 GACACCCT CTAACCGTCC AGCTAATAAC GGAAGTGGTA ATTACTTTAT GGGACGTCCT GATGGTTGGC
 T M D D T V Y V A A L L K T A G S V T V T S G
 1173 AAATATGGA TGACACCGTT TATGTTGCCG CACTTCTCAA GACTGCGGT AGCGTCACTG TCACGTCTGG
 G T T Q T F Q A N A G A N L F Q I P A S I G Q
 1243 CGGACCCT CAAACGTTCC AGGCCAACGC CGGAGCCAAC CTC TTCCAAA TCCCAGCCAG CATCGGCCAG
 Q K F A L T R N G Q T V F S G T S L M D I T N V
 1313 CAAAAATTCG CTC TAACTCG CAATGGTCAG ACCGTC TTTA GTGGAACCTC ATTGATGGAT ATCACCACCG
 C S C G I Y N F N P Y V G T I P A G F D D P L
 1383 TTTGCTCTTG CGGTATCTAC AACTTCAACC CATATGTTGG CACCATTCCT GCCGGCTTTG ACGACCCTCT
 Q A D G L F S L T I G L H V T T C Q A K P S L
 1453 ACAGGCTGAT GGTCTTTCT CT TTGACCAT CGGATTGCAT GTCACAAC TT GTCAGGCCAA GCCATCTCTT
 G T N P P V T S G P V S S L P A S S T T R A S S
 1523 GGAACCAACC CTCCTGTCAC TTCCGGCCCT GTGTCTCGC TTCCAGCTTC CTCCACCACC CGCGCATCCT
 P P P V S S T R V S S P P V S S P P V S R T S
 1593 CGCCGCTCC TGT TCTTCC ACTCGTGTCT CTTCTCCCC TGTCTCTCC CCTCCAGTTT CTCGCACC TC
 S T P P P A S S T P P S G Q V C V A G T V A D
 1663 TTCCACTCCC CCTCCAGCGA GCAGCAGGCC GCCATCGGGT CAGGTTGCG TTGCCGTAC CGTTGCTGAC
 G E S G N Y I G L C Q F S C N
 1733 GCGAGTCTG GCAACTACAT CGGCCGTGTC CAATTCAGCT GCAA GTCCC CATAACCCTT ACTTGC TTCC
Intron 2
 1803 TTAAC TAATC CTTCATAGTA Y G Y C P P G P C K C T A F G A P I
 N P P A S N G R N G C P L P G E G D G Y L G L
 1873 TCAACCACC GGCAAGCAAT GGC CGCAACG GCTGCCCTCT GCCGGGAGAA GGC GATGGTT ATCTGGGCTT
 C S F S C N H N Y C P P T A C Q Y C *
 1943 GTGCAGTTTC AGTTGTAACC ATAATTACTG CC CCCAACG GCATGTCAAT ACTGCTAAGA GAGAGATCAA
 2013 TCTTAGTATG AGTATATGGA GCGTGC CGAA GGACCAGTTA GCTGTTCTTA TCGGTAGACG AAACCCATAG
 2083 AGCAAGAAAT TAAA TAAAAT GCAATTAATT TGTTGTCAAT TCTGTTGCA TATACATGTA TATACCC TGA
 2157 ACATCATAAC ACCCTCCCG GAT

Fig. 1. The nucleotide sequence of the entire *T. harzianum* CCM F-340 *mutAW* gene.

Amino acid sequence of α -1,3-glucanase is presented as a single letter code. The leader sequence encoding the signal peptide is underlined and the sequences of introns 1 and 2 in the full-length gene are marked by boxes. The putative linker Pro-Ser-Thr region is highlighted in grey. This sequence has been deposited in the GenBank database and its accession number is HQ871941.

<u>Signal peptide</u>		
(1)	<u>M---LGVFRRRLRGALAAAALSSLGSAAPANVAIRSLEERASSADRLVFCHEMIGIVGDR</u>	<i>T.h.</i>
(1)	<u>M---LGVFRRRLRGALAAAALSSLGSAAPANVAIRSLEERASSADRLVFCHEMIGIVGDR</u>	<i>H.I.</i>
(1)	<u>MLpkLNLVRRRLGLGALAATALSSLSAAPANVAIRSLEERASSADRLVFCHEMIGIVGDR</u>	<i>T.a.</i>
(1)	<u>M-----IVKGLFLAALSA--MSALVQGYPGQ--ERLSERQST--DRLVFAHFMIGITSDR</u>	<i>P.m.</i>
(1)	<u>M-----KVSSAFAATLSAIIAACA--LPSDSMVSRSTSDRLVFAHFMVIGIVSDR</u>	<i>P.p.</i>
(58)	<u>GSSADYDDDMQRAKAAGIDAFALNIGVDGYTDQQLGYAYDSADRNGMKVVISFDFNWWSP</u>	<i>T.h.</i>
(58)	<u>GSSADYDDDMQRAKAAGIDAFALNIGVDGYTDQQLGYAYDSADRNGMKVVISFDFNWWSP</u>	<i>H.I.</i>
(61)	<u>GSSSDYDDDMQRAKAAGIDAFALNIGVDGYTDQQLGFAYDSANRNGMKVVISFDFNWWSP</u>	<i>T.a.</i>
(51)	<u>SSSADYDADMORAKSYGIDAFALNIGVDPYTDQQLGFAYDSAARNRNGMKVVISFDFNWWNT</u>	<i>P.m.</i>
(50)	<u>TSASDYDADMQAKAYGIDAFALNIGTDTFSDQQLGYAYESAANNRNGMKVVISFDFNWWST</u>	<i>P.p.</i>
(118)	<u>GNAVGVGQKIAQYANRPAQLYVDNRPFASSFAGDGLDVNLRSAAGSNVYFVFNHFGQS</u>	<i>T.h.</i>
(118)	<u>GNAVGVGQKIAQYANRPAQLYVDNRPFASSFAGDGLDVNLRSAAGSNVYFVFNHFGQS</u>	<i>H.I.</i>
(121)	<u>GNAAGVGQKIAQYANRPAQLYVDNRPFASSFAGDGLDVNLRSAAGSNVYFVFNHFGQS</u>	<i>T.a.</i>
(111)	<u>GOATAIGQKIAQYAGLPGLKVDGKVFVSSFAGDVNIGAMRSAAGVELFFAPNFHPSYG</u>	<i>P.m.</i>
(110)	<u>SQATEIGQKIAQYAGSLPGLQMLYDDKIFVSSFAGDGDVAALKSAAGGNVFFAPNFHPSYG</u>	<i>P.p.</i>
(178)	<u>S-PSNIDGALNWMWMDNDGNNKAPKPGQTVTVADGDNAYKNWLGKPYLAPVSPWFFTHF</u>	<i>T.h.</i>
(178)	<u>S-PSNIDGALNWMWMDNDGNNKAPKPGQTVTVADGDNAYKNWLGKPYLAPVSTWVFNHF</u>	<i>H.I.</i>
(181)	<u>S-TSNIDGALNWMWMDNDGNNKAPKPGQNTVADGDNAYKSWLGSKPYLAPISPEFFTHF</u>	<i>T.a.</i>
(171)	<u>TDMSNVDGLLNWMGWPSNGNNKAPTSAGATVSVVEQGDNDYISALAGKAYIAPASPWFSSTHF</u>	<i>P.m.</i>
(170)	<u>TDLSDVDGLLNWMGWPSNGNNKAPTANVTVEEGDEEYITALDGKPYIAPASPWFSSTHF</u>	<i>P.p.</i>
(237)	<u>GPEVSYSKNWVFPGGPLIYNRWQVQLQOGFPMVEIVTWNDYGESHYVGPLSKKHFDGNS</u>	<i>T.h.</i>
(237)	<u>GPEVSYSKNWVFPGGPLIYNRWQVQLQOGFPRVEIVTWNDYGESHYVGPLSKKHFDGNS</u>	<i>H.I.</i>
(240)	<u>GPEVSFSGNWVFPGGALIYNRWQVQLQOGFPMVEIITWNDYGESHYVGPLSKKHFDGNS</u>	<i>T.a.</i>
(231)	<u>GPEVSYSKNWVFPDILLWYNRWQELKLGPRFIEIVTWNDYGESHYIGPLSSPHDGDGAS</u>	<i>P.m.</i>
(230)	<u>GPEVSYSKNWVFPDILLFYQRWDLNLRGQFIEVVTWNDYGESQYVGPLNSPHDGDGSS</u>	<i>P.p.</i>
(297)	<u>KWVNDMPHDGFLDLSKPFIAAYKNRDTDI-SKYVQNEQLVYWYRRLKALDCDATDTTSN</u>	<i>T.h.</i>
(297)	<u>KWVNDMPHDGFLDLSKPFIAAYKNRDTDI-SKYVQNEQLVYWYRRLKALDCDATDTTSN</u>	<i>H.I.</i>
(300)	<u>KWTNDMPHDGFLDLSKPFIAAYKNRDTDV-SKYIQNEELVYWYRRLKALDCDATDTTSN</u>	<i>T.a.</i>
(291)	<u>KWVNDMPHDGWLDMAKPFIAAFKAGATTVDNYITNDELVYWYRPTLKNVNCDATDTCMN</u>	<i>P.m.</i>
(290)	<u>RWANDMPHDGWLDLAKPYIAAFHDGATSLSSSYITEDQLIYWYRQPRLMDCDATDTCM-</u>	<i>P.p.</i>
(356)	<u>RPANNGSGNYFMGRPDGWQTMDDTVYVAALLKTAGSVTVTSGGTTQTFQANAGANLFOIP</u>	<i>T.h.</i>
(356)	<u>RPANNGSGNYFEGRPDGWQTMDDTVYVAALLKTAGSVTVTSGGTTQTFQANAGANLFOIP</u>	<i>H.I.</i>
(359)	<u>RPANASGNYFEGRPDGWETMDDTVYVAALFLKSAGTVTVTSGGTTQSFQGNAGANIFOVP</u>	<i>T.a.</i>
(350)	<u>PSASNSGNYFLGRPNGYETMDSVVFVSLLTAPAQVTVTSGGNSQTFQASAGANAFQVP</u>	<i>P.m.</i>
(349)	<u>VAANNDTGNFEGRPNGWESMEDAVFVVALLOSAGTVQVTSGPNTETFDAPAGASAFQVP</u>	<i>P.p.</i>
(416)	<u>ASIGQQKFALTRNGQTVFSGTSLMDITNVCSCGIYNFNYPVGTIPAGFDDPLQADGLFSL</u>	<i>T.h.</i>
(416)	<u>ASIGQQKFALTRNGQTVFSGTSLMDITNVCSCGIYNFNYPVGTIPAGFDDPLQADGLFSL</u>	<i>H.I.</i>
(419)	<u>ASLQQQKFALTRNGQTVFSGVSLMDITDVCACGIYNFNTPYVGTIPAGFDDPLQDGLASL</u>	<i>T.a.</i>
(410)	<u>MGVGSQTFVTRNGATVFGQTSKQIINGCVCGLYNFNAYVGTIPAGFSDPLGPAGLASL</u>	<i>P.m.</i>
(409)	<u>MFGGQPSFSLSRDGETVLSGTSLKDIIDGCLCGIYNFNAYVGSLPATFSDPLEPPSLNAF</u>	<i>P.p.</i>
(476)	<u>TIGLHVTTTCQAKPSLG---TNPP--VTSGPVSSLPASSTTRASSPPVSVSTRVSSPPVSS</u>	<i>T.h.</i>
(476)	<u>TIGLHVTTTCQAKPSLG---TNPP--VTSGPVSSLPASSTTRASSPPVSVSTRVSSPPVSS</u>	<i>H.I.</i>
(479)	<u>TIGLHVTTTCQARPSLG---TNPP--VTSGPITSIPVSS--TQASS--PPVSSSTHASSPPVSP</u>	<i>T.a.</i>
(470)	<u>VOGLHVATCAATPSLG---TAPP--VVTAP-----PV-----PATTT</u>	<i>P.m.</i>
(469)	<u>SEGLKVSSTCSATPSLGLtsTTPPetIPTGTIT--PGSAITGAATTTSTIST--TSTISTTS</u>	<i>P.p.</i>
<u>Mutan-binding domain</u>		
(531)	<u>PPVSRSTSTPPPA-SSTPPSGQVCVAGTVADGESGNYIGLCQFSCNYGYCP-----FGPC</u>	<i>T.h.</i>
(531)	<u>PPVSRSTSSAPPPGNSSTPPSGQVCVAGTVADGESGNYIGLCQFSCNYGYCP-----FGPC</u>	<i>H.I.</i>
(532)	<u>PPSTSRSSSTSTPP-VSTPPSGQVCVAGTVADGESGNYIGLCNFSCNYGYCP-----FGPC</u>	<i>T.a.</i>
(502)	<u>TTIITTKTTTTST--STSTSG--TCIAGT---G-PGNYVGLCSFCCNFGYCP-----FGPC</u>	<i>P.m.</i>
(526)	<u>TFISTTTTTTSSAATST--TTG--TCIAGT---G-PDNYSGLCFCCNYGYCPgsdgsAGPC</u>	<i>P.p.</i>
(585)	<u>KCTAFGAPINPPASNGRNGCPLPGECDGYLGI CSFSCNHNYCPPTACQY-C</u>	<i>T.h.</i>
(586)	<u>KCTAFGAPISPPASNGRNGCPLPGECDGYLGLCSFSCNHNYCPPTACQY-C</u>	<i>H.I.</i>
(586)	<u>KCTAYGSPISPPATNGRNGCPLSCEGDGYLGLCSFSCNHGYCPPTACQY-C</u>	<i>T.a.</i>
(550)	<u>TCTAYGAPVPTPPVTGTVGLPLYNEDNSYGLCSFACNHGYCPNTACRV-A</u>	<i>P.m.</i>
(580)	<u>TCTAYGDPVPTPPVTGTVGVPLDGECDGYLGLCSFACNHGYCPSTACQVes</u>	<i>P.p.</i>

Catalytic domain (38 - 490 aa)

Fig. 2. Alignment of the amino acid sequence of *T. harzianum* CCM F-340 α -1,3-glucanase (*T.h.*) with homologous sequences of *H. lixii* CECT 2413 (anamorph: *T. harzianum*) (accession no CAC80493) (*H.I.*), *T. asperellum* CECT 20539 (accession no CAH04880) (*T.a.*), *P. marneffeii* ATCC 18224 (accession no EEA20869) (*P.m.*) and *P. purpurogenum* CBS 238.95 (accession no AF214481) (*P.p.*).

Amino acids identical to the *T. harzianum* CCM F-340 α -1,3-glucanase sequence are shaded. The sequences for signal peptide, catalytic domain and mutan-binding domain are underlined. The two regions of internal similarity in the polysaccharide-binding domain are boxed.

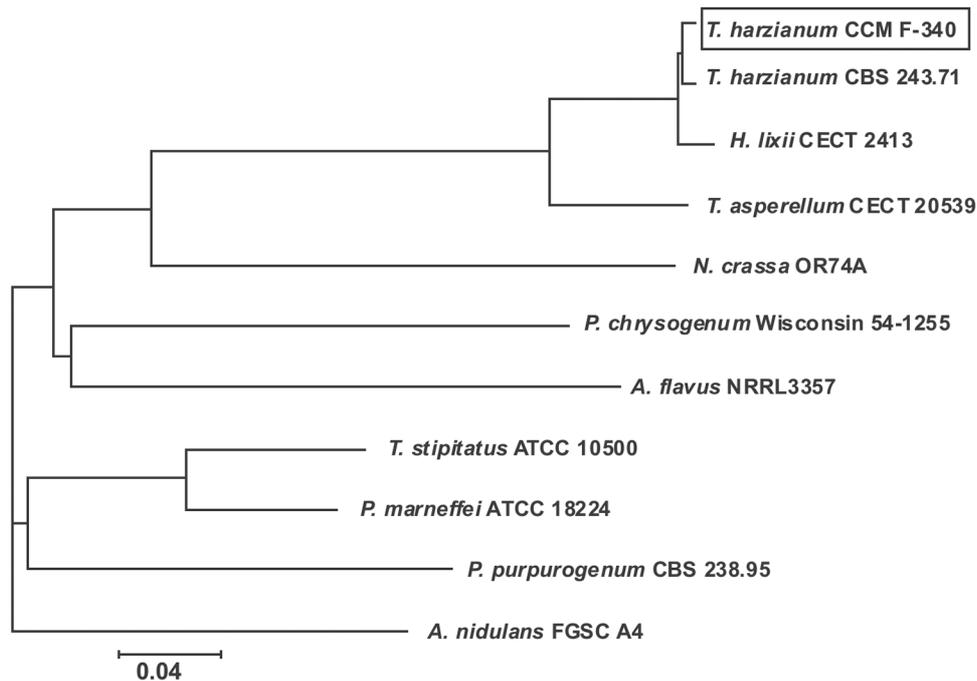


Fig. 3. Dendrogram showing the relationship between α -1,3-glucanases from *T. harzianum* CCM F-340 and other closely related organisms.

The amino acid sequences of α -1,3-glucanases used for phylogenetic tree construction were from *T. harzianum* CCM F-340 (accession no HQ871941), *T. harzianum* CBS 238.95 (accession no AAF27911), *H. lixii* CECT 2413 (accession no CAC80493), *T. asperellum* CECT 20539 (accession no CAH04880), *N. crassa* OR74A (accession no EAA29582), *P. chrysogenum* Wisconsin 54-1255 (accession no CAP80960), *T. stipitatus* ATCC 10500 (accession no EED20417), *Penicillium marneffeii* ATCC 18224 (accession no EEA20869), *P. purpurogenum* CBS 238.95 (accession no AF214481), *A. flavus* NRRL3357 (accession no EED56220), *A. nidulans* FGSC A4 (accession no CBF84404).

coding region for the *T. harzianum* mutanase without the signal peptide, was PCR amplified, digested with *Hind*III endonuclease, and cloned into *Hind*III site and previously blunted after digestion *Nco*I site of a pET15b expression vector. The resulting plasmid, pET15bTM, allowed the production of high levels of the recombinant mutanase using a T7 promoter (Fig. 4).

The pET15bTM plasmid was used to transform the *E. coli* BL21(DE3) plysS host and the expression of the mutanase gene was monitored as described in the Experimental section. Trials were conducted at several different temperatures and it was observed that the most effective enzyme production occurred when the temperature was lowered to 16°C (data not shown). To purify the recombinant enzyme, after expression, cells were suspended in buffer A, frozen and lysed by sonication. Insoluble fractions were separated by centrifugation and the supernatant was applied to a weak DEAE anionic resin previously equilibrated with buffer A. The protein was collected as the flow-through fraction. This fraction was condensed by diafiltration. In the next step, the enzyme was purified by molecular sieving as described in the Experimental section. Results for recombinant α -1,3-glucanase purification are summarized in Table I. The resulting protein had a molecular mass of ~68 kDa, which was in congruence with the theoretical molecular mass (63.86 kDa)

based on the amino acid sequence. Furthermore, the recombinant α -1,3-glucanase possesses a mutanolytic activity (0.097 U/mg protein). Figure 5 illustrates the activity of the recombinant mutanase in the streptococcal mutan degradation in the agar plate test. The mutan-removing effect of mutanase was significant, but lower than in its natural genetic background under inducible conditions (Wiater *et al.*, 2008). The

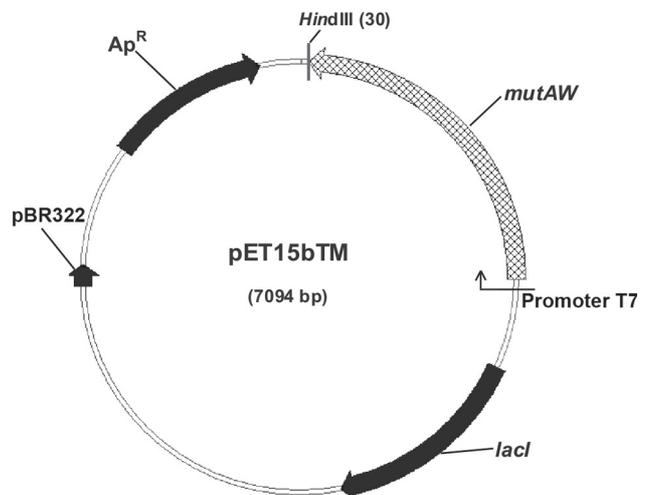


Fig. 4. Schematic diagram of the expression construct pET15bTM containing cDNA of *T. harzianum* CCM F-340 *mutAW* gene without the leader sequence for its signal peptide.

Table I
Activity of the recombinant α -1,3-glucanase of *T. harzianum* after purification steps

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold purification
Cell extract	580	21.4	0.036	100	1
DEAE-chromatography	112	10	0.089	46.7	2.5
Size exclusion – chromatography	40	3.9	0.097	18.2	2.7

mutanase naturally produced by the *Trichoderma* mold is in the form of glycoprotein. However, the unglycosylated protein demonstrated also mutanase activity. The recombinant protein showed a lower activity at 45°C than the native enzyme produced by the fungus (data not shown). This might indicate that proper glycosylation of mutanase is indispensable for the optimal activity of this enzyme. Fuglsang and co-workers (2000) reported that wild-type *T. harzianum* mutanase displayed a molecular mass of 75 kDa, both in SDS-PAGE and MALDI-MS. Carbohydrate composition analysis revealed the presence of glucose and mannose (18 and 32 mol/mol enzyme, respectively) that accounted for over 8 kDa of the protein mass. In general, mutanases demonstrate higher sequence similarity in the N-terminal and C-terminal parts of the mature enzymes, and are less conserved in the Pro-Ser-Thr region that is the supposed site for O-glycosylation. Similarly, Ser-Thr-rich linker region of *A. niger* glucoamylase was demonstrated to be the site for this protein modification (Coutinho and Reilly, 1994). The glycosylation level was sometimes even higher in comparison to the wild-type mutanases, as was described for the *T. harzianum*

and *P. purpurogenum* recombinant enzymes displaying molecular masses of 86 and 90 kDa, respectively, when they were produced in a heterologous expression system in *A. oryzae* (Fuglsang et al., 2000). The purified enzyme from *T. harzianum* CECT 2413 was present in the form of ~75 kDa after SDS-PAGE and 132 kDa after gel filtration chromatography. This suggests that this mutanase could be a dimeric protein in solution (Sanz et al., 2005; Ait-Lahsen et al., 2001), in contrast to the tetramer form for another α -1,3-glucanase from *T. harzianum* CCM F-470 (Wiater et al., 2001).

The mutanase 102 kDa from *P. purpurogenum* CBS 257.37 was probably N-glycosylated and O-mannosylated when generated using *Pichia pastoris* expression system (Shalom et al., 2008). This data indicated that the use of individual heterologous expression systems offers a number of advantages as well as disadvantages that affect the production of an active enzyme. We observed that extracellular production of *T. harzianum* CCM F-340 mutanase in the *P. pastoris* system resulted in its hyperglycosylation, and in effect a complete loss of activity. Removing the N-glycosylation site (NGS) by site directed mutagenesis (change of Asp-360 to Ala-360) also negatively affected the enzyme activity (data not shown). In an *E. coli* expression system, over-produced proteins often accumulate in the cells in insoluble forms as inclusion bodies, and as a consequence, are often inactive (Shu et al., 2007).

Conclusions. α -1,3-Glucanases (mutanases) have been reported in a number of bacteria, yeasts and fungi, but so far this class of enzymes is a relatively uncharacterized family of proteins. Because of their potential use in the treatment of dental caries, we were interested in the production of the *T. harzianum* mutanase in *E. coli* expression systems. For this purpose, the mutanase gene was identified, characterized, and used in the construction of a recombinant plasmid. Our data indicate that the obtained enzyme is conserved in *Trichoderma* and can be effectively produced in this heterologous expression system.

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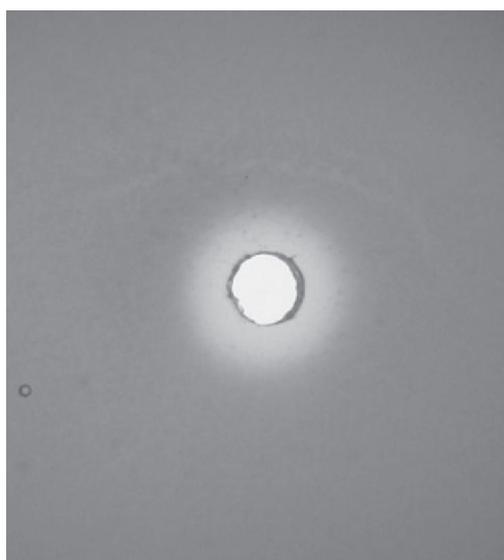


Fig. 5. Mutan-hydrolyzing activity of *T. harzianum* recombinant α -1,3-glucanase.

Mutan hydrolyzing activity was assayed on agar plate containing 0.25% streptococcal mutan after incubation for 24 hours at 37°C. The formation of the halo is indicative of mutan hydrolyzing activity.

Literature

- Ait-Lahsen H., A. Soler, M. Rey, J. de la Cruz, E. Monte and A. Llobell. 2001. An antifungal exo- α -(1 \rightarrow 3)-glucanase (AGN13.1) from the biocontrol fungus *T. harzianum*. *Appl. Environ. Microbiol.* 67: 5833–5839.
- Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–54.
- Budtz-Jørgensen E. and J. Kelstrup. 1977. Enzymes as denture cleansers. *Scand. J. Dent. Res.* 85: 209–215.
- Chung J., H.H. Kim, J.H. Shin, H.C. Lee, Z. Hee Lee and J.S. Oh. 2001. Identification of mutanase-producing *Microbispora rosea* from the soil of Chonnam Province. *J. Microbiol. Biotechnol.* 11: 677–684.
- Coutinho P.M. and P.J. Reilly. 1994. Structure-function relationships in the catalytic and starch binding domains of glucoamylase. *Protein Eng.* 7: 393–400.
- Ebisu S., K. Kato, S. Kotani and A. Misaki. 1975. Isolation and purification of *Flavobacterium* α -1,3-glucanase-hydrolyzing, insoluble, sticky glucan of *Streptococcus mutans*. *J. Bacteriol.* 124: 1489–1501.
- Fuglsang C.C., R.M. Berka, J.A. Wahleitner, S. Kauppinen, J.R. Shuster, G. Rasmussen, T. Halkier, H. Dalbøge and B. Henrissat. 2000. Biochemical analysis of recombinant fungal mutanases. *J. Biol. Chem.* 275: 2009–2018.
- García I., D. Jiménez, V. Martín, A. Durán and Y. Sánchez. 2005. The α -glucanase Agn1p is required for cell separation in *Schizosaccharomyces pombe*. *Biol. Cell* 97: 569–576.
- Hasegawa S., J.H. Nordin and S. Kirkwood. 1969. Enzymes that hydrolyze fungal cell wall polysaccharides. I. Purification and properties of an endo- α -D-(1 \rightarrow 3)-glucanase from *Trichoderma viride*. *J. Biol. Chem.* 244: 5460–5470.
- Inoue M., T. Yakushiji, J. Mizuno, Y. Yamamoto and S. Tanii. 1990. Inhibition of dental plaque formation by mouthwash containing an endo- α -1,3 glucanase. *Clin. Prevent. Dent.* 12: 10–14.
- Matsuda S., Y. Kawanami, H. Takeda, T. Ooi and S. Kinoshita. 1997. Purification and properties of mutanase from *Bacillus circulans*. *J. Ferm. Bioeng.* 83: 593–595.
- Meyer M.T. and H.J. Phaff. 1977. Survey for α -(1 \rightarrow 3)-glucanase activity among yeasts. *J. Bacteriol.* 131: 702–706.
- Papavizas G.C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annu. Rev. Phytopathol.* 23: 23–54.
- Pleszczyńska M., M. Marek-Kozaczuk, A. Wiater and J. Szczodrak. 2007. *Paenibacillus* strain MP-1: a new source of mutanase. *Bio-technol. Lett.* 29: 755–759.
- Reese E.T., A. Maguire and F.W. Parrish. 1972. α -1,3-Glucanases of fungi and their relationship to mycodextranase. Proceedings of the IVth International Fermentation Symposium. *Ferment. Technol. Today* 735–742.
- Sambrook J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: a Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
- Sanz L., M. Montero, J. Redondo, A. Llobell and E. Monte. 2005. Expression of an α -1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. *FEBS Journal* 272: 1–7.
- Schuster A. and M. Schmoll. 2010. Biology and biotechnology of *Trichoderma*. *Appl. Microbiol. Biotechnol.* 87: 787–799.
- Shalom G., J. Pratten, M. Wilson and S.P. Nair. 2008. Cloning, heterologous gene expression and biochemical characterization of the alpha-1,3-glucanase from the filamentous fungus *Penicillium purpurogenum*. *Protein Expr. Purif.* 60: 170–175.
- Shu Z.Y., Y.J. Yan, J.K. Yang and L. Xu. 2007. *Aspergillus niger* lipase: gene cloning, over-expression in *Escherichia coli* and *in vitro* refolding. *Biotechnol. Lett.* 29: 1875–1879.
- Viterbo A., O. Ramot, L. Chernin and I. Chet. 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie von Leewenhoek* 81: 549–556.
- Walker G.J. and M.D. Hare. 1977. Metabolism of the polysaccharides of human dental plaque. Part II. Purification and properties of *Cladosporium resinae* (1 \rightarrow 3)- α -D-glucanase, and the enzymic hydrolysis of glucans synthesized by extracellular D-glucosyltransferases of oral streptococci. *Carbohydr. Res.* 58: 415–432.
- Wei H., M. Scherer, A. Singh, R. Liese and R. Fischer. 2001. *Aspergillus nidulans* alpha-1,3-glucanase (mutanase), *mutA*, is expressed during sexual development and mobilizes mutan. *Fungal Genet. Biol.* 34: 217–227.
- Wiater A. and J. Szczodrak. 2002. Selection of strain and optimization of mutanase production in submerged cultures of *Trichoderma harzianum*. *Acta Biol. Hung.* 53: 389–401.
- Wiater A., J. Szczodrak and M. Pleszczyńska. 2008. Mutanase induction in *Trichoderma harzianum* by cell wall of *Laetiporus sulphureus* and its application for mutan removal from oral biofilms. *J. Microbiol. Biotechnol.* 18: 1335–1341.
- Wiater A., J. Szczodrak and J. Rogalski. 2001. Purification and characterization of extracellular mutanase from *Trichoderma harzianum*. *Mycol. Res.* 105: 1357–1363.