Biochemical Analysis of Recombinant Fungal Mutanases
A NEW FAMILY OF α,1,3-GLUCANASES WITH NOVEL CARBOHYDRATE-BINDING DOMAINS*

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Nucleotide sequence analysis shows that Trichoderma harzianum and Penicillium purpurogenum α,1,3-glucanases (mutanases) have homologous primary structures (55% amino acid sequence identity), and are composed of two distinct domains: a NH2-terminal catalytic domain and a putative COOH-terminal polysaccharide-binding domain separated by a β-glycosylated Pro-Ser-Thr-rich linker peptide. Each mutanase was expressed in Aspergillus oryzae host under the transcriptional control of a strong α-amylase gene promoter. The purified recombinant mutanases show a pH optimum in the range from pH 3.5 to 4.5 and a temperature optimum around 50–55 °C at pH 5.5. Also, they exhibit strong binding to insoluble mutan with Kd around 0.11 and 0.13 μM at pH 7 for the P. purpurogenum and T. harzianum mutanases, respectively. Partial hydrolysis showed that the COOH-terminal domain of the T. harzianum mutanase binds to mutan. The catalytic domains and the binding domains were assigned to a new family of glycoside hydrolases and to a new family of carbohydrate-binding domains, respectively.

Extracellular polysaccharides produced by microbial flora in the human oral cavity are believed to play an important role in the adherence and proliferation of bacterial aggregates on the surface of teeth (1). Consequently, these polysaccharides might have significance in the development of tartar, plaque, and possibly dental carries (2). Mutan is a major component of the surface of teeth (1). Consequently, these polysaccharides might indicate this fact. This paper is available on line at http://www.jbc.org

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF214480 (T. harzianum mutanase gene sequence previously listed as Geneseq™ accession number V12688) and AF214481 (P. purpurogenum mutanase gene sequence; previously listed as Geneseq™ accession number V81911).

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The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MALDI-MS, matrix-assisted laser desorption-ionization-mass spectrometry; PCR, polymerase chain reaction; bp, base pair(s); CAPS, 3-(cychoxyethylamino)propanesulfonic acid; nt, nucleotide(s); MU, mutanase unit; ORF, open reading frame.

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mycelium was collected, frozen in liquid N$_2$, and stored at −80°C. First-strand cDNA was synthesized from 5 µg of T. harzianum poly(A)$^+$ RNA as described earlier (9). A 387-bp fragment of the T. harzianum mutanase cDNA (10) was amplified using two mutanase-specific primers (100 pmol each), forward (5’-ACTACCTAGAGCATATCCGAGTTGAGT-3’), a DNA thermal cycler (Landgraf, Germany) and 2.5 units of Taq polymerase (Perkin-Elmer Cetus). Initially, two cycles of PCR were done using a cycle profile of denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 3 min, then the annealing temperature was increased to 55°C and 30 additional cycles were performed. The PCR fragment of interest was subcloned into pUC18 vector and sequenced as described previously (9).

Construction and Screening of the T. harzianum cDNA Library—
Total RNA was prepared from frozen, powdered mycelium of T. harzianum by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (11). The poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (12). Double-stranded cDNA was synthesized from 5 µg of T. harzianum poly(A)$^+$ RNA as described earlier (13), except that 25 ng of random hexanucleotide primers (Life Technologies, Inc.) were included in the first strand synthesis. A cDNA library, consisting of 1.5 × 10$^8$ independent clones was constructed in the yeast expression vector pYES2.0 (Invitrogen) as described (13), and screened by colony hybridization (14) using a random-primed (15) 32P-labeled (>1 × 10$^6$ cpm/µg) mutanase cDNA fragment as a probe. The hybridizations were carried out in 2 × SSC, 5 × Denhardt’s solution (14), 0.5% (w/v) SDS, 100 µg/ml denatured salmon sperm DNA for 2 h at 65°C followed by washes in 2 × SSC (2 × 15 min), 2 × SSC, 0.5% SDS (15 min), 0.2 × SSC, 0.5% SDS (15 min), and finally in 2 × SSC (2 × 15 min) at 65°C.

Cloning of P. purpureogenum Mutanase Gene—
Total cellular DNA was isolated from P. purpureogenum cells by a previously described method (16), and used for construction of genomic DNA libraries in the bacteriophage λ-ZipLox cloning system (Life Technologies Inc., Gaithersburg, MD) (17). Approximately 45,000 plaques from the library were screened by plaque hybridization (18) with a radiolabeled T. harzianum mutanase probe fragment using moderate stringency conditions (19). A 1790-nt fragment containing this mutation. This PCR fragment was created in a two-step procedure as reported in Ref. 23 using the following primers: Primer 1 (nt 2761, 5’-CAGGTTCCACATCAGGACC, nt 2779) and Primer 2 (nt 3306, 5’-CAAGAGGACGGTTTTCTCAGAGCC, nt 3281); Primer 3 (nt 3281 5’-CGCTCTCTGGAGAAGCTGCTTCT, nt 3306) and Primer 4 (nt 4276, 5’-GACATCTTGGATTTAGACC, nt 4257); nucleotide numbers refer to the pMT1802 plasmid.

Expression of Recombinant T. harzianum Mutanase in A. oryzae—
The A. oryzae host strain JA1225 was transformed using a polyethylene glycol-mediated protocol (24) and a DNA mixture containing 0.5 µg of a plasmid encoding the gene that confers resistance to the herbicide Basta (25) and 8.0 µg of the expression vector pMT1796. Transformants were selected on minimal plates containing 0.5% Basta and 50 mM urea as a nitrogen source. Each transformant was purified twice on selection media and conidia were harvested. Universal containers (20 ml, Nunc, catalog number 364211) containing 10 ml of YPM (2% maltose, 1% bactopeptone bactopeptone, and 0.5% yeast extract) were inoculated with spores from the transformants and incubated 5 days with shaking at 30°C. Culture supernatants were harvested after 5 days growth and assayed for the recombinant mutanase.

Expression of P. purpureogenum Mutanase in A. oryzae—Two synthetic oligonucleotide primers were designed to amplify the P. purpureogenum mutanase gene from plasmid pZL-Pp6A, 5’-cccttaattaaTTAGCTCTACTTGA- and 5’-eccttaattaaTTAGCTCTACTTGA-CAAGC (capital letters correspond to the sequence present in the cloned coding region). One hundred picomoles of each primer was used in a PCR reaction containing 52 ng of plasmid DNA, 1× Pwo polymerase buffer (Roche Molecular Biochemicals, Indianapolis, IN), 1 µM each dATP, dTTP, dGTP, dCTP, and 2.5 units of Pwo polymerase (Roche Molecular Biochemicals). The PCR conditions were 95°C 3 min, 25× (95°C 1 min, 60°C 1 min, 72°C 1.5 min) 3°C 5 min. The amplified 2.2-kilobase DNA fragment was purified by gel electrophoresis and cut with restriction endonucleases SauI and PcrI (using conditions specified by the manufacturers). The fragment was cloned into plasmid pBANe6 (26) that had been previously cut with SauI and PcrI and the resultant expression plasmid was named pJeRS35. This vector was introduced into A. oryzae host strain JA142 using a standard protoplast transformation procedure (24) and 40 transformants were selected by their ability to grow on COVE medium using acetamide as the sole nitrogen source. The transformants were grown in 20 ml of MY50N media (MY50N in glitner: Nutriose (Roquette), 62, MgSO$_4$ 7H$_2$O, 2.0; KH$_2$PO$_4$, 2.0; citric acid, 4.0; yeast extract, 8.0; urea, 2.0; trace metals, 0.5 ml; pH 6.0, and then add CaCl$_2$, 0.1) in shaker flasks for 3 days at 34°C with agitation. Mutan assay plates were prepared by blending a 1% (w/v) mutan, 1% agarose in 0.1 M sodium acetate buffer, pH 5.5, for 20 min at 4°C. The agarose was melted by heating and 150-mm Petri plates were poured. After solidification, small wells (about 40 µl of equivalent volume) were punched in the plates. To screen the transformants for ability to secrete mutanase, 35 µl of centrifuged culture broth from each transformant (and one untransformed control) were pipetted into the wells and the plates were incubated at 37°C. Mutanase activity in the broth samples caused formation of clear
zones around the wells.

Preparation of Mutan—Mutan was prepared by growing S. mutans CBS 350.71 at 37 °C, pH 6.5 (kept constant at a stirring rate of 75 rpm in a medium comprised of the following components: NZ-Case, 6.5 g/liter; yeast extract, 6 g/liter; (NH₄)₂SO₄, 20 g/liter; K₂PO₄, 3 g/liter; glucose, 50 g/liter; and 1.5× Penicillin-Streptomycin. After 35 h, the mutanase was harvested on a P30 filter (Scapa Filtration), and was washed with deionized water containing 1% sodium benzoate, pH 5 (adjusted with acetic acid). Finally, the insoluble mutan was lyophilized and ground.

Enzyme Assays—The production of soluble reducing sugars released from mutan was employed as a measure of enzyme activity. First, 0.1 ml of 5% mutan in 50 mM sodium acetate (allowed to swell at least for 1 h), pH 5.5, was added to 0.5 ml of enzyme sample (diluted in water) in a round-bottomed Eppendorf vial to ensure sufficient agitation and incubated for 15 min at 40 °C while shaking vigorously. The reaction was terminated by adding 0.1 ml of 0.4 M NaOH and the samples were centrifuged for 5 min at 14,000 × g and filtered through 0.45-μm HV filters (Millipore). To each filtrate (100 μl) in Eppendorf vials 750 μl of buffer (pH 5.5) and 0.2 μl of 10× glucose (sodium acetate, pH 4–5.5) were added and incubated 15 min at 85 °C. After allowing the samples to cool, the decrease in absorbance at 420 nm was measured. A dilution series of glucose was included as a standard. Proper controls (substrate and enzyme blanks) were always included. One mutanase unit (MU) was defined as the amount of enzyme releasing 1 mol of reducing sugar per minute at pH 5.5 and 40 °C. Temperature profiles were obtained by incubating the assay mixture (50 mM sodium acetate, pH 5.5) at various temperatures. The pH profiles were obtained by suspending the mutan in 50 mM buffer at various pH (glycine-HCl, pH 3–3.5; sodium acetate, pH 4–5.5; and sodium phosphate, pH 6–7.5).

Purification of Recombinant P. purpurogenum Mutanase—The fermentation broth (700 ml) containing 15.4 MU/ml was filtered using GF/A (Whatmann) and HV 0.45-μm filters (Millipore) prior to measuring absorbance at 420 nm. These filtrates were pooled, filtered (0.7 μm, Whatman), concentrated by ultrafiltration (approximately 600 μl), and loaded onto an S-Sepharose column (XL 50/22, Amersham Pharmacia Biotech) in 0.1× sodium acetate, pH 6.0. The mutanase was eluted in a linear gradient of NaCl. Fractions containing mutanase activity were pooled and concentrated on an Amicon YM10 and loaded onto a HiLoad Q-Sepharose column (Amersham Pharmacia Biotech) in 10 mM Tris-HCl, pH 8.0 (approximately 600 μl/cm), in three rounds. The mutanase was eluted in a linear gradient of NaCl. Pooled fractions (according to activity/purity) were concentrated and further purified by gel filtration on a Superdex 75 (16/60) column (Amersham Pharmacia Biotech) in 0.1× sodium acetate, pH 6.0.

Southern blotting analysis revealed only glucose and mannose but no N-acetylglucosamine, indicating O-glycosylation. The amount of glucose and mannose (18 and 32 mol/mol enzyme, respectively) accounts for over 8 kDa which, added to the theoretical mass (63.8 kDa), gives a molecular mass of about 72 kDa in close agreement with the 75 kDa measured by MALDI-MS and SDS-PAGE.

Isolation and Mutan Binding Activity of the COOH-terminal Domain from T. harzianum Mutanase—To obtain a cDNA probe for the T. harzianum mutanase, two oligonucleotides based on a genomic mutanase clone from T. harzianum (10) were designed. These primers were used to amplify a mutanase cDNA fragment from T. harzianum first-strand cDNA employing the PCR technique (28). Sequencing of the subcloned PCR fragment revealed a 387-bp cDNA with an open reading frame of 129 amino acids. In addition to the primer-encoded residues, the ORF was identical to the corresponding region in the T. harzianum mutanase amino acid sequence (10), confirming that the PCR had specifically amplified the desired cDNA species. Approximately 10,000 colonies from a T. harzianum cDNA library in E. coli were screened using the mutanase-specific PCR product as a probe. This yielded 12 positive clones with inserts ranging from 0.8 to 2.0 kilobase. These were further analyzed by sequencing the ends of the cDNAs with forward and reverse pYES polylinker primers, and determining the nucleotide sequence of the longest cDNA from both strands with synthetic oligonucleotide primers. The nucleotide sequence and the deduced amino acid sequence of the mutanase cDNA from T. harzianum are presented in Fig. 1. The 2062-bp cDNA clone contains a 1905-bp open reading frame initiating with an ATG codon at nucleotide position 29 and terminating with a TAG stop codon at nucleotide position 1931, thus predicting a 634-residue polypeptide. The open reading frame is preceded by a 28-bp 5′-noncoding region and followed by a 119-bp 3′-noncoding region and a poly(A) tail.

Cloning of P. purpurogenum Mutanase—Southern blotting experiments indicated that the T. harzianum mutanase cDNA could be used as a probe to identify mutanase gene-specific 2011
Fungal Mutanases, a New Family of α,1,3-Glucanases

FIG. 1. The nucleotide sequence and the deduced amino acid sequence of the α,1,3-glucanase (mutanase) cDNA from *T. harzianum*. The signal peptide and propeptide region are underlined, and the NH₂-terminal residues determined from the purified, recombinant *T. harzianum* mutanase are indicated by double underlines. The putative linker region (rich in Ser, Pro, and Thr) flanked by Cys residues at positions 484 and 553 is highlighted in gray. Non-coding sequences are in lowercase letters. This sequence has been deposited in the Geneseq™ database with the accession number V12368.
FIG. 2. DNA sequence and deduced amino acid sequence of *P. purpurogenum* mutanase gene. The signal peptide and propeptide region are *underlined*, and the NH₂-terminal residues determined from the purified, recombinant *P. purpurogenum* mutanase are indicated by *double underlines*. The putative linker region (rich in Ser, Pro, and Thr residues) flanked by Cys residues at positions 477 and 547 is highlighted in *gray*. Introns and noncoding regions are indicated by *lowercase letters*. Consensus lariat sequences (PuCTPuAC) with each intron are denoted by a *dashed underline*. This sequence has been deposited in the Geneseq™ database with the accession number T89024.
Pp6A) revealed that the region which hybridized to the *T. harzianum* mutanase cDNA was localized near one end of a 3.6-kilobase genomic DNA insert (not shown). DNA sequencing of a portion of this segment showed an open reading frame with clear homology to the *T. harzianum* mutanase cDNA and its deduced amino acid sequence (Fig. 2). The positions of introns and exons within the *P. purpurogenum* mutanase gene were assigned based on alignments of the deduced amino acid sequences to the corresponding *T. harzianum* mutanase gene product. On the basis of this comparison, the *P. purpurogenum* mutanase gene is composed of five exons (126, 532, 226, 461, and 548 bp) which are punctuated by four small introns (63, 81, 58, and 78 bp). These appear to be typical fungal introns with respect to size and composition in that all contain consensus splice donor and acceptor sequences as well as the consensus lariat sequence (PuCTPuAC) near the 3' end of each intervening sequence (29).

Comparison of *Trichoderma* and *Penicillium* Mutanase Primary Structures—The signal peptide and propeptide portions of *P. purpurogenum* mutanase and *T. harzianum* mutanase share little amino acid sequence similarity; however, the mature polypeptides (after removal of signal and propeptides) are approximately 53% identical. The regions of greatest identity are located in the NH2-terminal portion (residues 42 through 491; *T. harzianum* numbering) and over approximately the last 70 residues of these two proteins where 60 and 63% identity is observed, respectively. In both mutanases the NH2-terminal and COOH-terminal domains are separated by a Pro-Ser-Thr-rich linker region. Remarkably, the Pro-Ser-Thr-rich region of *P. purpurogenum* mutanase (residues 475 through 547) is composed of 69% Pro, Ser, and Thr, and is bordered roughly by Cys residues at positions 477 and 547. As the two mutanases appeared to have a modular structure, sequence comparisons using the BLAST algorithm to search the non-redundant GenBank CDS translations on the NCBI server (30) were therefore conducted on each domain separately. BLAST searches using the NH2-terminal domains did not produce any hits with known glycosidases, however, two ORFs of unknown function in *Schizosaccharomyces pombe* (C14C4.09 and BC646.06c, GenBank Z98596 and AL035216, respectively). Residues identical in 3 of the 4 sequences are printed in white on black background. The two regions of internal similarity are boxed.

**Fig. 3.** a, sequence alignment of the catalytic domains of *T. harzianum* (Triha) and *P. purpurogenum* (Penpu) mutanases (Geneseq™ protein data base accession numbers W44193 and W32213, respectively) with the two homologous *S. pombe* ORFs of unknown function (C14C4.09 and BC646.06c, GenBank Z98596 and AL035216, respectively). Residues identical in 3 of the 4 sequences are printed in white on black background. b, alignment of the mutan-binding domains of *T. harzianum* (Triha) and *P. purpurogenum* (Penpu) mutanases. Identical residues are printed in white on black background. The two regions of internal similarity are boxed.
An alignment of the sequences of the catalytic domains of the two mutanases with the two ORFs of *S. pombe* is shown in Fig. 3a. BLAST searches conducted with the COOH-terminal domains of the mutanases also failed to produce any significant hit in GenBank. Within the COOH-terminal domains of the two mutanases, two short regions display intriguing similarity (10 residues conserved out of 15) suggesting the existence of an internal duplication (Fig. 3b).

**Heterologous Expression of *T. harzianum* Mutanase in *A. oryzae*—**The *T. harzianum* mutanase coding region was amplified by PCR, and the amplicon was inserted into an *Aspergillus* expression vector so that the gene was under the control of an *A. oryzae* α-amylase gene promoter and an *A. niger* glaA terminator. The resulting expression construct, pMTH1802, was further modified by changing aa 35 from Glu to Lys resulting in the presence of a dibasic (KEX2-type) processing site at the amino terminus of the mature mutanase protein. This new expression vector, pMT1796 (Fig. 4a), was used to transform an *A. oryzae* strain, and 25 independent transformants were isolated. Mycelia from each transformant was used to inoculate 20-ml culture tubes containing 10 ml of YPM media and cultures were grown with shaking for 5 days at 30 °C. SDS-PAGE analysis revealed a dominant 85–90-kDa band indicating that these transformants were indeed expressing the recombinant mutanase gene.

**Heterologous Expression of *P. purpurogenum* Mutanase—**The *P. purpurogenum* mutanase coding region was amplified by PCR using primers that created 5’- and 3’-terminal restriction sites compatible with an *Aspergillus* expression vector. The amplified DNA segment was subsequently inserted into the vector which employed a strong *A. oryzae* α-amylase gene promoter. The resulting plasmid, designated pJeRS35 (Fig. 4b), was used to transform an *A. oryzae* recipient strain, and 40 transformants were isolated. Mycelia from each of the transformants were used to inoculate shaker flask cultures that were incubated for 3 days. Using a mutan agar plate assay, 14 of the transformants showed extracellular mutanase activity as indicated by opaque clearing zones (the control showed no clearing zone). Broth samples that were positive in the plate assay were subsequently analyzed by SDS-PAGE. These transformants showed a prominent band at approximately 90 kDa.

**Purification of and Molecular Properties of Recombinant Mutanases—**Recombinant *T. harzianum* mutanase was purified in a three-step procedure using cation-exchange chromatography, anion-exchange chromatography followed by size exclusion chromatography resulting in a yield of around 24%. The essentially pure mutanase exhibited a molecular mass around 86 kDa (Fig. 5a). A rather broad band was observed indicating some heterogeneity and/or heavy glycosylation. The NH₂-terminal amino acid sequence was determined by protein sequencing to be Ala-Ser-Ser—thus predicting a calculated molecular mass of 63.8 kDa for the mature enzyme (Table I). This obser-
viation suggests that the first 37 amino acid residues deduced from the gene sequence function as a secretory signal peptide and propeptide. This is supported by the fact that the NH₂ terminus of the mature mutanase is not preceded by a typical signal peptidase cleavage site (31, Fig. 1) but rather by a cleavage site for a monobasic processing enzyme. Furthermore, the mutanase cDNA encodes an apparent signal sequence of 24 amino acids, with a predicted signal peptidase cleavage site between Ala-24 and Ala-25 in the mutanase precursor (31). A simpler procedure for purification of the recombinant *P. purpurogenum* mutanase was established using the information that a putative COOH-terminal-binding domain is present in the enzyme. The enzyme was adsorbed to insoluble mutan and subsequently eluted in water. This procedure resulted in a 129-fold purification and a yield around 20%. The essentially pure mutanase had a molecular mass of about 90 kDa (Fig. 5b). NH₂-terminal amino acid sequencing revealed the following sequence: Ser-Thr-Ser-Asp-Arg-. Thus, the deduced amino acid sequence of the mutanase gene product (Fig. 2) predicts an amino-terminal extension of 30 amino acids which are not present in the mature enzyme and a molecular mass for the mature enzyme of 63.6 kDa (Table I). Based on the rules of von Heijne (31), the first 20 amino acids likely comprise a secretory signal peptide, and the next 10 residues probably represent a propeptide segment which is removed by a subsequent proteolytic cleavage following the dibasic Arg-Arg sequence.

**Characterization of the Purified Recombinant Mutanases —**

The two mutanases showed similar catalytic properties. They both exhibit slightly acidic pH optima in the range from pH 3.5 to 5.0 and pH 3.0 to 4.5 for the *T. harzianum* and *P. purpurogenum* mutanases, respectively. At pH 5.5 the two enzymes have specific activities of 16 and 12 MU/mg, respectively, on insoluble mutan at 40 °C. Also, the two mutanases have virtually identical temperature optima around 50–55 °C at pH 5.5. This correlates with DSC analysis of the thermal stability of the recombinant *T. harzianum* mutanase which shows a midpoint denaturation temperature (*T_m*) around 56 °C at pH 5.5 identical to that of the wild-type enzyme.

The binding properties of the two mutanases toward insoluble mutan were investigated at steady state conditions at pH 7 and 4 °C in order to limit hydrolysis. The kinetics of adsorption was followed by taking samples from the supernatant of mutanase incubated with mutan. The equilibrium was reached within 5 min, and then no further net adsorption was observed (data not shown). Varying concentrations of mutanase were incubated for 1 h under the above conditions with mutan and the amount of free mutanase was determined by fluorescence spectroscopy (concentrations verified by activity analysis) and the amount of bound enzyme was calculated. Thus, binding isotherms were generated, and the data fitted using the simple Langmuir model for adsorption to a surface (Fig. 6). Rather strong binding was observed with adsorption constants of 0.13 and 0.11 μmol/g for the *T. harzianum* and *P. purpurogenum* mutanases, respectively (Table II). A significant difference is observed in the maximum level of enzyme which can be adsorbed to the insoluble mutan 0.549 versus 0.244 μmol of enzyme/g of mutan for the *T. harzianum* and *P. purpurogenum* mutanases, respectively (Table II).

In order to probe the hypothesis that the homologous COOH-terminal domain of the two fungal mutanases constitutes a mutant-binding domain, the *T. harzianum* mutanase was subjected to limited proteolysis using chymotrypsin. The protease treatment resulted in a 41-kDa band on SDS-PAGE (Fig. 7), which was NH₂ terminally sequenced after being electroblotted onto a polyvinylidene difluoride membrane revealing the sequence Ser-Leu-Thr-Ile-Gly-Leu- corresponding to proteolytic cleavage following the dibasic Arg-Arg sequence.

**Discussion**

Nucleic acid sequences encoding extracellular mutanases from the filamentous fungi *T. harzianum* and *P. purpurogenum* were cloned and successfully expressed in *A. oryzae*. The primary translation products of these two DNA sequences appear to be preproenzymes, having both NH₂-terminal signal peptides and propeptides that are removed post-translationally. The two mutanases show deduced amino acid sequence identities of 53% overall. Further analyses of the protein sequences

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**Table I**

| Enzyme         | NH₂-terminal sequence | Starting at residue no. | m (calculated) | m (SDS) | pI  |
|----------------|-----------------------|-------------------------|----------------|---------|-----|
| *T. harzianum* | Ala-Ser-Ser-           | 38                      | 63,785 Da      | 86 kDa  | 5.24|
| *P. purpurogenum* | Ser-Thr-Ser-        | 31                      | 63,557 Da      | 90 kDa  | 3.88|

**Table II**

| Enzyme         | Ko (μM) | A_{max} (μmol/g mutant) |
|----------------|---------|------------------------|
| *T. harzianum* | 0.129 ± 0.021 | 0.549 ± 0.047          |
| *P. purpurogenum* | 0.111 ± 0.016 | 0.244 ± 0.012          |

**Fig. 6. Substrate binding isotherms of purified recombinant mutanases; 0.2% mutan in 0.1 M sodium phosphate, pH 7, 4 °C. a, recombinant *T. harzianum* mutanase; b, rec. *P. purpurogenum* mutanase.
reveal stronger similarity between the NH$_2$-terminal and COOH-terminal parts of the mature enzymes, separated by a less homologous Pro, Ser, and Thr-rich region. Consequently, like many cellulases, glucoamylases, and chitinases, the mature mutanases appear to be made of two distinct domains: a NH$_2$-terminal catalytic domain, and a putative COOH-terminal polysaccharide-binding domain separated by a Pro-Ser-Thr-rich linker peptide. MALDI-MS and carbohydrate analysis of the wild-type enzyme from *T. harzianum* suggest that the linker region is O-glycosylated in a manner similar to the Ser-Thr-rich linker region of *A. niger* glucoamylase (32). The glycosylation is even more pronounced in the recombinant enzymes which display molecular masses of 86 and 90 kDa for the *T. harzianum* and *P. purpurogenum* mutanases, respectively. Fungal polysaccharidases harboring a Pro-Ser-Thr-rich linker separating the catalytic from the carbohydrate-binding domain have long been known to undergo hyperglycosylation upon expression in yeast and other heterologous fungal systems (33).

Experiments showing that the chymotrypsin produced fragment of the *T. harzianum* mutanase adsors to mutan gave indirect indication that the COOH-terminal domain of the two fungal mutanases is responsible for binding to insoluble mutan. As a first step in an effort to further characterize the COOH-terminal mutan-binding domain of *T. harzianum* mutanase, two expression plasmids were constructed harboring (i) an internal deletion of the coding region encompassing residues 32–542 (i.e. coding for the isolated COOH-terminal binding domain without any linker) and (ii) the NH$_2$-terminal catalytic domain only. The transformants were tested by immunodiffusion using antibodies raised against the whole mutanase. Whereas the isolated catalytic domain was unaffected by preincubation with mutan, the first transformant became negative upon preincubation with mutan (data to be described elsewhere). The inability of the catalytic domain to bind to mutan was verified by activity analysis showing that no activity could be removed from the supernatant by preincubation with insoluble mutan.

Glycoside hydrolases and transglycosidases have been classified in a number of distinct families based on amino acid sequence similarities (34–36). BLAST searches (30) conducted with the NH$_2$-terminal catalytic domains of the two mutanases described here failed to display any similarity with known glycosidases from previously defined families. Families of glycoside hydrolases being defined with at least two related sequences (34), the two mutanases therefore allow the definition of a new family (designated family 71). Although, sequence similarities between the mutanase catalytic domains and the described ORFs of *S. pombe* are such that it is predictable that all these proteins adopt a similar fold and operate via the same catalytic mechanism using a similar catalytic machinery (37–39), the precise substrate specificity of the *S. pombe* proteins cannot be reliably ascertained as it has been shown that sequence-based families of glycosidases contain enzymes with sometimes widely different substrate specificity (34). Finally, it is worth mentioning that, unlike the two mutanases, none of the two *S. pombe* ORFs carries a COOH-terminal extension suggesting that the encoded proteins are made of a single domain.

Cellulases, xylanases, chitinases, and starch-degrading enzymes have long been recognized to have a modular structure with a catalytic domain carrying one or several ancillary modules whose function is often binding to insoluble polysaccharides (40). The best described of these ancillary modules are probably the cellulose-binding domains which have been classified in several distinct families based on sequence similarities (41, 42). The lack of sequence similarity of the two COOH-terminal domains of the mutanases with any known carbohydrate-binding domains together with their insoluble mutan binding activity allows the definition of a new family of carbohydrate-binding modules.

The pH optimum observed for the two mutanases is not exactly in agreement with the earlier reported pH optimum around pH 6.0 for the *T. harzianum* mutanase (7) but comparable to the pH optimum obtained for the *Trichoderma viride* (43) and the *Penicillium funiculosum* mutanases (3). For comparison, the bacterial mutanases from *Bacillus circulans* (44) and *Streptomyces chartreusis* (45) have slightly higher pH optima than the two fungal mutanases but similar temperature optima. Although, the pH in the oral cavity is around pH 6–7, the slightly acidic pH profile of the two fungal mutanases may be of importance in the application for plaque removal as low pH values have been observed locally in the plaque (46).

The substrate binding constants observed for the two mutanases to insoluble mutan are, although slightly higher, in the range of reported binding constants for cellulase adsorption to insoluble cellulose (27). The difference in the maximum binding capacity observed for the two mutanases may be explained by differences in the batches of mutan used for the experiment as these have been found to vary somewhat in quality/purity. Alternatively, a possible explanation would be that the *T. harzianum* mutanase is capable of dispersing the insoluble mutan (in analogy to cellulose-binding domains and cellulose) to a larger extent than the *P. purpurogenum* mutanase and thus revealing a larger surface area onto which the enzyme can adsorb. The strong adsorption of the fungal mutanases may be beneficial for their application in dentrifice as the enzymes are expected to bind to dental plaque and thus be retained in the oral cavity where it is supposed to exhibit its action in removing the dental plaque.

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