RESEARCH COMMUNICATION

A novel lactonohydrolase responsible for the detoxification of zearalenone: enzyme purification and gene cloning

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Zearalenone (ZEN) is converted into a far less oestrogenic product by incubation with *Clonostachys rosea* IFO 7063. An alkaline hydrolase responsible for the detoxification was purified to homogeneity from the fungus by a combination of salt precipitation and column chromatography methods. The purified enzyme was homodimeric with a subunit molecular mass of 30 kDa and contained an intra-subunit disulphide bridge. On the basis of the internal peptide sequences of the purified protein, we cloned the entire coding region of the gene (designated as *zhd101*) by PCR techniques. The ZEN degradation activity was detected in heterologous hosts (*Schizosaccharomyces pombe* and *Escherichia coli*) carrying the cloned gene. *Zhd101* could be a promising genetic resource for *in planta* detoxification of the mycotoxin in important crops.

Key words: endocrine disrupting chemicals, *Fusarium* mycotoxin, oestrogenic, transgenic wheat, trichothecenes.

INTRODUCTION

Zearalenone (ZEN), 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone (Figure 1, compound 1), is an oestrogenic mycotoxin produced by numerous *Fusarium* species in pre- or post-harvest corn, wheat and other cereals [1]. ZEN shows potent oestrogenicity in livestock, especially in female swine, and causes severe reproductive problems [2]. The economic loss associated with ZEN-contaminated feed has been a historical problem in agriculture. Regarding mutagenicity, ZEN was negative in both the *Salmonella typhimurium* assay and in the *Saccharomyces cerevisae* assay [3]. On the other hand, ZEN showed DNA-binding activity and genotoxicity in recombination-deficient *Bacillus subtilis* mutants [4] and in female mouse tissues [5]. ZEN was also reported to have carcinogenicity in mice [6].

Although the oestrogenic effect of ZEN in humans remains controversial, biochemical data indicate that it has adverse effects on humans. For example, ZEN was reported to bind to human oestrogen receptors [7] and to stimulate the growth of the human breast cancer cell lines [8]. Furthermore, an epidemiological study suggested the possible carcinogenicity of ZEN in humans [9]. Hence, increasing attention has been paid to the development of an effective strategy for the decontamination of ZEN.

Enzymic detoxification could offer a practical and efficient method of ZEN decontamination. Although such enzymes are currently unavailable, a fungal isolate of *Gliocladium roseum* NRRL 1859 was able to transform ZEN into 1-(3,5-dihydroxyphenyl)-10′-hydroxy-1′-undecen-6′-one (Figure 1, compound 2), which is far less oestrogenic than ZEN [10]. In the search for micro-organisms able to metabolize *Fusarium* mycotoxins, we also observed a similar degradation reaction with *Clonostachys rosea* (synonym *G. roseum*) IFO 7063, a near-isogenic strain of NRRL 1859. The present study deals with the purification of a novel lactonohydrolase responsible for the detoxification of ZEN; the cloning and characterization of the gene encoding the enzyme are also reported.

EXPERIMENTAL

Detection of compound 2, a ZEN degradation product, in a cell-free system

Each fraction (< 89 μl) was mixed with 25 μg of ZEN (in 1 μl of ethanol) and 10 μl of 1 M Tris/HCl (pH 9.5). The total volume was adjusted to 100 μl with water and the reaction mixture was

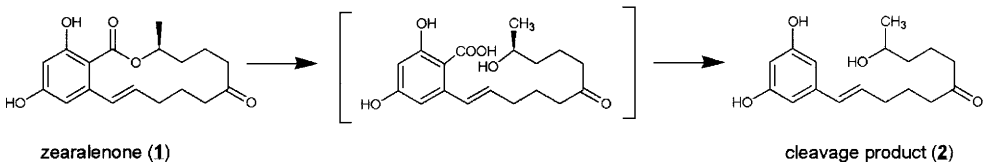

Figure 1 Detoxification of ZEN

A hypothetical pathway for the detoxification of ZEN [10]. Structures of ZEN (1) and 1-(3,5-dihydroxyphenyl)-10′-hydroxy-1′-undecen-6′-one (compound 2) are indicated. A putative unstable intermediate is shown in square brackets.

Abbreviations used: AP-I, *Achromobacter* protease I; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; PFG, pulse-field gradient; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcription PCR; ZEN, zearalenone.

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Table 1 Primers used in this study

Primers	Primer sequences (5′ to 3′)	Comments
AP-I-N-2	GGNATHACNTGGTAYTAYGARCAGGAAGG	Degenerate primer for RT-PCR
AP-I-34.5-2	ACRTCNGGRTGNGANACRTANGGRAAGTGCAT	Degenerate primer for RT-PCR
R-5F	GGGCTTCCCACGCAGAGCCTCCAGATCCTTAAC	Primer for 5′RACE
R-5N	CTCCGAGCCTCCAGACACGTCGTTCAACATTAC	Nested primer for 5′RACE
R-3F	ACCGCTGTGCTCGAAGACGAGGAAATCTCAAAG	Primer for 3′RACE
R-3N	GTAATGTTGAACGACGTGTCTGGAGGCTCGGAG	Nested primer for 3′RACE
V-5F	GCAATCTGCGACACGGAGCTGTCAAACATCTGG	Primer for vectorette PCR (promoter)
V-5N	ACCAGGTGATGCCATTCGGGGTCGAGATTGTGC	Primer for nested vectorette PCR (promoter)
V-3F	CTTTGACAACATTGTTACCGCTACCAAGGCTGG	Primer for vectorette PCR (terminator)
V-3N	TCGCTAAATATGTTGTGGAAACTACGCAGAAGC	Primer for nested vectorette PCR (terminator)
Zhd-U1	GCCCATATGCGCACTCGCAGCACAATC	Primer for expression vector
Zhd-D1	TCGGATCCGAGCTATCGTGAGCAGTG	Primer for expression vector

incubated at 37 °C. The product was extracted twice with an equal volume of chloroform and was developed on a TLC plate (Kieselgel F_{254}; Merck, Darmstadt, Germany) with chloroform/acetone (80:20, v/v).

We determined the structure of the detoxification product and it proved to be identical with compound 2 (Figure 1) by its physicochemical properties, detailed NMR spectral analysis, including ^{1}H-, ^{13}C-, pulse-field gradient (PFG)-double-quantum filtered COSY, PFG-heteronuclear multiple quantum coherence ('HMQC') and PFG-heteronuclear multiple bound coherence ('HMBC') spectra, and mass spectroscopies (fast-atom-bombardment MS, electron impact ionization MS, and GC–MS of the trimethylsilyl derivatives). The fragment patterns in electron impact ionization MS were as follows: m/z 292 (M^+; 8%), 274 ($M^+ - H_2O$; 24%), 162 (100%), 161 (86%), 112 (30%). The data were identical with those reported previously [10].

Purification of ZHD101

C. rosea IFO 7063 was incubated on 100 ml of YG medium (0.5% yeast extract and 2% glucose) containing 100 μg/ml ZEN (Sigma, St. Louis, MO, U.S.A.) and was shaken at 250 rev./min. After 1 week at 25 °C, the culture was transferred to 1 litre of YG medium containing 25 μg/ml ZEN and was incubated at 25 °C for a further 1 week. Mycelia were collected by filtration, crushed in liquid N_2, transferred to 100 ml of buffer A [10 mM Tris/HCl (pH 7.5)], and sonicated on ice. The debris was removed by centrifugation at 5000 ***g***, and ammonium sulphate was added to the supernatant at 40–60% saturation. The precipitant, obtained by centrifugation at 10000 ***g*** for 1 h at 4 °C, was then dialysed against buffer A. The sample was applied on to a 5 ml HiTrap Q column (Amersham Biosciences, Little Chalfont, Bucks., U.K.) and was eluted with a linear gradient of 0–1 M NaCl in 100 ml of buffer A. Active fractions were size-fractionated by Superdex 75 HR 10/30 (Amersham Biosciences) with buffer A containing 0.1 M NaCl. Catalytic fractions were then applied on to an anion-exchange column (Mono Q HR 5/5; Amersham Biosciences) pre-equilibrated with buffer A, and eluted with 20 ml of an NaCl gradient (0–0.5 M, with an active fraction eluted at 0.25 M). The last Mono Q step was repeated to remove a faint contaminant protein. All of the above purification steps were carried out at 4 °C.

Amino acid sequence analysis

Purified ZHD101 was digested by *Achromobacter* protease I (AP-I; TaKaRa, Kusatsu, Japan) in the presence of 2 M urea, and applied on to a reversed-phase HPLC column (VP304-1251; Senshu Kagaku, Tokyo, Japan). The peptide fragments were eluted with a linear gradient of acetonitrile [0–60% in 60 ml of 0.1% (v/v) trifluoroacetic acid]. Seven major peaks, detected at 225 nm, were collected manually, and their sequences were determined using an automated protein sequencer (Procise HT 492; Applied Biosystems, Foster City, CA, U.S.A.). The undigested protein was also analysed to obtain the N-terminal sequence.

Cloning of *zhd101* by PCR

Total RNA was isolated from the mycelia using the RNeasy Mini kit (Qiagen, Cologne, Germany). The first-strand cDNA was generated from 5 μg of total RNA and an oligo(dT) primer by using the Superscript First-Strand Synthesis kit for reverse-transcription (RT)-PCR (Invitrogen, Carlsbad, CA, U.S.A.). For amplification of the *zhd101* cDNA, degenerate primers were paired with all possible combinations. The remaining parts of the cDNA were cloned by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification kit (ClonTech Laboratories, Palo Alto, CA, U.S.A.). The promoter and terminator regions of *zhd101* were cloned from the genomic DNA of the fungus using the LA PCR *in vitro* Cloning kit (TaKaRa). On the basis of the cDNA sequence, gene-specific primers were used for the vectorette PCR (Table 1).

DNA sequence analysis

The PCR products were cloned into pGEM-TEasy (Promega, Madison, WI, U.S.A.) and sequenced by an ABI 377 DNA sequencer using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). For the sequence determination, at least five independent clones were analysed for each amplified product in order to eliminate any PCR errors. In the PCR with a set of primers specific to *zhd101*, the amplified products were directly sequenced after purification with the QIAquick PCR Purification kit (Qiagen).

Expression of *zhd101* in heterologous hosts

The coding region of *zhd101* was amplified by PCR with primers zhd-U1 (containing an *Nde*I site) and zhd-D1 (containing a *Bam*HI site) (Table 1), and was cloned into pGEM-TEasy. After sequence confirmation, *zhd101* was cloned into the *Not*I site of pcDSP21 [11] and between the *Nde*I–*Bam*HI sites of pET-12a (Novagen, Madison, WI, U.S.A.). The resulting plasmids were used to produce recombinant ZHD101 in heterologous hosts *S. pombe ura4⁻972h⁻* [12] and *E. coli* BL21 (DE3) respectively.

For protein expression, fission yeast was grown on a minimal medium and the bacterial culture was subject to the gene induction by 0.1 mM isopropyl β-D-thiogalactoside.

The ZEN degradation assay (in a total volume of 100 μl) was initiated by adding concentrated crude protein extract of the fission yeast or bacterial transformant, i.e. equivalent to 50 ml or 10 μl of culture respectively. For identification of ZEN and its metabolite, we also used a reversed-phase column (Pegasil ODS; Senshu Kagaku) with 60 % acetonitrile as an elution solvent. The absorbance was detected at 254 nm.

Nucleic acid hybridizations

The coding region of *zhd101* was labelled with the PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany) and used for Southern-blot analysis. For Northern-blot analysis, an RNA probe was generated from the *zhd101* cDNA cloned in pGEM-TEasy using a DIG RNA labelling kit (SP6/T7; Boehringer Mannheim). Standard hybridization conditions were used for both Southern- and Northern-blot analyses [13].

RESULTS AND DISCUSSION

Purification of ZHD101

To achieve efficient purification of the enzyme(s) involved in the detoxification of ZEN, it was essential to use starting materials abundant in the active fraction(s) and to establish a sensitive assay for the detection of activity. As shown in Figure 1, the detoxification involves cleavage of the lactone ring and subsequent decarboxylation of the resulting product. We were able to reconstitute these reactions for the first time with a cell-free extract by: (1) starting with fungal cultures incubated in the presence of ZEN, and (2) carrying out the reaction under alkaline conditions (pH 9–10). The active fraction was attributed to a single enzyme, as shown below.

In the crude extract of mycelia, we were able to detect very weak ZEN degradation activity using the above assay conditions. This activity was concentrated by ammonium sulphate fractionation to a precipitate of 40–60 % saturation. At this stage, the dialysed crude extract completely converted ZEN ($R_F = 0.8$) into a product with an R_F value identical with that of compound 2 ($R_F = 0.2$) within 12 h (Figure 2A). The structure of compound 2 was determined unequivocally by ^{1}H- and ^{13}C-NMR spectral analysis, including two-dimensional NMR techniques and MS. The active fraction was subjected to a series of further column chromatography separations. The enzyme was relatively stable during these procedures. Anion-exchange chromatography with a Mono Q column was particularly effective in eliminating the contaminant proteins. Finally, after five purification steps (Figure 2B, lanes 1–5), a single polypeptide of approx. 30 kDa was obtained. The specific activity of the purified enzyme (lane 5) was estimated to be approx. 200-fold, in comparison with that of crude extract after the salt precipitation step (lane 1). The enzyme appeared to occur as a homodimer, since its molecular mass was estimated to be 60 kDa by the gel-permeation chromatography and 68 kDa by native PAGE (results not shown).

Although the purified enzyme was able to detoxify ZEN at pH 7, its maximum activity was observed at pH 9–10. Considering that ZEN is stable at pH 9.5 (Figure 2A, lane 3), the enzyme responsible for the detoxification appeared to be a hydrolase (designated as ZHD101) that cleaves the lactone ring. It is possible that spontaneous decarboxylation of the resulting product may have led to compound 2 with which the irreversibility of the detoxification reaction is guaranteed (Figure 1). At low pH ($<$ pH 4.5), ZHD101 was unstable and irreversibly inactivated.

Cloning and characterization of *zhd101*

Six major peptide fragments of AP-I-digested ZHD101 were sequenced. We designed degenerate primers on the basis of their sequences and amplified a portion of the cDNA with primers AP-I-N-2 and AP-I-34.5-2 (Table 1). On the basis of the partial sequence of the cDNA, the 5′ and 3′ portions of *zhd101* were cloned by RACE using the gene-specific primers (Table 1). After cloning into pGEM-TEasy, six and twelve independent clones were sequenced for the 3′- and 5′-RACE products respectively. The assembled nucleotide sequence of the *zhd101* cDNA revealed an open reading frame that encodes an acidic protein of 264 amino acids with a calculated average molecular mass of 28 749 Da. Before the translation initiation codon, an adenine nucleotide appeared at position -3; this position is highly conserved among fungal genes [14]. The deduced amino acid sequence of *zhd101* contained all of the partial amino acid

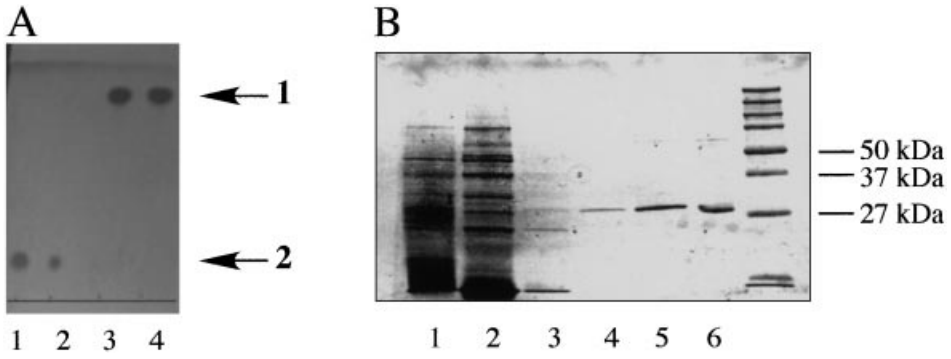

Figure 2 Purification of ZHD101

(**A**) Detection of ZEN degradation in a cell-free system. Lane 1, compound 2 standard; lane 2, ZEN incubated with crude enzyme (40–60% ammonium-sulphate-precipitated fraction) at pH 9.5; lane 3, ZEN incubated at pH 9.5 (negative control); lane 4, ZEN standard. The positions of ZEN (compound 1) and compound 2 are indicated. (**B**) SDS/PAGE of active fractions during purification. Proteins were denatured in SDS sample buffer in the presence (lanes 1–5) or absence (lane 6) of 2-mercaptoethanol, and separated by SDS/PAGE on a 12.5% (w/v) gel. The proteins on the gel are active fractions from the following purification steps: lane 1, ammonium sulphate precipitation (18.5 μg of protein); lane 2, HiTrap Q separation (23.2 μg of protein); lane 3, Superdex 75 separation (3.1 μg of protein), lane 4, the first Mono Q separation (0.5 μg of protein); and lanes 5 and 6, the second Mono Q separation (2.3 μg of protein each). Molecular-mass-marker proteins (in kDa; Amersham Biosciences) are shown in the last lane.

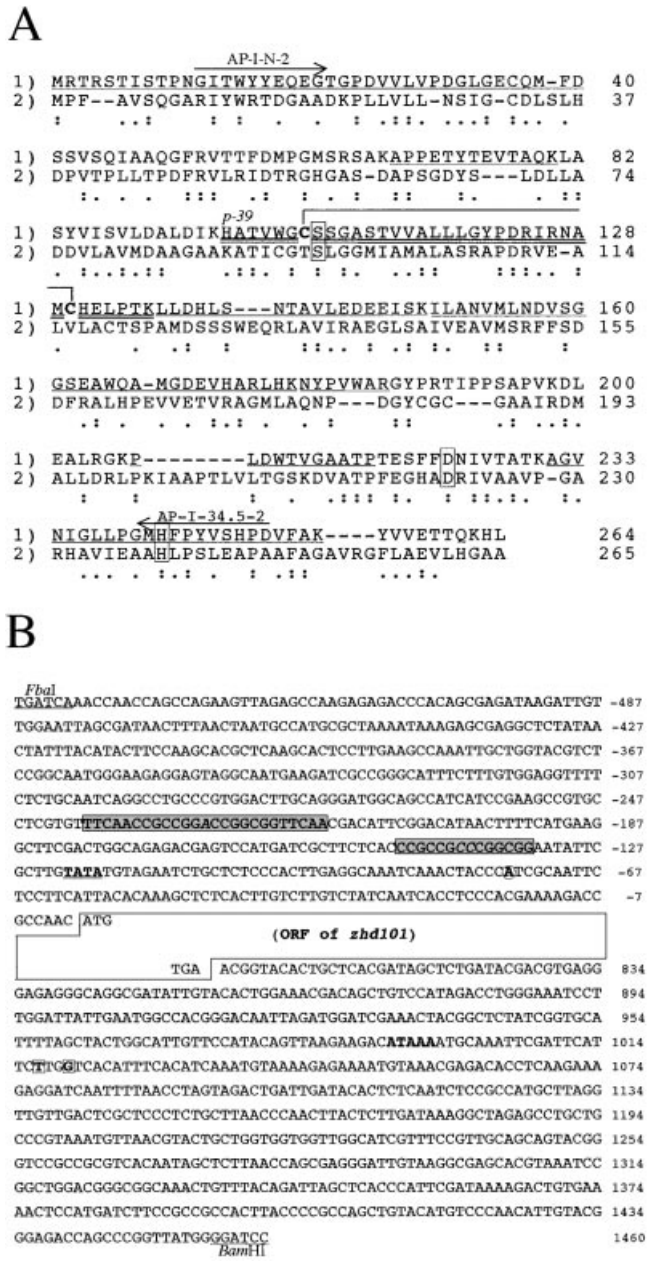

Figure 3 Primary structures of *zhd101*

(**A**) Alignment of the deduced amino acid sequences of *zhd101* (1, upper line) with the N-terminal portion (265 residues out of a total of 393 residues) of 3-oxoadipate enol-lactone hydrolase/4-carboxymucolactone decarboxylase from *C. crescentus* (2, lower line). Identical (**:**) and similar (**.**) amino acid residues are shown. Residues that comprise the catalytic triad of a serine hydrolase family are boxed. The intra-subunit disulphide bond is shown as a bridge above the two cysteine residues, which are shown in bold. The peptide sequences determined by the amino acid sequence analyses are underlined, and the peptide p-39 is indicated by double underlines. The locations of the amino acids corresponding to the primers used for RT-PCR (AP-I-N-2 and AP-I-34.5-2) are shown. (**B**) The promoter and terminator sequences of *zhd101*. Only the first and last codons of the open reading frame (ORF) of *zhd101* are shown. The nucleotide sequence (the complete sequence is available from GenBank® with the accession number AB076037) is numbered on the basis of the translation initiation site of the coding region (the first adenine is taken as +1). Imperfect palindrome sequences (mismatched bases are in italics) are shown in the shaded boxes. The sequence at −121 (bold and underlined) is most likely to be a TATA box element. The sequence at +993 to +997 (shown in bold) appears to be the most likely candidate for a canonical polyadenylation signal (AATAAA). The bases at +1017 and +1020 (boxed) may represent the polyadenylated start sequences. The adenine at −76 (circled) indicates the start of the 5′-RACE products. The locations of the *Bam*H1 and *Fba*I restriction digestion sites are underlined.

sequences determined from the purified enzyme (underlined in Figure 3A).

On the basis of the cDNA sequence, the coding region of *zhd101* was amplified by PCR from the genomic DNA of the fungus. Sequence analysis of the product showed that the coding region was not interrupted by introns. We also amplified the promoter and terminator regions by vectorette PCR from the *Fba*I and *Bam*HI cassette libraries respectively. The combined sequence of these regions is deposited in the GenBank® database with the accession number AB076037.

The genomic DNA sequence thus obtained was compared with that of the RACE products. As shown in Figure 3(B), all of the 5′-RACE products started with adenine at position −76 (circles), suggesting that this position could be a candidate for the transcription initiation site. In consideration of the above results, the sequence at position −121 (bold and underlined) is most likely to be a TATA box element. Further upstream of the region, two imperfect palindromic sequences (shaded boxes) were found. Among the 3′-RACE products analysed, the polyadenylated sequences started after thymine (+1017) in one clone, and after guanine (+1020) in five clones. Although a canonical polyadenylation signal (AATAAA) was not found, the sequence at +993 to +997 (shown in bold in Figure 3B) appeared to be the most likely candidate.

Structural features of ZHD101

The molecular mass of the purified enzyme was determined by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI-TOF)–MS. The value (molecular mass = 28754.0 Da) was practically identical with the theoretical value, thereby indicating the absence of sugar residues covalently linked to the polypeptide. Interestingly, the enzyme showed a slightly different mobility on SDS/PAGE when the reducing agent was omitted from the denaturation buffer (Figure 2B, lane 6). This result is suggestive of the presence of an intra-subunit disulphide bond. The molecular mass of the peptide fragment p-39 containing the two cysteine residues (Figure 3A, Cys^{101} and Cys^{124}, shown in bold) was determined by MALDI-TOF–MS. The obtained experimental value was in good agreement with the theoretical value calculated on the assumption that these two cysteine residues are cross-linked by a disulphide bond.

A search of the protein sequence databases at the National Center for Biotechnology Information revealed that ZHD101 demonstrates a significant level of similarity (25% of identity) to a 3-oxoadipate enol-lactone hydrolase/4-carboxymuconolactone decarboxylase from *Caulobacter crescentus* (a protein found in the *C. crescentus* complete genome sequence project, accession number AAK24382) [15]. The conserved domain search suggested that the two enzymes share a common secondary structural feature designated as an α/β hydrolase fold [16]. These enzymes belong to the serine hydrolase family, which is characterized by the presence of a catalytic triad [17]. The sequence alignment revealed that ZHD101 possessed a catalytic triad consisting of Ser^{102}, His^{242} and Asp^{223} (Figure 3A, boxed residues), where the active site serine was embedded in the consensus sequence Sm-Xaa-Ser-Xaa-Sma-Sma (where Sma is small amino acid residue, e.g. glycine) [16]. The ester hydrolysis is supposed to be mediated by the nucleophilic attack of the serine on the carbonyl of the substrate in a charge-relay system.

Expression of *zhd101* in heterologous hosts

Our initial attempts to detect ZEN detoxification with *S. pombe* transformants were not successful, and compound 2 was not

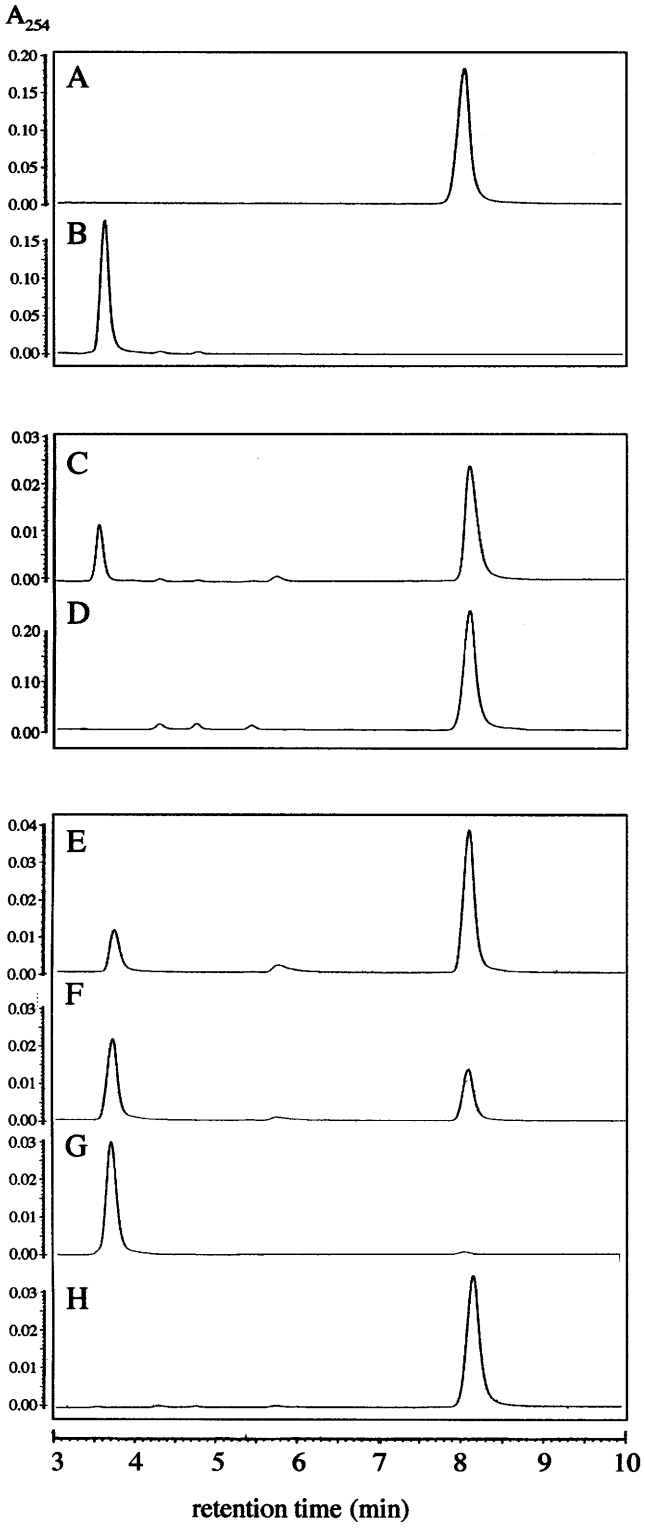

Figure 4 Detoxification of ZEN by ZHD101 expressed in heterologous hosts

Recombinant ZHD101 was expressed in *S. pombe* and *E. coli* using the expression vector pcDSP21 and pET-12a respectively. HPLC profiles are as follows: ZEN standard (**A**); compound 2 standard (**B**); the acetonitrile extract of the reaction mixture after incubation for 12 h with the concentrated crude protein extract of transformed (**C**) and wild-type (**D**) *S. pombe*; the reaction mixtures after incubation for 5 min (**E**), 15 min (**F**) and 30 min (**G**) with the *E. coli* transformant protein extract; and the reaction mixture after incubation for 24 h with the protein extract of wild-type (i.e. untransformed) *E. coli* (**H**).

detected in the culture of transformants incubated with 100 μg/ml ZEN. This result is in contrast with our previous observations with *bsd* from *Aspergillus terreus* [11] and *Tri101* from *Fusarium graminearum* [18]. In either case, the resistance gene on pcDSP21 was able to detoxify a sufficient amount of the antibiotic (blasticidin S and trichothecenes respectively) to be monitored when expressed in fission yeast. The observed failure to detoxify ZEN in *S. pombe* culture may be attributed to the instability of pcDSP21 in the absence of selective pressure [11], since this mycotoxin did not significantly inhibit the growth of the organism (also see the next subsection).

Although the lactonohydrolase activity was not detected *in vivo* under the above conditions, the activity was detected *in vitro* with the ZEN degradation assay. Using the crude cell extracts of the transformant, a small amount of compound 2 was detected in the reaction mixture after incubation for 12 h (Figure 4). Under the same conditions, extracts from control fission yeast cells did not show ZEN degradation activity (Figure 4, compare C and D).

To augment further evidence that the cloned gene does code for an enzyme responsible for the detoxification of ZEN, we also used the powerful T7 expression system for production of *zhd101* in *E. coli*. The crude extract of the induced bacterial culture, but not the extract of the non-induced culture, revealed a strong band that corresponded with the molecular mass of ZHD101 in SDS/PAGE (results not shown). This protein extract from the induced culture (6.6 μg of total protein) efficiently converted ZEN into a product with a retention time identical with that of compound 2 (Figures 4E–4G). In addition, the trimethylsilyl derivative of the product showed exactly the same total ion monitoring (TIM) chromatogram in GS–MS analysis (results not shown). The amount of compound 2 in the reaction mixture correlated well with the incubation time, which is suggestive of the catalytic reaction by a gene product derived from the *zhd101* expression plasmid. The control *E. coli* extract (i.e. without the expression plasmid) did not yield compound 2, even after 24 h of incubation with ZEN (Figure 4H).

Nucleic acid hybridizations

Southern-blot analysis was performed in order to examine whether or not there are homologous regions of *zhd101* in the genome of *C. rosea*. When DNA was digested with restriction enzymes that do not cut the probe DNA, a single band was detected on the blot with all of the ten enzymes used (Figure 5A; only five of these are shown in lanes 1–5). Such single hybridization signals were split into two when restriction enzymes with one recognition site were used for the Southern-blot analysis (Figure 5A, lanes 6–8). These results indicate that *zhd101* is a single-copy gene and that there are no evolutionarily similar hydrolase genes in the genome of this fungus.

Interestingly, the expression level of *zhd101* was significantly elevated upon addition of ZEN to the culture. In the Northern-blot analysis, the *zhd101* transcript was detected from mycelia grown in the presence of ZEN (100 μg/ml), but not from those grown without the mycotoxin (Figure 5B). In fact, lactonohydrolase activity was not detected even after concentration by ammonium sulphate fractionation (results not shown). A similar result was obtained by RT-PCR; ZEN significantly induced the expression of *zhd101*. The RT-PCR assay was so sensitive that we were able to detect trace amounts of the transcript from untreated mycelia (Figure 5C).

Induction of the antibiotic-inactivating genes by the antibiotics themselves is known to occur in some fungal genes. For example, the transcription levels of *bsd* and *Tri101* are significantly elevated

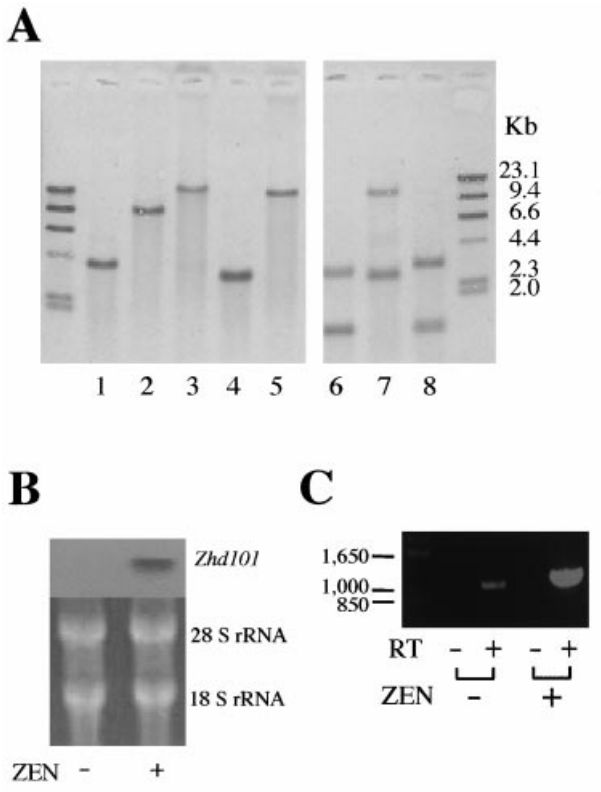

Figure 5 Analyses of genomic DNA and RNA of *zhd101*

(**A**) In the Southern-blot analysis, genomic DNA was digested by the following restriction enzymes: lane 1, *Bam*HI; lane 2, *Bgl*II; lane 3, *Eco*RI; lane 4, *Fba*I; lane 5, *Hin*dIII; lane 6, *Nco*I; lane 7, *Xho*I; and lane 8, *Pst*I. DNA-size markers (in kb) are indicated on the right. (**B**) Northern-blot analysis. *C. rosea* was incubated with or without 100 p.p.m. ZEN on YG medium for 2 days. Total RNA samples were run on a 0.8% formaldehyde-agarose gel and stained with ethidium bromide (lower panel). RNA was then transferred on to a nylon membrane and hybridized with the *zhd101* riboprobe (upper panel). The positions of 18 S and 28 S rRNA are indicated as loading controls. (**C**) Detection of a trace amount of the *zhd101* transcript by RT-PCR. A portion (5 μg) of RNA samples prepared in (**B**) was used for RT. To eliminate the possibility of trace amounts of genomic DNA contamination, control PCR was performed in parallel without (−) RT. Size markers are indicated on the left.

upon addition of blasticidin S and trichothecenes respectively, which are protein synthesis inhibitors [11,19]. Distinct from these instances, the *zhd101* expression was enhanced in *C. rosea* (but not in recombinant *S. pombe*; see also the previous section) by the addition of an oestrogenic compound ZEN without antifungal activity. Furthermore, there were no consensus sequences of mammalian oestrogen-responsive elements upstream of *zhd101*. These unique features may suggest the existence of a type of hormone receptor in this fungus, although other physiological roles of the system (if any) are still not understood.

Conclusions and perspectives

We have purified a novel lactonohydrolase that is responsible for the detoxification of ZEN and cloned the encoding gene *zhd101*. Although enzyme activity was maximal at pH 9–10, ZHD101 was still able to degrade the mycotoxin at pH 7. The gene is expected to serve as a useful genetic resource in the decontamination of wheat and other small grains, where *Fusarium* species cause serious problems of food pollution associated with mycotoxins. Together with *Tri101*, which inactivates trichothecenes (another mycotoxin of the ZEN producer, which is also known as a virulence factor [20,21]), *zhd101* is now being transformed into wheat in our laboratory.

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