

Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn²⁺

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Summary

The white-rot fungus *Pleurotus ostreatus* produces both manganese-dependent peroxidase (MnP) and versatile peroxidase (VP) in non-manganese-amended peptone medium (PM). We studied the effect of Mn²⁺ supplementation on MnPs and VPs in *P. ostreatus* by analysing the enzymatic and transcript abundance profiles of the peroxidases, as well as the lignin mineralization rate. The fungus was grown in PM under solid-state conditions using perlite as an inert solid support. Mn²⁺ amendment resulted in a 1.7-fold increase in [¹⁴C]-lignin mineralization relative to unamended medium. Anion-exchange chromatography was used to resolve the fungal peroxidase's enzymatic activity profile. Five peaks (P1–P5) of VP and one peak (P6) of MnP activity were detected in unamended medium. In Mn²⁺-amended medium, a reduction in the activity of the VPs was observed. On the other hand, a sharp increase in the MnP activity level of peak P6 was detected. The P6 isoenzyme was purified and showed manganese-dependent peroxidation of phenolic substrates. Internal sequence analysis of the purified enzyme revealed 100% identity with the deduced amino acid sequence of *P. ostreatus* MnP3 (GenBank AB016519). The effect of Mn²⁺ on the relative abundance of gene transcripts of three VPs and one MnP from *P. ostreatus* was monitored using reverse transcription–polymerase chain reaction (RT–PCR) with oligonucleotide primer sets synthesized on the basis of non-conserved sequences of the different peroxidases. The reduction in VP gene transcript abundance and the increase in *mnp3* transcript level were collinear with the changes observed in the enzyme activity profiles. These results indicate that

the activity of peroxidases is regulated at the transcriptional level. We suggest that the expression of MnP and VP may be differentially regulated by the presence of Mn²⁺.

Introduction

Lignin degradation is an important step in carbon cycling, mediated by oxidative enzymes such as manganese peroxidase (MnP). The white-rot fungus *Pleurotus ostreatus* preferentially degrades lignin in cotton stalks and wheat straw (Kerem and Hadar, 1993; Martinez *et al.*, 1994). *P. ostreatus* has been found to produce laccase (Platt *et al.*, 1984; Sannia *et al.*, 1986), MnP (Becker and Sinitsyn, 1993; Kerem and Hadar, 1996; Sarkar *et al.*, 1997) and aryl-alcohol oxidase (Pelaez *et al.*, 1995). No typical lignin peroxidase (LiP) has been described in this or any other *Pleurotus* species. MnP preferentially oxidizes Mn²⁺ to Mn³⁺, the former being an obligatory co-substrate for this enzyme, as it is required to complete the catalytic cycle (Gold and Alic, 1993). A new family of ligninolytic peroxidases has been described in the genera *Pleurotus* and *Bjerkandera* (Heinfling *et al.*, 1998; Camarero *et al.*, 1999). The new enzymes combine catalytic properties of LiP (oxidation of veratryl alcohol, methoxybenzenes and non-phenolic lignin model dimers) and MnP (oxidation of Mn²⁺). These enzymes have been designated as versatile peroxidases (VPs). Accordingly, we have used this term in the current report to designate peroxidases that exhibited Mn²⁺-independent peroxidase activity.

The degradation and mineralization of [¹⁴C]-lignin under conditions of solid-state fermentation (SSF) in basidiomycete-selective medium are enhanced when Mn²⁺ is added, suggesting the importance of MnP in the process (Kerem and Hadar, 1993). Camarero *et al.* (1996) described strong stimulation of lignin mineralization (SSF of wheat straw) by *Pleurotus pulmonarius* after the addition of Mn²⁺. Martinez *et al.* (1996) studied the catalytic properties of MnP isoenzymes from liquid peptone medium (PM) of *Pleurotus eryngii*. High MnP activity was detected in this medium, even though peroxidase production was strongly inhibited by Mn²⁺ concentrations above 10 µM (Martinez *et al.*, 1996). Two isoenzymes (MnPL1 and MnPL2) were purified and described as VPs, capable of oxidizing Mn²⁺ as well as phenolic and non-phenolic aromatic substrates (Martinez

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Table 1. Oligonucleotide sequences used in this study.

Gene	Primer designation ^a	Sequence	Expected amplicon		Reference
			cDNA	gDNA	
<i>mnp1</i>	MnP1F MnP1R	CGGCACCCTCGTGTTACCAA CATGGCGTCCTGGTTCTGTCC	510	960	Asada <i>et al.</i> (1995)
<i>mnp2</i>	MnP2F MnP2R	ATCTTCAGTGACACCGAGCC GGCGGTCCCTTGCAACATTGT	422	686	Giardina <i>et al.</i> (2000)
<i>mnp3</i>	MnP3F MnP3R	CTCCTGACTTTGGCATCTCAA CATAGCAGCCTTGAAAGCGG	828	1264	Irie <i>et al.</i> (2000)
<i>mnp4</i>	MnP4F MnP4R	CGCCGACGGACGCACCACC GGGGAGGCGGCCACGGCATC	394	552	Ruiz-Dueñas <i>et al.</i> (1999a)
Generic MnP	MnP MnP	ACCTTCCACGACGCTATT GACATCGGAGCAATCGAT	713	1264	Sarkar <i>et al.</i> (1997)
β-Tubulin	TubF1	TGGGCT(G/C/A)AAG(A)GGT (G/C/A/CAT(C)TAT(C)AC	970	1300	Yarden and Katan (1993)
	TubR	CGGAATTC(T/C)TG(G/A)TA (T/C)TG(T/C)TG(G/A)TA(T/C)TC	900	1200	Kim <i>et al.</i> (1997)
	TubF2	GTGCGT AAGGAAGCTGAGGG			

a. 'F' and 'R' represent the forward and reverse primers respectively.

et al., 1996). VPs were also isolated from a *Bjerkandera* sp. showing Mn²⁺-independent activity and the ability to oxidize non-phenolic substrates. It was suggested that this enzyme is a LiP–MnP hybrid (Mester and Field, 1998). In recent years, five peroxidase genes have been isolated from *Pleurotus* species. The first gene (*mnp1*) was isolated from *P. ostreatus* (Asada *et al.*, 1995), encoding a peroxidase that oxidizes Mn²⁺ to Mn³⁺ and has the ability to degrade the beta-O-4 lignin substructure model compound (Kofujita *et al.*, 1991). *mnp2*, encoding the isoenzyme MnP2, was isolated from *P. ostreatus* grown on wood sawdust (Giardina *et al.*, 2000)

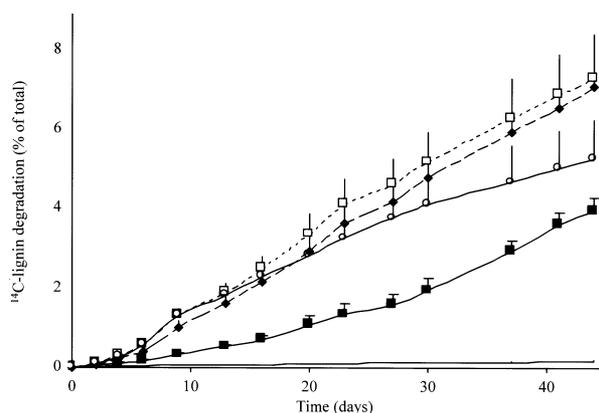


Fig. 1. Effect of various manganese concentrations on the mineralization of [¹⁴C]-lignin by *P. ostreatus* grown in peptone medium under solid-state fermentation conditions on perlite. Percentage of ¹⁴CO₂ emitted from the total initial radiolabelled [¹⁴C]-lignin is presented. —, Control; -■-, non-Mn²⁺-amended; -◆-, 73 μM Mn²⁺; -◊-, 730 μM Mn²⁺; -○-, 4500 μM Mn²⁺. Bars represent the standard error and when not visible are smaller than the symbol.

and was found to exhibit manganese-independent activity. Irie *et al.* (2000) described the isolation of a cDNA and genomic fragment of *mnp3* from *P. ostreatus* grown on peptone- and Mn²⁺-amended medium. The *P. eryngii mnp1* gene was isolated from cultures grown in peptone and low manganese (Ruiz-Dueñas *et al.*, 1999a). Two alleles (*mnp11* and *mnp12*) of this gene were isolated and shown to differ by a single metal response element (MRE) in the promoter (Ruiz-Dueñas *et al.*, 1999a). Camarero *et al.* (2000) compared mature protein sequences of 29 fungal peroxidases. The *Pleurotus* peroxidases were clustered in one group together with *Trametes versicolor*. This group is much closer to the *Phanerochaete chrysosporium* LiP group than to the MnP group. Expression of MnP is regulated at the transcriptional level by various regulators, such as Mn²⁺ (Bonnamme and Jeffries, 1990; Brown *et al.*, 1990; Ruiz-Dueñas *et al.*, 1999b), nutrient nitrogen (Pribnow *et al.*, 1989; Ruiz-Dueñas *et al.*, 1999b), heat shock (Brown *et al.*, 1993), H₂O₂, reduced oxygen species (Ruiz-Dueñas *et al.*, 1999b) and other chemical stresses (Li *et al.*, 1995). MREs have been reported in *mnp* genes (Gold and Alic, 1993).

Recent studies on *P. ostreatus* peroxidases have clearly indicated an effect of Mn²⁺ on their activity levels. However, the relationship between the expression of specific peroxidase-encoding genes, the activity of their corresponding products and lignin degradation is not well understood. In this study, we describe the regulation of peroxidase activity and gene expression by Mn²⁺, as well as the lignin mineralization rate by *P. ostreatus* in PM under SSF conditions. The activity profiles of the peroxidases and the effect of Mn²⁺ on transcript

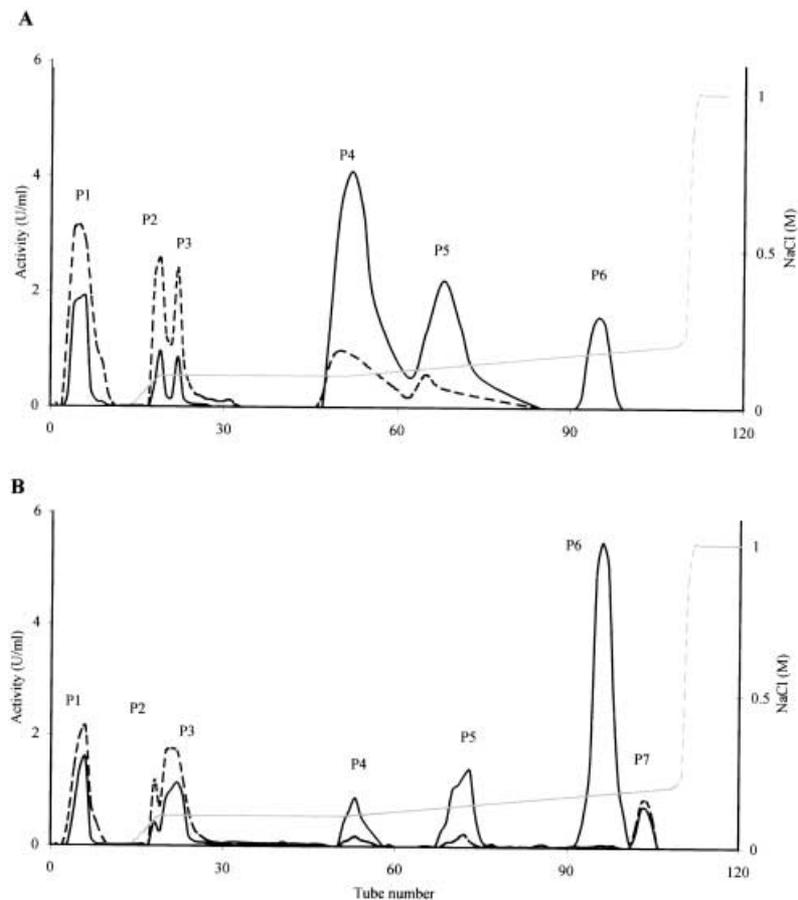


Fig. 2. Enzymatic profiles of manganese-dependent peroxidase (MnP) and versatile peroxidase (VP) after anion-exchange chromatography (HiTrapQ, 5 ml fraction size). *P. ostreatus* was grown under solid-state fermentation conditions on perlite in unamended peptone medium (PM) (A) or 500 μM Mn^{2+} -amended PM (B). MnP and VP activities (U ml^{-1}) were determined by the oxidation of phenol red with (—) or without (---) Mn^{2+} in the reaction mixture. Elution was obtained using a 1 M NaCl gradient (grey line).

abundance of the different *mnp* genes are described, and the functional definition of the corresponding enzymes is discussed.

Results

Effect of Mn^{2+} on [^{14}C]-lignin mineralization

We conducted a study using *P. ostreatus* cultured on PM under SSF conditions to follow the mineralization of [^{14}C]-lignin prepared from cotton branches. Mineralization rate was determined after the addition of various concentrations of Mn^{2+} to the PM over the course of 44 days. Figure 1 shows the rate of [^{14}C]-lignin mineralization in culture amended with 0, 73, 730 or 4500 μM Mn^{2+} . Manganese concentration in the unamended medium was determined by atomic absorption spectroscopy and found to be less than 0.1 μM . Mineralization of [^{14}C]-lignin in the unamended medium was found to be 0.35% and 4% after 9 and 44 days respectively. The addition of 730 μM Mn^{2+} increased lignin degradation significantly to 1.3% and 7.3% (3.7- and 1.84-fold respectively) after 9 and 44 days respectively. The addition of 4500 μM Mn^{2+} also increased the degradation rate, but to a lesser extent

than 730 μM Mn^{2+} (Fig. 1). Although Mn^{2+} is known as an inhibitor of MnP production in media containing peptone (Martinez *et al.*, 1996), our results suggest an important role for Mn^{2+} in lignin degradation by *P. ostreatus* under SSF conditions using rich PM.

Effect of Mn^{2+} on peroxidase activity profiles

MnP activity may play a predominant role in lignin oxidation by white-rot fungi. We determined the effect of Mn^{2+} on MnP and VP enzyme activity profiles after anion-exchange chromatography. The activity profiles were determined in either unamended medium or medium containing 500 μM Mn^{2+} . The filtrate supernatant of 6-day-old SSF cultures (5 mg of protein) was passed through an anion-exchange column, and the eluted fractions were tested for MnP and VP activity (Fig. 2). In the unamended medium, five peaks (P1–P5) of VP were detected, whereas only one peak (P6) was found that exhibited only MnP activity (Fig. 2A). By increasing the level of Mn^{2+} in the growth medium to 500 μM , the MnP activity level of peak P6 increased significantly. In contrast, the total

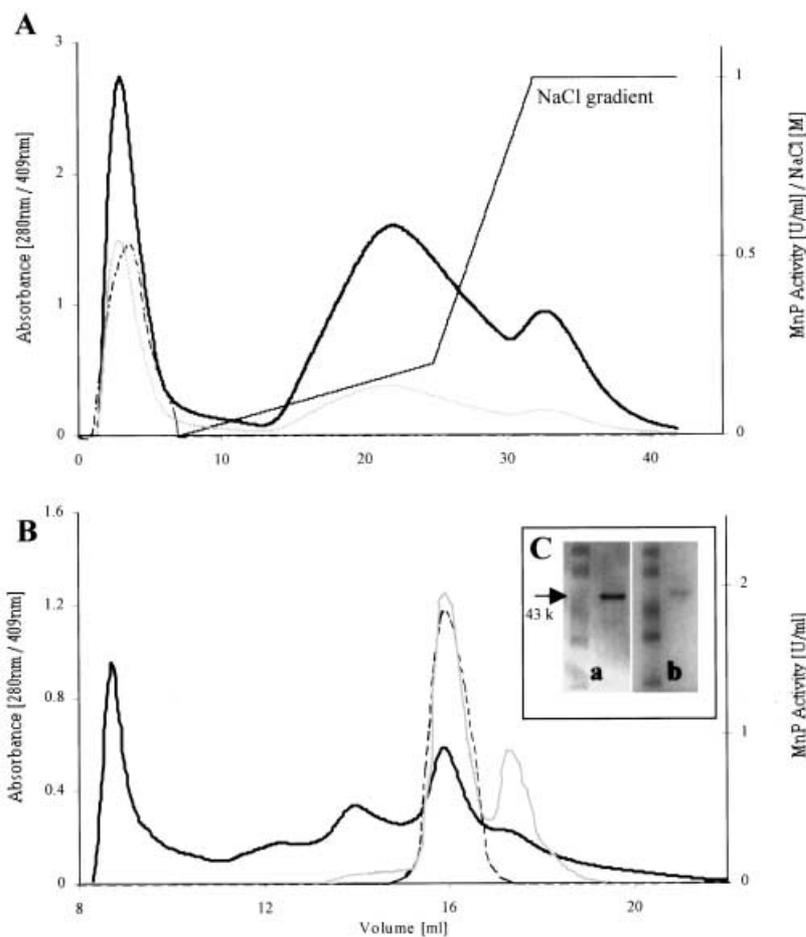


Fig. 3. Purification of the P6 isoenzyme from *P. ostreatus* grown in Mn²⁺-amended (500 μ M) peptone medium (PM) under solid-state fermentation conditions on perlite. A. Elution profile of anion-exchange chromatography (MonoQ) of P6. B. Elution profile of gel-filtration chromatography (Superdex 200). Bold line, A_{280} ; grey line, A_{409} ; dotted line, MnP activity (U ml^{-1}), determined by the oxidation of phenol red. Chromatography conditions are described in *Experimental procedures*. C. Native PAGE (12%) of purified P6; (a) activity staining in 0.1 M sodium tartrate, pH 5, including 4-chloro-1-naphthol as a substrate, 100 μ M Mn²⁺ and 50 μ M H₂O₂; (b) Coomassie blue (R-250) staining. Left lanes contain prestained markers.

activity of five of the VP peaks (P1–P5) was significantly reduced, and P7, exhibiting a low level of VP activity, appeared under these conditions. The increased activity of P6 in Mn²⁺-amended medium together with the positive effect of Mn²⁺ on [¹⁴C]-lignin degradation suggests positive regulation of MnPs in lignin degradation in *P. ostreatus*.

Purification and characterization of the MnP isoenzyme P6

To determine the molecular nature of the P6 isoenzyme, we first purified it from *P. ostreatus* cultures grown on PM-SSF containing 500 μ M Mn²⁺ (Fig. 2). Fractions of isoenzyme P6 containing MnP activity from the first purification step (HiTrapQ) were purified further by anion-exchange chromatography (MonoQ) at pH 3.6 in order to concentrate the enzyme in the unretained fractions while trapping other proteins on the column (Fig. 3A). MnP activity was detected in the unretained fractions that were subsequently loaded on to the last purification step using gel-filtration chromatography

(Superdex 200 H) (Fig. 3B). A purification factor of 34 was determined after the three purification steps. An A_{409}/A_{280} ratio of 3.2 suggested the presence of a haem-containing protein (Martinez *et al.*, 1996). Activity gels using native PAGE in 12% polyacrylamide gels and 4-chloro-1-naphthol as the substrate confirmed the activity of the purified P6 as MnP (Fig. 3C), as activity staining was detected only in reactions containing Mn²⁺ and H₂O₂. Based on its elution from the gel-filtration column, the molecular mass of P6 was determined to be 42 kDa. P6 oxidized Mn²⁺ to Mn³⁺ in the presence of H₂O₂ and exhibited Mn²⁺-dependent activity with phenol red and 2,6-dimethoxyphenol as substrates. The K_m of P6 for Mn²⁺ was 25 μ M, calculated by the formation of Mn³⁺-tartrate.

The N-terminal sequence of the purified P6 was ATCADGRTTANAACCVL, which is identical to those of isoenzymes MnP3 (Irie *et al.*, 2000), MnP of *P. pulmonarius* (Martinez *et al.*, 1996), MnP of *P. ostreatus* (Sarkar *et al.*, 1997) and MnPL1 of *P. eryngii* (Ruiz-Dueñas *et al.*, 1999a). Because the N-terminal sequences were identical, we obtained an internal sequence for P6 that was 100% identical to the deduced amino acid

MnP1	MTFASLSALV	LVFAVTQVA	Q.AVSLPQKR	ATCAGGQVTA	NAACCVLFPL	MEDLQKNLFD	DGACGEDAHE	ALRL TFHDAI	GFSPSRGVM.	...GGADGS	94																		
MnP2	MAFAKLSALV	LALGATV..A	LGA.PSLNKR	VTCATGQTTA	NEACCALFFI	LEDIQTNLFD	GAQCCEEVHE	SLRL TFHDAI	AFSPALTNAG	QFGGGADGS	97																		
MnP3	MAFKHSSSLV	<u>L.LTLASQAV</u>	RGAA...VMKR	ATCADGRTTA	NAACCVLFPI	LDDIQEALFD	GAECCEEVHE	SLRL TFHDAI	GFSPTK....	..GGGGADGS	90																		
MnP4	MSFKTLSALA	LALGAAVQFA	SAAVPLVQKR	ATCADGRTTA	NAACCVLFPI	LDDIQENLFD	GAQCCEEVHE	SLRL TFHDAI	GFSPTL....	..GGGGADGS	94																		
Cons	M	F	S	L	L	A	KR	TCA	G	TA	N	ACC	LFP	DIQ	LFD	CGE	HE	LRL TFHDAI	FSP	GGADGS									
MnP1	VITFSDTEVN	FPANLGIDEI	VEAEKPFILAR	HNISAGDLVH	<u>FAGT</u> LAVTNC	PGAPRIPFFL	GRPPAKAASP	IGLVPEPFDT	ITDILARMDD	AGFVSVEVVW	194																		
MnP2	<u>MI</u> FSDTEPN	FHANLGIDEI	VEAQKPFILAR	HNISAADFIQ	FAGAIGVTNC	AGAPRLNFFL	GRPDATQIPP	DGLVPEPFDS	VDKILSRMGD	AGFSTVEVVW	197																		
MnP3	I ^V T ^F DEIETA	FHANGGIDDI	VDAQKPFILAR	HNISAGDFIQ	FAGAVGVSN	PGAPRLNFFL	GRPPATAASP	NGLIPEPFDT	VTDILARMGD	AGFSPPEEVA	190																		
MnP4	IIAFDTIETN	FPANAGIDEI	VSAQKPFVAK	HNISAGDFIQ	FAGAVGVSN	PGGVRIPFFL	<u>GRPD</u> VAASP	DHLVPEPFDS	VDSILARMSD	AGFSPVEVVW	194																		
Cons	F	E	F	AN	GID	I	V	A	KPF	AR	HNISA	D	FAG	V	NC	G	R	F	L	GRP	A	P	L	PEPFD	IL	RM	D	AGF	EVV
MnP1	LLSAHSVAAA	DHVDETIPGT	PFDPSTPNLFD	SQIFIETQLR	GISFPGTGGN	HGEVQSPKLG	EMRLQSDHFL	ARDDRTSCEW	QSMTNDQQKI	QDRFSDTLFK	294																		
MnP2	LLSHTIAAAA	DLVDPISIPGT	PFDPSTPSTFD	SQFFLE <u>TMLO</u> <u>GTA</u>	FPFGTPGN	QGEVESPLAG	EMRLQSDFLL	ARDSRSACEW	QSMVNNMPKI	QNRFTQVMRK	297																		
MnP3	LLASHSVAAA	DHVDETIPGT	PFDPSTPGEFD	SQFFIETQLR	GTAFPGVGGN	QGEVESPLAG	EIRIQSDHDL	ARDSRTACEW	QSFVNNQAKL	QSAFKAAMDK	290																		
MnP4	LLASHSIAAA	DKVDPISIPGT	PFDPSTPGVFD	SQFFIETQLK	GRLFPGTADN	KGEAQSPLOG	EIRLQSDHLL	ARDPQTACEW	QSMVNNQPKI	QNRFAATMSK	294																		
Cons	LL	H	AAA	A	VD	IPGT	PFDPSTP	FD	SQ	F	ET	G	FPG	N	GE	SPL	G	E	R	QSD	ARD	CEW	QS	N	K	Q	F	K	
MnP1	MSMLGQND <u>A</u>	<u>MIDCS</u> DVIPV	PAALVTKPHL	PAGKSKTDVE	QACATGAFPA	LGADPGPVT	VPRVPPA	364																					
MnP2	L ^S L ^L G ^H N ^Q A ^D	<u>LIDCS</u> DVIPV	P ^K T ^L T ^K A ^A T ^F	P ^A G ^K S ^Q A ^D V ^E	I ^V V ^A A ^T P ^F F ^A	L ^A S ^D P ^G P ^V T ^A	V ^P P ^V P ^P S	367																					
MnP3	L ^A T ^L G ^Q D ^R S ^K	<u>LIDCS</u> DVIPV	P ^K P ^L Q ^S K ^A H ^F	<u>PAGL</u> T ^M N ^N I ^E	Q ^A C ^A S ^T P ^F F ^A	L ^T A ^D P ^G P ^V T ^T	V ^P P ^V P ^P S	360																					
MnP4	M ^A L ^L G ^Q D ^K T ^K	<u>LIDCS</u> DVIP ^T	P ^P A ^L V ^G A ^A H ^L	P ^A G ^F S ^L S ^D V ^E	Q ^A C ^A A ^T P ^F F ^A	L ^T A ^D P ^G P ^V T ^S	V ^P P ^V P ^G S	364																					
Cons	LG	IDCS DVIP	P	L	PAG	E	A	FPA	L	DGPVT	VP	VP																	

Fig. 4. Multiple alignment of deduced peroxidase protein sequence from different *Pleurotus* species as indicated in Table 1. The bottom line (designated 'Cons') represents the full consensus amino acids found in all the aligned proteins. The amino acid sequences used to design primer pairs for the amplification of specific peroxidase genes are indicated below the specific sequence. The 5' primers are underlined, the 3' primers are doubled underlined. Residues in bold represent the conserved region among many peroxidases (designated 'Generic *mnp*' in Table 1).

sequence of MnP3 from *P. ostreatus* grown on peptone-containing medium (Irie *et al.*, 2000). The three internal sequence peptides obtained corresponded to amino acid residues 70–82, 231–253 and 254–262. Thus, we

concluded that the *mnp3* gene (GenBank AB016519) encodes the P6 isoenzyme purified in this study. Overall sequence homology analysis of MnPs is described in Fig. 4. Comparison between the amino acid sequences of

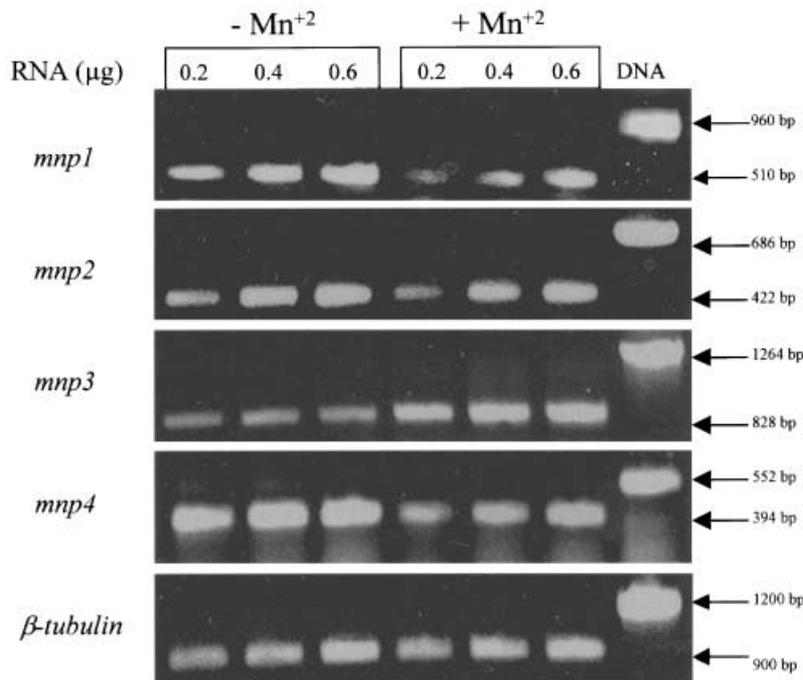


Fig. 5. Relative transcript abundance of *mnp* and β -tubulin genes using RT-PCR of total RNA extracted from 6-day-old peptone medium under solid-state fermentation cultures of *P. ostreatus* in unamended or Mn^{+2} -amended (500 μ M) medium. Aliquots (10 μ l) of products obtained from RT-PCRs containing varying amounts of RNA templates were loaded on agarose gels and stained with ethidium bromide. Primers used for the specific amplification of the different genes are listed in Table 1. A template of genomic DNA was used as a control (right lanes). Product size is indicated on the right.

MnP3 and other MnPs revealed similarities of 78% to MnP4 (Table 1), 73% to MnP1 and MnP2 (Table 1), 62% to MnP3 of *P. chrysosporium* (Alic *et al.*, 1997) and the MnP of *T. versicolor* (Collins *et al.*, 1999) and 59.3% to LiP GLG4 of *P. chrysosporium* (De Boer *et al.*, 1987). We concluded that the P6 isoenzyme is an MnP that is activated and positively induced by the presence of Mn²⁺ and that the *mnp3* gene encodes this isoenzyme.

Effect of Mn²⁺ on transcript levels of MnP and VP genes

As the presence of Mn²⁺ imposed a clear difference in MnP and VP activity levels, we determined the effect of Mn²⁺ on the relative abundance of different *mnp* gene transcripts using a relative reverse transcription–polymerase chain reaction (RT–PCR) approach. To analyse the effect of Mn²⁺ on the transcript level of MnP- and VP-encoding genes, we used available sequence information concerning *P. ostreatus mnp1*, *mnp2* and *mnp3*, as well as *P. eryngii mnp4*. *mnp1* has been reported to encode a peroxidase that exhibits manganese-dependent activity (Asada *et al.*, 1995), whereas *mnp2* and *mnp4* encode peroxidases that have manganese-independent activity (Ruiz-Dueñas *et al.*, 1999a; Giardina *et al.*, 2000). The transcript level of β -tubulin was used as a reference. As the different peroxidases share a relatively high degree of amino acid conservation (71–80% identity among the isoenzymes studied here; Fig. 4), we designed the oligonucleotide primers on the basis of non-conserved sequences. Different lengths of amplicons were obtained, by RT–PCR, corresponding to the different amplicon targets (Table 1, Figs 4 and 5). Based on related transcript abundance (Fig. 5), it appears that *mnp* transcription is involved in the regulation of peroxidase activity. Transcription products of *mnp1*, *mnp2*, *mnp3* and *mnp4* were observed in both Mn²⁺-amended or unamended cultures. However, Mn²⁺ did not affect all the *mnp* genes analysed in a similar fashion. The transcript levels of *mnp3* were significantly higher when Mn²⁺ was present in the medium; conversely, *mnp1*, *mnp2* and *mnp4* transcript abundance was reduced. We concluded that the different *mnp* genes undergo a manganese-dependent counter-regulation process. The abundance of the β -tubulin transcript was not affected by the presence of Mn²⁺ (Fig. 5).

Isolation and characterization of *P. ostreatus* MnP, VP and β -tubulin gene fragments

To verify further the nature of the reaction product, direct sequencing of the amplified DNA was performed. In all cases, the sequence obtained confirmed that the target

amplicon had been produced. During the course of this study, we obtained three new *P. ostreatus* cDNA fragments. The sequence of the *mnp2* RT–PCR product (GenBank AF326204) confirmed the predicted intron length and boundaries suggested by Giardina *et al.* (2000). Four differences in the nucleic acid sequence were detected (when compared with the genomic clone). However, in only one case did these differences have any influence on the predicted amino acid sequence.

The *P. ostreatus mnp4* RT–PCR product (GenBank AF326203), amplified on the basis of the *P. eryngii* homologue sequence for *mnp1* (Ruiz-Dueñas *et al.*, 1999a), proved to be highly similar to the *P. eryngii* gene. The differences between the *P. ostreatus* and *P. eryngii* gene fragments amounted to 12 nucleotides, one of which conferred an amino acid substitution.

The abundance of β -tubulin transcript was used as a relative control for the RT–PCR experiment. With the conserved β -tubulin degenerate primers designed by Yarden and Katan (1993), an amplicon was obtained when RT–PCR was used, but not when a DNA template was used. In subsequent experiments, we determined that the *P. ostreatus* β -tubulin gene fragment (GenBank AF332138) harbours an intron within the TuBF1 primer (Table 1). We therefore replaced it in subsequent experiments with TubF2, designed on the basis of a *Pleurotus sajor-caju* β -tubulin gene sequence (Kim *et al.*, 1997).

Discussion

In recent years, the emphasis on analysing peroxidases has increased, as members of this enzyme family are produced by most white-rot fungi, including species that lack lignin peroxidase (LiP) (Hatakka, 1994). Different types of peroxidases have been characterized, including manganese-dependent peroxidase (MnP), manganese-independent peroxidase (Mester and Field, 1998) and a versatile peroxidase (VP) that combines both LiP and MnP properties (Camarero *et al.*, 1999). The isolation of different genes encoding MnP and VP isoenzymes in *Pleurotus* species raised the question of what effect Mn²⁺ has on the expression of these genes and subsequent lignin degradation.

Media composition and growth conditions are important factors affecting the production of extracellular ligninolytic enzymes and lignin degradation. As lignocellulosic substrates such as cotton stalks and wheat straw interfere with the extraction of active enzymes, the use of perlite as an inert solid support greatly benefits the study of ligninolytic enzymes under solid-state conditions (Kerem and Hadar, 1993) and was therefore adopted in the current study. *Pleurotus* produces very low levels of MnP under culture conditions used to produce MnP in *P. chrysosporium* (ammonium as N source). As high MnP

and VP activity was found in liquid peptone cultures of *P. eryngii* (Martinez *et al.*, 1996) and *P. ostreatus* (Sarkar *et al.*, 1997), we also used this growth medium. The fact that MnP production is inhibited by Mn^{2+} amendment (Martinez *et al.*, 1996) seems to be a biological contradiction, as Mn^{2+} is known to be a requirement for the expression of *mnp* genes and protein production in several white-rot fungi (Brown *et al.*, 1991; Gold and Alic, 1993). Here, we demonstrate the positive effect of Mn^{2+} on [^{14}C]-lignin degradation and on the expression of a manganese-dependent isoenzyme. These data are in agreement with the results obtained for lignin degradation by *P. ostreatus* in poor medium containing asparagine as a nitrogen source (Kerem and Hadar, 1993), suggesting the importance of Mn^{2+} in lignin degradation by this fungus regardless of its being cultured in poor or rich (PM) media. To evaluate the molecular basis of the enhanced degradation of lignin in the presence of Mn^{2+} , we characterized the profiles of enzymatic activity and gene transcription in Mn^{2+} -amended and unamended media. Owing to the presence of several different members of the peroxidase family, enzymatic activity in crude supernatants of the different cultures is not an effective reflection of the role of the specific isoenzymes. Thus, we conducted a study using enzyme activity profiles of MnP and VP after anion-exchange chromatography of the crude supernatants. Although Mn^{2+} amendment decreased the overall activity of MnP, the level of one MnP isoenzyme (designated P6) increased. To compare the P6 isoenzyme with other peroxidases and to find the gene corresponding to this isoenzyme, it was purified from Mn^{2+} -amended medium by anion-exchange chromatography and gel filtration. Oxidation of phenolic substrates was detected only in reaction mixtures containing both Mn^{2+} and H_2O_2 . N-terminal and internal sequencing of the purified P6 revealed 100% identity to the isoenzyme MnP3 described by Irie *et al.* (2000). They grew *P. ostreatus* in peptone and Mn^{2+} liquid medium and described the isolation of cDNA and a genomic sequence encoding MnP3. Based on these data, we concluded that the protein described as P6 is an MnP, which is encoded by the *mnp3* gene.

In *P. chrysosporium*, MnP occurs as a series of isoenzymes encoded by a family of closely related genes (Gettemy *et al.*, 1998). To date, five peroxidase genes have been isolated from *Pleurotus* species, and sequences of cDNA and genomic clones have been determined (Asada *et al.*, 1995; Camarero *et al.*, 1999; Ruiz-Dueñas *et al.*, 1999a; Giardina *et al.*, 2000; Irie *et al.*, 2000). To study the effect of Mn^{2+} on the transcript levels of the various peroxidases, we determined the relative transcript abundance of the different peroxidases (MnP and VP). In terms of studying the effect of different factors on transcript level, RT-PCR has been shown to be 10^3 - to

10^4 -fold more sensitive than Northern blotting (Gilliland *et al.*, 1990; Sooknanan *et al.*, 1993). Moreover, RT-PCR can distinguish between very similar mRNAs, a situation that cannot be achieved by Northern analysis using very similar probes. Thus, the use of specific primers, designed on the basis of non-conserved regions, to analyse the transcripts of closely related genes is required. Using relative RT-PCR with specific primer pairs for each gene, we evaluated the effect of Mn^{2+} on the differential profiling of the different transcripts of *mnp1*, *mnp2*, *mnp3* and *mnp4*. We demonstrated that Mn^{2+} differentially regulates the abundance of MnP and VP transcript. In Mn^{2+} -containing cultures, the abundance of *mnp3* transcript increased significantly relative to the unamended medium, and a concomitant reduction in manganese-independent gene transcript levels was obtained. Ruiz-Dueñas *et al.* (1999b) studied the regulation of *P. eryngii mnp12* (corresponding to the *P. ostreatus mnp4* gene analysed here) transcript levels in peptone liquid culture and in isolated mycelia. In that study, Northern blot analysis was used with a 648 bp *mnp12* cDNA probe corresponding to 212-amino-acid residues of the peroxidase polypeptide. In liquid cultures, no transcript was detected when a Mn^{2+} concentration of 25 μM or higher was present in the medium. However, in isolated mycelia treated with Mn^{2+} , *mnp12* transcript was present, most likely because of induction of transcript that had occurred when the samples were first cultured in peptone medium (Ruiz-Dueñas *et al.*, 1999b). Gettemy *et al.* (1998) described the differential regulation by manganese of the transcript levels of three genes encoding MnP isoenzymes in *P. chrysosporium* using RT-PCR. In nitrogen-limited, stationary cultures supplemented with 180 μM Mn^{2+} , the levels of two *mnp* gene transcripts increased, and the level of one *mnp* transcript did not change. LiP was reported to be adversely regulated by Mn^{2+} in liquid cultures of *P. chrysosporium* (Bonnarme and Jeffries, 1990).

Additional evidence supports the biochemical and gene expression data, suggesting that MnP3 has characteristics of MnP rather than of VP. Multiple sequence alignment analysis of the different MnPs (Fig. 4) shows that MnP1, MnP2 and MnP4 have a conserved Trp residue within a Glu-Val-Val-Trp-Leu motif at amino acid residues 192-197. In contrast, in MnP3, this Trp residue is substituted by a non-aromatic Ala residue. Camarero *et al.* (1999) suggested, on the basis of structural analyses, that this Trp residue may be required for veratryl alcohol oxidation enabling this enzyme to oxidize both Mn^{2+} and non-phenolic substrates. Indeed, *P. chrysosporium* LiPH8 enzymes, in which Trp-171 had been substituted, lost all capability to oxidize veratryl alcohol (Doyle *et al.*, 1998).

In contrast to analyses of overall peroxidase activity,

there is a need to identify the number of peroxidase family members in a given organism. The enzymatic activity of each can then be assessed along with the involvement of environmental factors (nutrition, substrates, cofactors) in the regulation of expression of their genes. This information can then be used to determine the peroxidase's role in, and relative contribution to, lignin degradation. In essence, this was done here to show that Mn²⁺ regulates MnP and VP gene expression in opposite ways during SSF of *P. ostreatus*. These considerations are in agreement with studies on members of other microbial gene/enzyme families that have been shown to be differentially regulated, such as those participating in cell wall biosynthesis and degradation (Choi *et al.*, 1994; Haran *et al.*, 1996). Thus, following both message levels and enzymatic activity appears to be a rational approach to analysing collinear as well as divergent expression levels of multigene family members.

Experimental procedures

Solid-state fermentation on PM

Stock cultures of *P. ostreatus* Florida F6 were maintained on PM containing 20 g l⁻¹ glucose, 5 g l⁻¹ peptone, 1 g l⁻¹ KH₂PO₄, 2 g l⁻¹ yeast extract, 0.5 g l⁻¹ MgSO₄·7H₂O, 1.2 g l⁻¹ Ca(NO₃)₂·4H₂O and 0.012 g l⁻¹ FeSO₄ at pH 5.5. Mn²⁺ was added as MnSO₄ to the required concentrations.

The fungus was cultured for 6 days in sterile cups (100 ml) containing 6 g of perlite and 24 ml of inoculum at 28°C. The inoculum was prepared in PM containing 10% homogenized cultures grown for 24 h. The homogenized cultures were prepared by homogenization of 10 disks (8 mm diameter) of fungi grown for 3 days in 60 ml of liquid PM.

Perlite (agricultural grade) was purchased from Agrical and used as the solid support for the SSF. Before the addition of growth media, the solid particles were washed free of impurities with 5% nitric acid followed by distilled water.

[¹⁴C]-lignin mineralization

[¹⁴C]-lignin mineralization during SSF was studied according to the methods described by Kerem and Hadar (1993). [¹⁴C]-radiolabelled substrate (44 × 10⁴ d.p.m. [¹⁴C]-lignin, 20 ± 0.1 mg) was added to each of five replicates of 1.5 g of perlite in 20 ml polyethylene cups. Each polyethylene cup was then sterilized, inoculated as described, sealed in a 300 ml biometer flask with two gas-tight caps and incubated at 28°C. [¹⁴C]-lignin, mixed as described already with 1.5 g of perlite in a plastic cup, was autoclaved and then incubated with media to serve as a control. To study the effect of Mn²⁺ concentration on [¹⁴C]-lignin mineralization, three Mn²⁺ concentrations were used: 73, 730 and 4500 μM Mn²⁺, added to the medium as MnSO₄.

¹⁴CO₂ evolved in each flask was trapped daily for 2 h on GF/C 25-mm-diameter filter paper (Whatman) presoaked with 0.5 ml of 5 N NaOH. After the removal of the filters, the flasks were flushed for 1 min with moistened, sterile,

atmospheric air. Subsequently, the amount of trapped ¹⁴CO₂ was measured by liquid scintillation counting (Insta-Gel; Packard).

Enzyme profiles after anion-exchange chromatography

The extracellular fluid of 6 day SSF cultures was extracted by soaking the perlite gently in 10 mM sodium acetate buffer (pH 6) for 20 min followed by 0.45 μm filtration. The fluid was then concentrated by ultrafiltration with a PM-10 membrane (10 kDa; Amicon). The extracts were equilibrated with 10 mM sodium acetate (pH 6), and the concentrated supernatant was loaded onto a HiTrapQ anion-exchange column (Pharmacia) using fast protein liquid chromatography (FPLC; Äkta, Pharmacia); 6 ml fractions were collected (at 4 ml min⁻¹). Proteins were eluted with a linear gradient of sodium acetate (pH 6) containing 1 M NaCl, and MnP and VP activities were determined in the eluted fractions.

MnP (P6) purification

FPLC was used for the purification of MnP, carried out on 6-day-old Mn²⁺-amended (500 μM) SSF cultures. The extraction and the first step in the purification (HiTrapQ) were performed as described for obtaining the enzyme profiles. Fractions of P6 (Fig. 2B) containing MnP activity were collected, then washed, concentrated by ultrafiltration and dialysed against 10 mM sodium acetate, pH 3.6. After the concentrated fluid was loaded onto a MonoQ anion-exchange column (Pharmacia), using 10 mM sodium acetate, pH 3.6, 1 ml fractions were collected (1.5 ml min⁻¹). Proteins were eluted with a linear gradient of the same buffer containing 1 M NaCl. The unretained fractions containing MnP activity were collected, then washed and concentrated by ultrafiltration. The concentrated fluid was loaded onto a Superdex 200 HR 10/30 (10 × 300 mm, volume 24 ml) gel filtration column (Pharmacia). The enzyme was eluted with 10 mM sodium acetate buffer (pH 6) containing 150 mM NaCl at a flow rate of 0.3 ml min⁻¹ in 0.5 ml fractions.

Characterization of purified MnP

Protein concentration was determined by means of Bradford reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard. Protein homogeneity was verified by native PAGE in 12% polyacrylamide gels. Protein bands were stained with Coomassie blue R-250. The molecular mass of the enzyme was determined by gel filtration using a Superdex 200 HR 10/30 column and calibration kit (Pharmacia). MnP activity staining was performed with 4-chloro-1-naphthol (Sigma) as the substrate (Ruttimann *et al.*, 1994), 5 mg of which was dissolved in 1.7 ml of methanol and mixed with 8.3 ml of 20 mM Tris-HCl (pH 7.5)–0.5 M NaCl. The resulting solution (8 ml) was mixed with 32 ml of 50 mM sodium tartrate (pH 5.0) containing 100 mM MnSO₄. After the gel had been soaked in this solution, H₂O₂ was added to a final concentration of 50 μM.

The internal sequence of the enzymatically active band of the purified protein was analysed. In-gel proteolysis using

trypsin was performed, followed by peptide separation using reverse-phase chromatography. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (MS; LCQ, Finnigan). MS was performed in the positive ion mode using a repetitive full MS scan, followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The MS data were compared with simulated proteolysis and CID of the proteins in the 'genpept' database using SEQUEST software (J. Eng, University of Washington, and J. Yates, Finnigan). N-terminal sequencing was performed on a peptide sequencer (494A, Perkin-Elmer).

Enzyme assays

Enzyme activity was determined as described by Martinez *et al.* (1996). MnP activity was determined by the production of a Mn^{3+} tartrate complex ($\epsilon_{238} = 6.5 \text{ mM}^{-1} \text{ cm}^{-1}$) from 0.1 mM MnSO_4 with 0.1 mM H_2O_2 in 0.1 M sodium tartrate (pH 5). MnP was also determined with 0.01% (w/v) phenol red (610 nm) as the substrate ($\epsilon_{610} = 22000 \text{ M}^{-1} \text{ cm}^{-1}$) (Kerem and Hadar, 1996), under the conditions described by Kuwahara *et al.* (1984). VP activity was determined with phenol red (as described for MnP) or with 0.1 mM 2,6-dimethoxyphenol (Sigma) in a reaction mixture that did not contain Mn^{2+} .

Nucleic acid isolation and manipulation

Genomic DNA was isolated from *P. ostreatus* mycelia using the method described by Yatzkan and Yarden (1995). Total RNA was extracted from mycelia grown on perlite after a rapid wash with double-distilled water, filtration and quick freezing in liquid nitrogen. Samples were transferred to a 2 ml screw-cap tube containing 1 ml of TRI reagent (Sigma) and 2 g of zirconium beads (Biospec Products). Cells were disrupted by two 30 s rounds of shaking (4200 r.p.m.) in a bead beater (Biospec Products), and total RNA was extracted according to the TRI reagent protocol. RNA samples were treated with RNase-free DNase (37°C for 1.5 h) to remove DNA residues from the samples. The DNase was removed by standard protocols (Sambrook *et al.*, 1989). RNA samples were stored at -80°C until use. As a first step, to compare the transcript abundance of different genes, the kinetics of the RT-PCR reactions were calibrated. The abundance of the RT-PCR products obtained from different transcripts and treatments was compared in a reaction that was terminated well within the linear phase of product accumulation. Preliminary results (data not shown) indicated that, under the reaction conditions chosen (see below), 30 cycles of amplification were appropriate. RT-PCR amplifications were performed on RNA samples from different culture conditions in order to compare the relative abundance of *mnp1*, *mnp2*, *mnp3*, *mnp4* and β -tubulin gene transcripts (Table 1). Oligonucleotides used for MnP and VP RT-PCR reactions, as well as those used for sequencing, were designed on the basis of available genomic or cDNA sequences identified in *Pleurotus* species (Table 1). The primers were designed on the basis of the unconserved regions of the predicted MnP/VP polypeptides (in order to

reduce the possibility of non-specific MnP/VP priming and amplification). To help to distinguish between RT-PCR and potential DNA contaminant amplicon products, the expected amplicons were designated to span regions in which predicted introns had been identified. Oligonucleotide primers were synthesized by BTG. RT-PCR reactions were performed using the Access RT-PCR system (Promega). For cDNA preparation, 50 U of AMV reverse transcriptase was added to different amounts of total RNA (0.2, 0.4 and 0.6 μg), and the mixtures were incubated for 45 min at 48°C in the presence of 28 U of RNasin (Promega). For PCR, 50 U of *Tff* DNA polymerase, 1 mM MgSO_4 , 25 pmol of each primer and 1 mM deoxynucleoside triphosphates were used. The amplification programme was: 94°C for 2 min, 55°C for 1 min and 68°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 68°C for 2 min, and a final 7 min extension at 68°C . After amplification, a 10 μl aliquot of each PCR product was analysed by agarose gel electrophoresis. The PCR and RT-PCR products were gel purified using Jet Sorb (Genomed) and then sequenced directly using the appropriate primer (Table 1). Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using the *Taq* Dideoxy Terminator cycle sequencing kit and an automated sequencer (Applied Biosystems 373A). Sequence analyses were carried out with the aid of PILEUP and BESTFIT from the GCG package (Devereux *et al.*, 1984) and BLAST software.

Acknowledgements

We thank the Smoler Protein Center of the Israel Institute of Technology, Haifa, for the protein sequencing analysis. The work described in this communication was partially supported by a grant from the European Union in the frame of INCO-DC (International Co-operation with Developing Countries, contract no. ERBIC18CT970186), and by a grant from the NEDO/RITE agency of the Government of Japan.

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