A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member

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*Pleurotus ostreatus* produces several extracellular proteases which are believed to be involved in the regulation of the lignonolytic activities of this fungus. Recently, purification and characterization of the most abundant *P. ostreatus* extracellular protease (*PoSI*) have been reported. The sequence of the *posl* gene and of the corresponding cDNA has been determined, allowing the identification of its pre- and pro-sequences. A mature protein sequence has been verified by mass spectrometry mapping, the N-glycosylation sites have been identified and the glycosidic moieties characterized. Mature *PoSI* shows a cleaved peptide bond in the C-terminal region, which remains associated with the catalytic domain in a non-covalent complex. Reported results indicate that this enzyme is involved in the activation of other *P. ostreatus* secreted proteases, thus suggesting its leading role in cascade activation mechanisms. Analyses of the *PoSI* sequence by homology search resulted in the identification of a DNA sequence encoding a new protease, homologous to *PoSI*, in the *Phanerochaete chrysosporium* genome. A new subgroup of subtilisin-like proteases, belonging to the pyrolysin family, has been defined, which includes proteases from ascomycete and basidiomycete fungi.

INTRODUCTION

Subtilisin-like serine proteases, subtilases, have been classified into six families on the basis of their amino acid sequences (Siezen & Leunissen, 1997). Members of these families have been extensively studied and the crystallographic structures of some of them have been determined (Bode et al., 1987; Jain et al., 1998; Wright et al., 1969). Site-directed mutagenesis and protein engineering have provided intimate knowledge of structure–function relationships in this class of enzymes (Sroga & Dordick, 2001; Ness et al., 2002). The majority of the subtilases are synthesized as a precursor with a pre- and pro-sequence extension of the N-terminus of the mature protein (Bryan et al., 1995). The pre-sequence acts as a signal peptide, driving translocation through the cell membrane, whilst the pro-sequence acts both as an intramolecular chaperone that guides the correct folding of the mature protein and as a protease self-inhibitor (Yabuta et al., 2001, 2003). The pro-sequence is usually cleaved from the mature protein by autoproteolysis to produce active mature protease. In addition to a myriad of prokaryotic subtilases, many members of this superfamily have been identified in eukaryotes such as fungi, plants, insects and mammals.

*Pleurotus ostreatus* and *Phanerochaete chrysosporium* are white-rot basidiomycetes, which belong to different sub-classes of lignonolytic micro-organisms, producing distinct patterns of lignonolytic enzymes. They produce extracellular proteases, which are believed to be involved in the regulation of the lignonolytic activities of these fungi (Palmieri et al., 2000, Dosoretz et al., 1990). In particular, it has been demonstrated that different laccase isoenzymes from *P. ostreatus* can be specifically degraded or activated during fungal growth by proteases present in the culture broth (Palmieri et al., 2000, 2003). In a recent study, we reported the purification and characterization of the main *P. ostreatus* extracellular protease *PoSI* (Palmieri et al., 2001).

On the basis of structural and kinetic properties, *PoSI* appears to be a serine protease belonging to the subtilase family. This enzyme seems to play a key role in the regulation process of *P. ostreatus* laccase activities. A similar relationship was observed for lignin peroxidases (LiPs) in *Ph. chrysosporium*; in this case, the extracellular proteases caused an almost complete disappearance of LiP activity due to degradation of all LiP isoenzymes (Dosoretz et al., 1990).
This paper reports evidence concerning the role played by PosI in the activation of Pl. ostreatus extracellular proteases. The posl gene and cDNA were cloned and sequenced, and mass spectrometric analysis was employed to validate the deduced amino acid sequence and to identify post-translational modifications. Furthermore, analyses by homology search allowed us to define a new subtilase subfamily which includes proteases from ascomycete and basidiomycete fungi.

METHODS

Organisms and culture conditions. White-rot fungi, Pleurotus ostreatus (Jacq.Fr.) Kummer (type: Florida) (ATCC no. MYA-2306) and Phanerochaete chrysosporium Burdshall M1 (DSM 13583) were maintained through periodic transfer at 4 °C in potato glucose (2-4 %) agar plates (Difco) in the presence of 0.5 % yeast extract (Difco).

Pl. ostreatus cultures were carried out in the basal medium as previously described (Palmeri et al., 1997), or with the addition of 150 μM copper sulphate or 1 mM vanillic acid (4-hydroxy-3-methoxybenzoic acid). Ph. chrysosporium cultures were carried out in 0.24 % potato glucose broth in the presence of 0.5 % yeast extract (Difco).

Protease assay. Protease activity was assayed using SucAAPFpNA (N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide; Sigma) or azoalbumin as substrates as follows:

a) The assay mixture contained 5 mM SucAAPFpNA, 10 mM CaCl₂ and 50 mM Tris/HCl buffer, pH 8.4. After electrophoresis, gels were incubated for 16 h at 37 °C in 50 mM Tris/HCl, pH 7.6, buffer containing 200 mM NaCl and 5 mM CaCl₂. Gels were then stained for 30 min with 30 % methanol/10 % acetic acid containing 0-5 % Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis.

Zymographic analysis. Samples underwent electrophoresis in 10 % gelatin-containing polyacrylamide gel at alkaline pH under non-denaturing conditions. The separating and stacking gels contained 12-5 % acrylamide solution in 50 mM Tris/HCl, pH 9.5, and 9 % acrylamide solution in 18 mM Tris/HCl, pH 7.5, respectively. The electrode reservoir solution was 25 mM Tris/190 mM glycine, pH 8.4. After electrophoresis, gels were incubated for 16 h at 37 °C in 50 mM Tris/HCl, pH 7.6, buffer containing 200 mM NaCl and 5 mM CaCl₂. Gels were then stained for 30 min with 30 % methanol/10 % acetic acid containing 0-5 % Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis.

Cloning of the posl gene. Amplification experiments of Pl. ostreatus genomic DNA were performed using the oligonucleotide pair 1, as primers (Table 1). The 800 bp fragment obtained was cloned into the pGEM-T Easy Vector (Promega) and sequenced. This fragment, labelled by the random priming method, was used as probe to screen a Pl. ostreatus genomic library (Giardina et al., 1995). A further screening of the genomic library was performed using, as probe, a 700 bp fragment obtained by KpnI digestion of the amplified cDNA. Colony hybridization experiments were carried out in 5 × SSC at 65 °C (where 1 × SSC is 0-15 M NaCl, 0-015 M sodium citrate).

Cloning of posl and pcsI cDNAs. Mycelia from Ph. chrysosporium and copper-supplemented Pl. ostreatus cultures were collected after 2 days of growth. Total RNA was extracted from lyophilized mycelia, as described by Lucas et al. (1977). Reverse transcription

### Table 1. Oligonucleotide pairs used in amplification experiments

<table>
<thead>
<tr>
<th>Oligonucleotide pair</th>
<th>Sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 posl nterm</td>
<td>5’-GGECCPGAYGAYCCPGC-3’</td>
<td>56 °C</td>
</tr>
<tr>
<td>1 posl down</td>
<td>5’-TCGTCACCCCRTCPCG-3’</td>
<td>64 °C</td>
</tr>
<tr>
<td>2 pep segn posl</td>
<td>5’-TGAAGGGGCTACGTGTTGG-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>2 nter rev posl</td>
<td>5’-GACCAGGATGCAGGGGC-3’</td>
<td>60 °C</td>
</tr>
<tr>
<td>3 posl spec nterm</td>
<td>5’-GCCTCTCAGAAGAAGGGTG-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>4 posl 1D down</td>
<td>5’-GACCCTTTTCCTTCGAGGC-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>5 posl 1D up</td>
<td>5’-CAATCCGGGCTCTTTC-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>6 5’ pcsI phan</td>
<td>5’-CCGCTACACCATGAGG-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>6 pcsI phan rev</td>
<td>5’-GAGCGAGAATTCTCCGTCG-3’</td>
<td>58 °C</td>
</tr>
<tr>
<td>7 pcsI phan fw</td>
<td>5’-TCAGGAGCTCCGGGGCAT-3’</td>
<td>65 °C</td>
</tr>
<tr>
<td>7 nter rev posl</td>
<td>5’-BTCAGCCGCATGCTAGGGGC-3’</td>
<td>65 °C</td>
</tr>
<tr>
<td>8 nter rev posl</td>
<td>5’-GACCAGGATGCAGGGGC-3’</td>
<td>65 °C</td>
</tr>
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</table>
reactions were performed using Super Script II Rnase H- Reverse transcriptase (Invitrogen) and oligonucleotide dT-NotI as primer, following the manufacturer’s instructions. Amplification experiments for each posl cDNA fragment were performed using oligonucleotide pairs 2, 3 and 4 at the corresponding annealing temperatures (Table 1). Amplification experiments for each posl cDNA fragment were performed using oligonucleotide pairs 5 and 6 at the corresponding annealing temperatures (Table 1). The amplified fragments were cloned in the pGEM-T Easy Vector and sequenced.

First-strand cDNA synthesized from Pl. ostreatus total RNA using the gene-specific primer posl 1D down (Table 1) was used to perform rapid amplification of 5’ cDNA end (5’-RACE). Terminal transferase (Roche) was used to add a homopolymeric A-tail to the 3’ and specific oligonucleotide primers. Sequencing by the dideoxy chain-termination method was performed using oligonucleotide pair 8 (Table 1), cloned in pGEM-T Easy Vector and sequenced.

DNA preparation, subcloning and restriction analyses were performed by standard methods according to Sambrook et al. (1989). Sequencing by the dideoxy chain-termination method was performed by the CEINGE Sequencing Service (Naples, Italy) using universal and specific oligonucleotide primers.

Amino acid sequence analysis. Automated N-terminal degradation of the protein was performed using a Perkin-Elmer Applied Biosystem 477A pulsed liquid protein sequencer equipped with a model 120A phenylthiohydantoin analyser for the on-line identification and quantification of phenylthiohydantoin (PTH) amino acids.

Matrix-assisted laser desorption ionization–mass spectrometry (MALDIMS) analysis. MALDIMS analyses were carried out with a Voyager DE MALDI Time of Flight mass spectrometer (PerSeptive Biosystems) on both the protein and the peptide mixtures obtained from proteolytic hydrolysates.

Molecular mass determination of whole protease was performed by loading a mixture of sample solution and 3,5-dimethoxy-4-hydroxycinnamic acid (CHCA) solution on a sample slide and drying by loading a mixture of sample solution and 3,5-dimethoxy-4-hydroxycinnamic acid (CHCA) solution on a sample slide and drying in vacuo. Mass range was calibrated using apomyoglobin from horse heart (mean molecular mass, 16952±5 Da) and human serum albumin (mean molecular mass, 66431±0 Da).

Peptide mixtures derived from each hydrolysis were lyophylized and successively dissolved in 0-2% trifluoroacetic acid and 1 μl sample solution was mixed with 1 μl 10 mg ml-1 x-cyano-4-hydroxycinnamic acid (CHCA) solution on a sample slide and left to dry under vacuum. The CHCA solution was prepared in acetonitrile:0-1% TFA, 70:30 (v/v). Mass spectra were acquired in a linear mode, and calibrated from 379-35 and 5734-59, the m/z values of the CHCA dimer and bovine insulin, respectively.

Enzymic hydrolysis. A PoSl sample (1 nmol) was reduced with 100 mM DTT at 37 °C for 2 h under a nitrogen atmosphere in 0-25 M Tris/HCl (pH 8-5), 1-25 mM EDTA, containing 6 M guanidinium chloride, and then alkylated for 30 min by using an excess of iodoacetamide at room temperature in the dark. The protein sample was desalted by loading the reaction mixture onto a PD-10 prepacked column (Pharmacia), equilibrated and eluted in 0-4% ammonium bicarbonate, pH 8-5.

Enzymic digestions with trypsin and endoproteinase Glu-C were carried out in 0-4% ammonium bicarbonate, pH 8-5, at 37 °C overnight using an enzyme/substrate molar ratio of 1:50.

The tryptic mixture was deglycosylated by incubation with 0-15 U of peptide N-glycosidase F (Boehringer Mannheim) in 0-4% ammonium bicarbonate, pH 8-5, at 37 °C for 16 h. Samples were fractionated on a prepacked cartridge Sep-Pak C18 (Waters); peptide fractions were collected manually.

RESULTS

Protease induction and activity regulation

Different substances, already tested as inducers of Pl. ostreatus laccase production, were analysed with respect to their ability to induce protease production; among them, vanillic acid was shown to be the best protease inducer. Fig. 1 shows the time-course of extracellular protease activity from Pl. ostreatus culture broth supplemented with 1 mM vanillic acid compared to that of basal or copper-supplemented culture media. SucAAPFpNa, a subtilisin-like protease substrate, or azoalbumin, a non-specific

Fig. 1. (A) Time-course of Pl. ostreatus extracellular protease activity from cultures supplemented with copper sulphate (□) or vanillic acid (▲), in comparison to basal medium culture (●). Protease activity was determined using SucAAPFpNa as substrate (dashed line) or azoalbumin (solid line). (B) Gelatin zymography of samples collected at different growth times from vanillic acid-supplemented culture.
protease substrate, was used as substrate. The gelatin zymography of samples from vanillic acid culture is shown in the same figure. The protease activity determined using SucAAPFPpNa as substrate, as well as the zymogram analysis, indicated that the maximum activity of PoSI was reached in the first days of growth. New active protease bands appeared at longer growth times, and a remarkable increase of total protease activity (assayed with azoalbumin) was observed. The occurrence of these new protease activities at prolonged growth times could arise from their late production or from maturation of inactive precursors. In order to distinguish between these two hypotheses, samples of culture broth at the third day of fungal growth were filtered through a 0.2-μm-pore-size membrane and incubated for 24 h at 28 °C in the presence of 1 mM PMSF (an irreversible inhibitor of serine proteases) or 10 mM CaCl₂, which strongly increases PoSI activity (Palmieri et al., 2001). Fig. 2 shows the zymogram analysis of these samples together with culture broth samples collected after 3 or 9 days of growth. No PoSI activity signal was detected in the PMSF-treated sample, and the protease activity bands of this sample were unaltered after 24 h incubation. On the other hand, when the incubation was performed in the presence of CaCl₂, new protease activity bands were switched on, displaying a pattern similar to that observed at prolonged fungal growth time (i.e. the ninth day).

**Cloning and sequencing of the posl gene and cDNA**

Sequences of PoSI N-terminus and of three tryptic peptides have previously been reported (Palmieri et al., 2001). On the basis of the sequences of the N-terminus and that of a tryptic peptide (boxed peptides in Fig. 3), oligonucleotide primer mixtures were designed (posl nterm and posl 1 down, Table 1) and used in amplification experiments performed using Pl. ostreatus genomic DNA as template. The amino acid sequence encoded by the 800 bp amplified fragment contained the sequence of another tryptic peptide, homologous to the conserved sequence around the Asp residue of the catalytic triad in the subtilase family. To amplify the PoSI-encoding cDNA, two amplification experiments were performed using oligonucleotide pairs 3 and 4 (Table 1), designed on the basis of the nucleotide sequence of the amplified gene fragment. Two amplified fragments of 500 bp and 2000 bp, corresponding to 5’ and 3’ regions of posl cDNA, respectively, were cloned and sequenced.

A Pl. ostreatus genomic library was screened using the gene-amplified fragment. One of the positive clones analysed (named 6C4, 2500 bp) encompassed the 5’ coding sequence and extended 750 bp upstream from the codon corresponding to the N-terminal of the mature protein. To complete the 3’ coding region of the posl gene, a 700 bp fragment of the amplified 2000 bp cDNA was used as probe for further screening of the genomic library. One of the positive clones analysed (5–13) overlapped clone 6C4 for 1600 bp and extended 60 bp at the 3’ non-coding region. Thus, the whole posl gene sequence was determined. A putative start codon (ATG 166–168) was identified in the 5’ gene region, 600 bp upstream from the codon corresponding to the N-terminal of the mature protein. The corresponding cDNA fragment was amplified (oligonucleotide pair 2, Table 1), cloned and sequenced. The strategy used to clone the posl gene and cDNA is shown in Fig. 4. All known PoSI peptide sequences were identified in the encoded sequence. Comparison of cDNA and gene sequences allowed determination of the gene structure, with the coding sequence interrupted by 20 introns (Fig. 4).

A 5’-RACE experiment was performed allowing the identification of the transcription initiation site at nucleotide 145, 21 bp upstream from the ATG 166–168, thus confirming it as the translation start codon.

**Validation of the PoSI primary structure and characterization of its glycoside moiety**

Comparison of the PoSI amino acid sequence with that of the mature protein N-terminus allowed us to identify the putative PoSI pre-propeptide. The putative pre-peptide (−124 −108) was identified as a secretion signal by the program SignalP; consequently the propeptide was established as peptide (−107 −1). The mature protein N-terminal sequence had previously been determined from the electrophotographed purified protein (GPDDPALPPD...) (Palmieri et al., 2001). Edman degradation of the purified native protein actually gave rise to two equimolar PTH amino acids for each step. Other than the above N-terminal sequence, a second one (AQVPTLGTF-EL), corresponding to a polypeptide chain starting from A690 of PoSI, was detectable. Hence, it can be inferred that the F689-A690 peptide bond is hydrolysed in the mature protein and the cleaved peptide remains associated with the

![Fig. 2. Zymogram of filtered culture broth (supplemented with vanillic acid) incubated with 1 mM PMSF or 10 mM CaCl₂. Lanes: 1, 3-day-old filtered culture broth (sample A); 2, 3, sample A after PMSF addition (lane 2) and 24 h incubation (lane 3); 4, sample A after CaCl₂ addition and 24 h incubation; 5, 9-day-old filtered culture broth.](image-url)
Fig. 3. PoSI sequence. The numbers represent the positions of the amino acid residues starting from the N-terminus of the mature PoSI protein. Regions verified by peptide mass mapping are underlined. N-Glycosylated asparagine residues are marked with one star, asparagine residues found both glycosylated and non-glycosylated are marked with two stars. Boxed peptide sequences were used to design oligonucleotide-primer mixtures for the amplification experiments. The arrow indicates the peptide bond cleaved in the mature protein.

Fig. 4. (A) Schematic representation of posI and pcsI cDNAs. Arrows show the positions of oligonucleotide primers used for cDNA amplifications. KpnI restriction sites at the ends of the probe used for genomic library screening are also shown. The positions of introns are shown by vertical bars. (B) PoSI domains. SP, signal peptide; PRO, propeptide; CATALYTIC, catalytic domain; PA, PA domain; D, H, S, catalytic active site residues.
catalytic domain in a non-covalent complex, since no Cys residues in the cleaved peptide are present.

The PoSl amino acid sequence is characterized by seven putative glycosylation sites. As previously reported (Palmieri et al., 2001), MALDIMS analysis of permethylated N-linked sugar released by hydrolysis with N-glycosidase F showed the occurrence of several high mannose moieties with molecular mass ranging from 1580·0 and 2603·6 m/z and identified as Hex$_3$HexNAc$_2$ and Hex$_{10}$HexNAc$_2$. The other molecular ions at m/z 1784·0, 1987·5, 2191·5 and 2394·6 were identified as homologous structures having between three and six mannose residues linked to the pentasaccharide core.

To verify the deduced PoSl amino acid sequence, the PoSl protein was reduced, alkylated and then digested with trypsin, and the peptide mixture was directly analysed by protein was reduced, alkylated and then digested with trypsin, and the peptide mixture was directly analysed by protein mass spectrometry. This analysis also showed three different clusters of signals each characterized by a pattern of molecular mass ranging from 1580·0 and 2603·6 m/z and identified as Hex$_3$HexNAc$_2$ and Hex$_{10}$HexNAc$_2$. The other molecular ions at m/z 1784·0, 1987·5, 2191·5 and 2394·6 were identified as homologous structures having between three and six mannose residues linked to the pentasaccharide core.

To verify the deduced PoSl amino acid sequence, the PoSl protein was reduced, alkylated and then digested with trypsin, and the peptide mixture was directly analysed by MALDIMS, allowing the identification of several peptides (Fig. 3). This analysis also showed three different clusters of signals each characterized by a pattern of molecular mass ranging from 1580·0 and 2603·6 m/z and identified as Hex$_3$HexNAc$_2$ and Hex$_{10}$HexNAc$_2$. The other molecular ions at m/z 1784·0, 1987·5, 2191·5 and 2394·6 were identified as homologous structures having between three and six mannose residues linked to the pentasaccharide core.

The assignments of the different molecular masses to the corresponding peptides (Table 2) led to the identification of Asn106, Asn479 and Asn728 as N-glycosylation sites. As shown in Fig. 3, the two residues Asn479 and Asn728 were present in both glycosylated and unglycosylated state. A further picture of PoSl N-glycosylation sites was obtained after deglycosylation of peptide mixture by N-glycosidase F. Indeed, new signals were detected by MALDIMS analysis at 3440·1, 5087·1, 5484·1 and 6361·2 m/z, corresponding to the expected molecular mass of peptides 707–738, 438–485, 59–112 and 59–120, respectively, each increased by 1 Da, since the N-glycosylated Asn residues are converted to Asp following N-glycosidase F treatment. Moreover, an additional peak at m/z 1793·0 was present after deglycosylation. This mass value is in agreement with the expected molecular mass of the peptide 628–642 (containing the potential N-glycosylation site Asn628) increased by 1 Da. This result led to the assignment of Asn628 as an N-glycosylation site, even though the MALDIMS spectrum of the tryptic peptide mixture did not allow us to detect the cluster of m/z peaks related to the Asn628 glycoforms. Hence, two out of seven putative PoSl N-glycosylation sites were found to be glycosylated, Asn479 and Asn728 were found to be both glycosylated and unglycosylated, whilst Asn238 and Asn301 were only found unmodified. MALDIMS analyses did not allow mapping of the last putative N-glycosylation site, Asn335. This could be due to an incomplete extraction of the peptides from the gel or to the well-known MALDI suppression phenomena.

To validate further regions of the protein sequence, the tryptic peptide mixture was submitted to enzymic hydrolysis with the endoproteinase Glu-C. The data obtained allowed us subsequently to verify 84% the PoSl primary structure, as shown in Fig. 3.

**Homology search analysis and pcsI cDNA cloning and sequencing**

PoSl shows a high level of identity with a hypothetical protein from *Neurospora crassa* (38%, BLAST E score = 2·1 × 10$^{-12}$), a subtilisin-like serine protease from the fungus *Metarhizium anisopliae*, Pr1C (39%, E score = 2·0 × 10$^{-11}$), and a minor extracellular serine protease from *Bacillus subtilis*, Vpr (31%, E score = 7·1 × 10$^{-41}$).

*Phycomyces chrysosporium* is the only basidiomycete whose genome has been sequenced to date. BLAST analysis of this genome

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**Table 2. Theoretical and measured molecular mass values for PoSl glycopeptides**

MH$^+$, singly protonated ion. Values in the theoretical and measured MH$^+$ columns are in Da.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Glycosylation site</th>
<th>Theoretical MH$^+$</th>
<th>Measured MH$^+$</th>
<th>Glycoforms</th>
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<tr>
<td>628–642</td>
<td>Asn628</td>
<td>1792·0</td>
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</tr>
<tr>
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<td>Asn728</td>
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(http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) resulted in the identification of two PoSl homologous sequences, about 112 kb from each other. One of these, designated pc.18.58.1, appeared to encode a protein which was very similar to PoSl. RNA was extracted from Ph. chrysosporium and specific cDNA was amplified using oligonucleotide pairs designed on the basis of the genomic sequence and protein homology. The amplified cDNA was longer than pc.18.58.1 at its 5’ terminus, even if it did not contain the start translational codon. Moreover, the alignment between pc.18.58.1 and AJ748587 showed a 6 aa insertion in the former, due to an incorrect exon end. The deduced amino acid sequence corresponding to the amplified cDNA showed an identity of 65% with PoSl. Fourteen out of 15 introns in the gene sequence were in the same positions as those of the posl gene (Fig. 4).

**DISCUSSION**

The extracellular subtilisin-like protease PoSl from *Pl. ostreatus* was believed to be involved in the activation/degradation mechanism of laccase isoenzymes (Palmieri *et al.*, 2000, 2001). This mechanism seemed to be indirectly controlled by PoSl, whose activity appeared to be necessary but not sufficient for laccase post-translational regulation. In this paper we investigated the effect of PoSl on other *Pl. ostreatus* extracellular proteases. The addition of vanillic acid to the fungal culture led to an increase both of total protease activity and of PoSl activity, with respect to any of the other conditions examined, such as copper-supplemented culture (Fig. 1). In vanillic acid-amended cultures, some protease activities were only detectable at long fungal growth times (Fig. 1). These proteases were also found to be activated after incubation of the early growth time culture broth in the absence of mycelia, but in the presence of active PoSl. Therefore these proteases were secreted as inactive forms and subsequently activated in a process governed by PoSl.

The PoSl coding sequence was determined and the deduced protein primary structure confirmed by MALDIMS mapping. This approach has proven useful for the identification of the N-glycosylation sites and characterization of the glycoside moiety of the protein. PoSl is homologous to many other serine proteases, especially in the areas surrounding the amino acids that are known to be involved in the active site of subtilisin (D, H and S). The first amino acid (G1) of the mature protein was identified, allowing determination of the PoSl pro-sequence. Pro-sequences are usually removed from the catalytic domain by self-digestion or by another protease upon completion of protein folding. The peptide bond K–1L–G1, which connects the PoSl pro-sequence to its catalytic domain, should be cleaved by a protease with trypsin-like specificity different from the known PoSl substrate preference (Palmieri *et al.*, 2001). Moreover, no significant activity of PoSl towards the chromogenic peptide D-Val-Leu-Lys-p-nitroanilide was observed (data not shown). On the other hand, PoSl has a D residue in the position corresponding to E156 in subtilisin BPN’. Subtilases with a negative charge on this residue are reported to have an additional ability to cleave after K at the P1 position (Gron *et al.*, 1992; Voorhorst *et al.*, 1997). Furthermore, all the interactions of the substrate P4–P4’ residue side chains with the S1 pocket can contribute to binding; thus it cannot be excluded that PoSl zymogen can undergo autolysis to self-remove the pro-peptide from unprocessed protein during the maturation process.

PoSl shows a high identity with hypothetical proteins from *N. crassa* and *Trichoderma reesei*, and Pr1C, a ‘bacterial-type’ subtilisin-like serine protease from the fungus *M. anisopliae*. Eleven subtilisin-like proteins were identified in the latter fungus (Bagga *et al.*, 2004), ten of which displayed identities ranging from 93 to 98%, being classified as proteinase K-like subtilisins. The unique exception was the ‘bacteriotype’ subtilisin Pr1C, which is largely divergent from the others. Among the bacterial subtilases, Vpr, a minor extracellular serine protease from *B. subtilis* (Sloma *et al.*, 1991), shows the highest identity with PoSl. This bacterial subtilase belongs to the pyrolysin family, according to the classification of Siezen & Leunissen (1997).

Interestingly, a *Ph. chrysosporium* genomic sequence encoding a hypothetical protein, PoSl, showing the highest identity with PoSl (65%), has been identified and the corresponding cDNA was amplified and sequenced, thus demonstrating the expression of the pcsl gene in this fungus. The *posl* and *pcsl* genes shared 57% of their sequences and their intron/exon structures were very similar (Fig. 4). Furthermore, all the glycosylated Asn identified in PoSl was located in consensus sequences conserved in *PcSl*, suggesting common structural features between the two proteins. Indeed, a 76 kDa protease, inhibited by PMSF, has previously been reported in *Ph. chrysosporium* culture broth. This enzyme is the most abundant protease produced in the presence of both excess and limiting nitrogen source (Dass *et al.*, 1995). These data suggest that this protease is the one encoded by the *pcsl* gene. Hence, a new subgroup of subtilisin-like proteases from ascomycete and basidiomycete fungi, belonging to the pyrolysin family, can be defined, including the proteases PoSl and *PcSl* from the basidiomycete fungi *Pl. ostreatus* and *Ph. chrysosporium*, the hypothetical protein from *N. crassa*, and Pr1C from *M. anisopliae*. In Fig. 5 the alignment of these protease sequences is shown.

The spacing between the catalytic H and S residues in PoSl, as well as for several other members of the pyrolysin family and all those of the new fungal pyrolysin subfamily, is approximately 150 aa longer than that of subtilisins (Voorhorst *et al.*, 1996). This insert region has significant similarity to sequences of other protein classes (M8/M33 zinc peptidases, Ring-type Zinc Finger proteins, vacuolar sorting receptor, transferrin receptor), and has been named as a protease-associated (PA) domain (Mahon & Bateman, 2000). The role of the PA domain remains unclear; however, it has been supposed that the position and the size
of the inserted domain might allow interference with substrate access and thus it could be involved in substrate specificity (Luo & Hofmann, 2001).

All the members of the new fungal pyrolysin subfamily have a long C-terminal extension, which is less conserved than the catalytic region (Siezen & Leunissen, 1997; Voorhorst et al., 1996). Mature PoSI shows a cleaved peptide bond in this region, probably caused by an autocatalytic event, on the basis of its specificity. The cleaved peptide remains associated with the catalytic domain in a non-covalent complex, as demonstrated by the two N-terminal sequences obtained from the native protein and confirmed by the MALDIMS characterization of the protein. This finding suggests that the cleaved bond belongs to an exposed loop of the protein and that, after the proteolytic event, the newly generated peptide remains tightly bound to the core of the protein.

The occurrence of this class of proteases in a wide variety of fungal genera could be suggestive of the crucial physiological role played by these proteins in the micro-organisms which produce them. With regard to P. ostreatus, the role proposed for PoSI, a member of this subfamily, would be to start a cascade of proteolytic reactions, leading to the activation of other extracellular proteases.

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