Gene Expression of a New Metalloprotease from *Pleurotus ostreatus* is Developmentally Regulated

JOONG-HO JOH NAM-KUK KIM HYE-RAN PARK JONG-HYUN LIM KYUNG-JUNG BANG YUN-SUN CHOI GAE-NAM JUNG BONG-GUM CHO AND CHANG-SOO LEE*
Department of Applied Biochemistry, College of Natural Science, Konkuk University, Chung-Ju, Korea.
E-mail: cslee@kku.ac.kr

Abstract: A metzincin family metalloprotease cDNA (PoMTP) was cloned from *Pleurotus ostreatus*. Full-length cDNA sequence (1,140bp) of PoMTP contained an open reading frame (ORF) of 870bp encoding a protein product of 290 amino acids. The deduced amino-acid sequences of PoMTP contained an extensive zinc-binding consensus sequence and a so-called Met-turn sequence that are typical for the metzincin family of metalloproteases. Four cysteine residues were also observed in the zinc-binding region of PoMTP amino-acid sequence, which are known to be important for the structure and the function of some subfamilies of the metzincins. Searches of the GenBank protein database showed that the amino-acid sequence of PoMTP over the whole amino-acid sequence has a high identity with metalloproteases or hypothetical proteins from one basidiomycete of *Ustilago maydis*, and four ascomycetes of *Magnaporthe, Metarhizium anisopliae var. anisopliae, Aspergillus nidulans* and *Neurospora*. Although no information about the function of these proteins is known at present, all these proteins have an extended zinc-binding sequence and the Met turn sequence. Furthermore, the putative metzincin PoMTP metalloprtease indicated no significant homology with any other pre-reported mushroom metalloproteases, which are fibrinolytic metalloprotease, mitochondrial intermediate peptidase and mitochondrial processing peptidase. Real-time PCR, quantitative RT-PCR and Northern blot analyses indicated the PoMTP mRNA to be abundant at primordial and fruit body stages, but scarce at the mycelial stage, suggesting that the PoMTP metalloprotease plays an important role in mushroom fruiting.

Key words: *Pleurotus ostreatus*, metalloprotease, mushroom, real-time PCR

1 Introduction

P. ostreatus, known as oyster mushroom, is one of the most widely cultivated edible mushrooms. Because of many cultivating advantages and their nutritional properties, the world production of oyster mushroom has increased tremendously and now is ranked second to the button mushroom, *A. bisporus*, and this mushroom was recognized as a very good dietary food.[1] Recently, the effects on increasing macrophage and lymphocyte activities,[2] reducing cholesterol levels,[3] and increasing antihepatoma and antisarcoma activities[4] of *Pleurotus* were also reported. Bioactive materials such as Lovastin, Lectin, (1,3)-beta-D-glucans were identified.[5-7] Its pharmaceutical applications[2, 8, 9] were reported. Although *P. ostreatus* is an important pathogen causing root and wood rot of forest trees, use of *P. ostreatus* in biobleaching, catalysis of difficult chemical conversions, and the environmental bioremediation.[10-12] were most attention getting. The wide ranges of enzymes related with the catalytic ability of *Pleurotus* spp. were reported.[13-15] The high ability to degrade lignocellulose of *Pleurotus* spp. was also used in eliminating the xenobiotic pollutants such as pentachlorophenol (PCP), dioxin, polycyclic aromatic hydrocarbons (PAHs) through cycles of extracellular oxidation, followed by intracellular reduction.[11] Recently, its ability to degrade toxic substances such as bisphenol A has been noted.[12]
Since almost al the interesting substances of *P. ostreatus* are specific to the fruiting body, and humans used the fruiting body as food, studies on the fruiting mechanism are important. Fruit body formation is initiated by

nutritional and environmental factors such as light, temperature, humidity and carbon dioxide. However, the molecular mechanism of fruiting body initiation and maturation is still poorly understood. For molecular studies of fruiting in *P. ostreatus*, several specially expressed genes in fruiting bodies were isolated by differential screening methods. The developmentally regulated genes were isolated for molecular studies of developmental process in *P. ostreatus*. Most of the genes isolated encode for abundant structural elements, like hydrophobins, laccase, manganese peroxidase and lectin, which are involved in hydrophobic aggregation, ligninolytic and antitumor activities.[4, 16-22] These genes were involved in various physiological functions and a role during mushroom development was suggested. Mating of *P. ostreatus* is controlled by a bifactorial tetrapolar system composed by two unlinked incompatibility factors, *A* and *B*.[23, 24] Mating type genes related with the compatibility of *Pleurotus* spp. and their genetic structure were analyzed. It was suggested that the genetic mechanism for mating type determination is the same as in the model mushroom species.[25, 26]
For molecular studies of the complete developmental process of *P. ostreatus*, large-scale developmentally regulated genes are required. But only a limited amount of research has been performed on this species. A recent study analyzed the genomic constitution of *P. ostreatus* by using pulsed-field gel electrophoresis (PFGE), and the probe hybridization experiments using the RFLP markers mapping to different linkage groups of *P. ostreatus*.[26, 27] These studies have determined that this fungus contains 11 pairs of chromosomes with sizes ranging from 1.4 to 4.7 Mb, and the total dikaryotic genome size for *P. ostreatus* is approximately 70 Mb. Recently, ESTs derived from cDNA libraries of mycelia and fruit bodies of *P. ostreatus* have been reported. In this study, 1256 unigenes were isolated during mycelium and fruiting body stages, and the changes of the gene expression were compared during two stages.[28] Besides EST sequences during mycelia and fruiting body stages, the relationship between mycelial growth and mating type.[29] and their corresponding QTL[30] were analyzed. In the course of analysis of the *P. ostreatus* genome, a genetic linkage map that consists of 178 RAPD and 23 RFLP markers, phenotypic characters and cloned genes has been constructed.
Metalloproteases are involved in numerous physiological functions, depending on their substrate specificity and the tissues and cellular compartments in which they are expressed. Most metalloproteases have a zinc-binding consensus sequence (His-Glu-Xaa-Xaa-His or HExxH) for their catalytic activity, and the protease with this active site motif is predicted to have metalloprotease activity. The group possessing the zinc-binding site motif is called the zincins superfamily and this superfamily has been classified into several major families of metzincins, gluzincins and aspzincin according to the structural topology around the active site. Furthermore, the metzincins are divided into several subfamilies based on their overall structure or the source of origin such as astacins, reprolysins, serralysins, matrixins and pappalysins.[31-35] Although various metalloproteases are widely distributed from bacteria to mammals, metalloproteases of higher basidiomycetes have received relatively little attention. Only a few metalloproteases have been purified or cloned from mushrooms, which are lysine-specific proteinases of *Armillaria mellea, Grifola frondosa, P. ostreatus and Tricholoma saponaceum*, with a potent fibrinolytic activity,[36-39] mitochondrial processing peptidase of *Lentinula edodes*[40] and mitochondrial intermediate peptidases of *Coprinus bilanatus, P. djamor and Schizophyllum commune*.[25, 41, 42] Some of them were characterized to belong to gluzinsins (HExxH + E motif), aspzincins (HExxH + D motif), pitrilysins (HxxEH motif) or thimet oligopeptidase (HExxH motif) family, but not to the metzincins (HExxHxxGxxH + M motif) family.
Since metalloprotease members have a critical role in a broad spectrum of developmental processes,[33] we reported the metalloprotease gene (PoMTP) belonging to the new eucolysins subfamily and the characterization by quantitative analysis of mRNA.[43] In the present study, we report the confirmation of the family of metalloprotease genes and of their developmental expression by taxonomical analyzing program and real time PCR method, respectively.

2 Materials and Methods

2.1 Strain and culture conditions

P. ostreatus strain ASI2029 (Korean specific strain) was used throughout this study. The strain was routinely grown on mushroom complete media (MCM: 2% glucose, 0.05% $MgSO_4$, 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.2 % yeast extract and 0.2 % bacto peptone) at 28°C. Mycelia were grown in liquid MCM medium for 5-10 days and the fruiting process was performed in cotton waste substrate at 18°C for 25 days.

2.2 Nucleotide sequence of *P. ostreatus* metalloprotease (PoMTP)

EST databases prepared from mycelia and fruiting bodies cDNA libraries of *P. ostreatus* strain ASI2029 were searched for the presence of metalloprotease cDNA clones. EST clone highly homologous with metalloprotease of *Metarhizium anisopliae var. anisopliae* which is only reported in GenBank database was isolated.[44] The nucleotide sequence of a putative metalloprotease cDNA was constructed by comparison of cloned sequences. In order to obtain full-length cDNA, 5' rapid amplification of cDNA ends (RACE) was performed using the TaKaRa RACE-Core Kit (Takara). Total RNA from fruit bodies of *P. ostreatus* was isolated using Trisol (Invitrogen). The nucleotide sequences of primers used for RACE reactions and their locations on the cloned cDNA are shown in Figure 1. As primers, P primer (5'-gaactcggtcatgcactc-3') was used for 1st cDNA synthesis, PS1 (5'-aaatgggaacgttgcgtatc-3') and PA1 (5'-acaccttccaaggaggttgc-3') for 1st PCR amplification, and PS2 (5'-agcgttgactgtacgtgtgg-3') and PA2 (5'-gcctttggatgccccgttgg-3') for 2nd PCR amplification. PCR products were cloned into pGEM-T vector (Promega) and sequenced. The nucleotide sequence of cloned cDNA was confirmed by PCR amplification with primers P1 (5'-ataagaaggggtcctcgatg-3') and P2 (5'-acattagactttcggcacaga-3') as shown in Figure 1. In order to confirm the gene family, phylogenetic trees were generated by MegAlign tree drawing software for windows (DNASTAR, Madison, WI) from aligned nucleotide sequence by CLUSTAL W program.

2.3 RNA analysis

Total RNA were isolated from mycelia, primordia, fruiting bodies and spores of *P. ostreatus*. For Northern blot analysis, 50μg total RNA was electrophoresed in formaldehyde gel and transferred to Hybond N+ membranes according to the manufacturer (Amersham). The DNA probe (527bp) was prepared by PCR amplification with the cloned cDNA as a template and primers PL (5'-atccccgattcccaaattac-3') and PR (5'-gtccatgtagttatggatgg-3') as shown in Figure 1. Hybridizations with radiolabeled probes were performed at 65°C in 0.5 M sodium phosphate buffer, 7% SDS, and 1 mM EDTA (pH 7.2). The signals were detected by autoradiography. For real-time PCR analysis of PoMTP genes, SMART CYCLER (Cepheid, SC1000-1) and SYBR Green II were used for amplification and detection. The cDNA was synthesized from total RNA (1 or 3μg) that was treated with DNase I to digest DNA contamination, and with RNase H to digest RNA. These cDNAs were used as templates in a PCR reaction with two specific primers PL (5'-atccccgattcccaaattac-3') and PR (5'-gtccatgtagttatggatgg-3'). The endpoint used in the real-time PCR quantification, Ct, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold.

3 Results and Discussion

Development of fruiting bodies from vegetative mycelia is a very dramatic and scientifically and economically interesting process in basidiomycetes. Therefore, many mycologists have studied environmental factors (temperature, light, nutrients, and fruiting inducing substances) and genetic factors (mating type genes, specifically expressed genes, and identification of mutants) of fruiting body formation in several basidiomycetes.[45-47] However, how gene expression is regulated and how environmental signals are interpreted in the cell during fruiting body formation is not well understood. Genes that show increased expression in one stage and decreased in the other stage were key genes, which play an important part in fruiting body formation. It is suggested that the alteration of expression patterns during the fruiting process reflect what genes are involved in fruiting and how the genes work for fruiting. So many developmentally regulated genes were isolated for molecular studies of fruit body formation. Most of the genes isolated encode for abundant structural elements, like hydrophobins, laccase and lectin, that are involved in hyphal aggregation.[48] The putative transcription factors of pri A and B,[49] a cell-adhesion protein[50] and a mitochondrial processing peptidase[41] were also developmentally regulated. To add more informative genes of mushroom, expressed sequence tags (ESTs) have been generated from cDNA libraries of mushroom at different developmental stages.[51, 52]

3.1 The metalloprotease gene and comparison with related protein sequences

Recently, we have reported ESTs derived from cDNA libraries of mycelia and fruit body of the oyster mushroom *P. ostreatus*.[51] In this study, 15 unigenes in sequence redundancy level were preferentially expressed at the fruit body stage than during the mycelial stage. One among seven identified unigenes showed a high homology (41.7%) with the metalloprotease MEP1 from a basidiomycete *Metarhizium anisopliae var. anisopliae* (GenBank accession no. CAB63909); this nucleotide sequence has been reported only in the GenBank database.[43] We named this EST cDNA as PoMTP. The PoMTP cDNA was sequenced and the sequence included a poly(A) tail and lacked a residue of 5'-end. The upstream 5'-end sequence was extended by the RACE method. The PoMTP cDNA sequence and its deduced amino-acid sequence are shown in Figure 1. The full-length PoMTP cDNA was 1,140 nucleotides long (Accession no. AY640032). The nucleotide sequence revealed an ORF of 870 bp which was preceded by 99 bp of 5'-untranslated sequence and followed by 171 bp of 3'-untranslated sequence terminating with a poly (A) tail. Translation most likely starts at the ATG codon at nt 100 and stops at nt 970, so that the ORF encodes for a polypeptide of 290 amino acids with a predicted molecular weight of 31, 896 daltons. The first 20 amino acid residues from the translation start site were expected to be a signal peptide.[53] The PoMTP amino-acid sequence has an extended zinc-binding consensus sequence (HExxHxxGxxH) and a methionine-containing turn of similar conformation (the Met-turn), which are typical for the metzincin metalloprotease family (Figure 2). Searches of the GenBank protein database showed that the amino-acid sequence of PoMTP over the whole amino-acid sequence has a high identity with metalloprotease or hypothetical protein from the basidiomycete *Ustilago maydis* (GenBank accession no. EAK86013, 50.9%), four ascomycetes (*Magnaporthe grisea* (Accession no. EAA57072, 44.2%), *Metarhizium anisopliae var. anisopliae* (Accession no. CAB63909, 41.7%), *Aspergillus nidulans* (Accession no. EAA58448, 40.7%) and *Neurospora crassa* (Accession no. XP327486, 38.5%)), and one bacterium (*Gloeobacter violaceus*) (Accession no. NP924609, 52.6%). Although no information about the function of these proteins is known at present, all these proteins have an extended zinc-binding sequence and the Met turn sequence except for *A. nidulans* (N residues instead of G in zinc-binding consensus sequence), suggesting that these proteins belong to the metzincin metalloprotease family (Figure 2). Furthermore, the putative metzincin PoMTP metalloprotease indicated no significant homology with any other pre-reported mushroom metalloproteases, which are fibrinolytic metalloprotease (*A. mellea, G. frondosa, P. ostreatus* and *T. saponaceum*),[36-38] mitochondrial intermediate peptidase (*C. bilanatus* and *S. commune*)[25, 41, 42] and mitochondrial processing peptidase (*L. edodes*).[40]

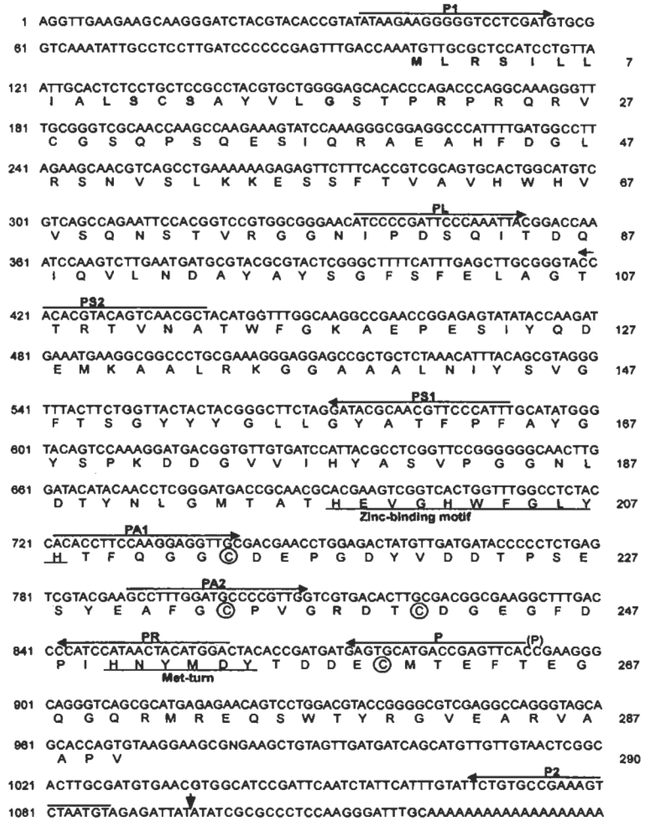


Fig.1. Nucleotide sequence and deduced amino-acid sequence of a *P. ostreatus* metalloprotease (PoMTP)

The nucleotide sequence of PoMTP was constructed from the EST sequence and by 5'-RACE PCR cloning. The sequences and directions of the primers for cloning and sequencing are indicated by horizontal arrows. The deduced amino-acid sequence is noted by one-letter symbols. The putative signal peptide is shown in bold. The extensive zinc-binding and Met-turn sequences are underlined. Four cysteine residues within the zinc-binding domain that are presumed to form two intramolecular disulfate bonds are circled. The putative polyadenylation site is marked by a vertical arrow.

The metzincins family can be divided into five subfamilies of astacins, reprolysins, serralysins, matrixins and pappalysins, based on the residue following the third histidine zinc ligand and the residues surrounding the metionine in the Met-turn.[31, 54] The subfamily-specific residue following the third histidin zinc ligand was a glutamate (E) for astacins, an aspartate (D) for reprolysins, a proline (P) for serralysins, a serine (S) for matrixin and a valine (V) for pappalysins (Figure 2). However, the subfamily-specific residue was a threonine (T) in all metalloprotease members having a high homology with the PoMTP except for *U. maydis* with a valine (V) residue. In the Met-turn consensus sequence (UBMOx), U and O are conserved family-specific residues, while B is a hydrophobic side chain of the hydrophobic core protein. In the PoMTP and this type metalloproteases, U was an asparagine (N) residue which was observed only in the pappalysins family, while O was an aspartate (D) residue which was not observed within the metzincins family except for *M. anisopliae var. anisopliae*. From these observations, the PoMTP-type metalloproteases could be defined as a sixth subfamily and we proposed a new subfamily name 'eucolysins'.[43] The PoMTP-type metalloprotease from a basidiomycete *U. maydis* might belong to either the pappalysins based on the residue following the third histidine of zinc-binding motif, or to the eucolysins or astacins subfamily based on the conserved residues in the Met-turn. The eucolysins members have not shared with the fungalysin family containing fungal metalloproteases in amino-acid sequence and

zinc-binding sequence.[55] To confirm the genetic distances of these genes, all sequence data were aligned to construct phylogenetic trees using MegAlign software, and are shown in Figure 3. The phylogenetic positions of PoMTP type metalloproteases were then compared with PoMTP in a dendrogram. Becasues the phylogenic position of the PoMTP-type metalloprotease from *U. maydis* was closed with PoMTP, this gene might belong to the eucolycins subfamily. But other metalloproteases were identically distinguished with subfamily based on the sequence of amino acid residues.

Proteinase	**Zinc binding motif**										**Gap**	**Met-turn**			
Astacins															
Astacin	H	E	L	M	H	A	I	G	F	Y	H	**E**	- 44 -	S	I	M	H	Y
BMP-1	H	E	L	G	H	V	V	G	F	W	H	**E**	- 46 -	S	I	M	H	Y
Reprolysins															
Adamalysin II	H	E	L	G	H	N	L	G	M	E	H	**D**	- 13 -	C	I	M	R	P
ADAM12	H	E	L	G	H	N	F	G	M	N	H	**D**	- 19 -	C	I	M	N	A
Serralysins															
Alk. Proteinase	H	E	I	G	H	T	L	G	L	S	H	**P**	- 27 -	S	V	M	S	Y
Serralysin	H	E	I	G	H	A	L	G	L	S	H	**P**	- 27 -	S	L	M	S	Y
Matrixins															
Collagenase	H	E	F	G	H	S	L	G	L	A	H	**S**	- 7 -	A	L	M	Y	P
Matrilysin	H	E	L	G	H	S	L	G	M	G	H	**S**	- 7 -	A	V	M	Y	P
Pappalysins															
PAPP-A	H	E	I	G	H	S	L	G	L	Y	H	**V**	- 63 -	N	F	M	S	Y
PAPP-A2	H	E	V	G	H	V	L	G	L	Y	H	**V**	- 63 -	N	Y	M	S	Y
MEP of *U. maydis*	H	E	V	G	H	W	V	G	L	Y	H	**V**	- 41 -	S	Y	M	D	Y
Eucolycins															
PoMTP	H	E	V	G	H	W	F	G	L	Y	H	**T**	- 41-	N	Y	M	D	Y
MTP of *M. grisea*	H	E	V	G	H	W	F	G	L	Y	H	**T**	- 42 -	N	Y	M	D	Y
MTP of *M. anisopliae*	H	E	V	G	H	W	F	G	L	F	H	**T**	- 40 -	N	Y	M	S	Y
MTP of *N. crassa*	H	E	V	G	H	W	L	G	L	Y	H	**T**	- 42 -	N	Y	M	D	Y
MTP of *G. violaceus*	H	E	V	G	H	W	F	G	L	Y	H	**T**	- 42 -	N	F	M	D	Y

Fig. 2. Sequence alignment of representatives of each subfamily of the metzincins

Only the zinc-binding and Met-turn sequences from representatives of five established subfamilies of the metzincins are aligned.[54] The corresponding sequences of putative metalloprotease (MEP) of five fungi and one bacterium from GenBank database are shown. These members are classified as a sixth subfamily, named eucolycins, except for the MEP of *U. maydis*. The histidine zinc-ligands, the catalytically active glutamic acid residue, the conserved Met residue are boxed. Residue strictly conserved within the subfamilies of the metzincins is underlined and in bold. The numbers indicate the intervening residues. BMP1, bone morphogenetic protein 1. ADAM, a disintegrin and metalloprotease. PAPP-A, pregnancy-associated plasma protein. PoMTP, *P. ostreatus* metalloprotease.

All PoMTP-type metalloproteases belonging to the eucolysins subfamily reserved well four cysteine residues with the almost residues spacing in zinc-binding and M-turn sequences ($HExxHxxGxxHT^{12}$—C^{17}—C^{37}—C^{44}-$NYMDY^{58}$—C^{63}-in PoMTP). These conserved cysteine residues probably form disulfide bridges and are important for the general structures and functions of metalloproteases.[33, 34] The pattern of disulfide loop in zinc-binding domain is apparently conserved in astacins, reprolysins and pappalysins subfamilies of the metzincins, but not in serralysins and matrixins. Only two disulfide loops are well conserved throughout the astacins members, although diverse patterns are observed in reprolysins and pappalysins. Although the phylogenetic position of serralysins is more close to eucolycins then astacins (Figure 3), these observations for functional relationship suggested that the eucolysins subfamily possibly more closely related to the astacins than to the serralysins subfamily. All known members of the astacins are from animals, whereas the serralysins are in Gram-negative bacteria, indicating that the function of the PoMTP-type metalloproteases may be conserved more in higher fungi and animal systems than in bacteria.

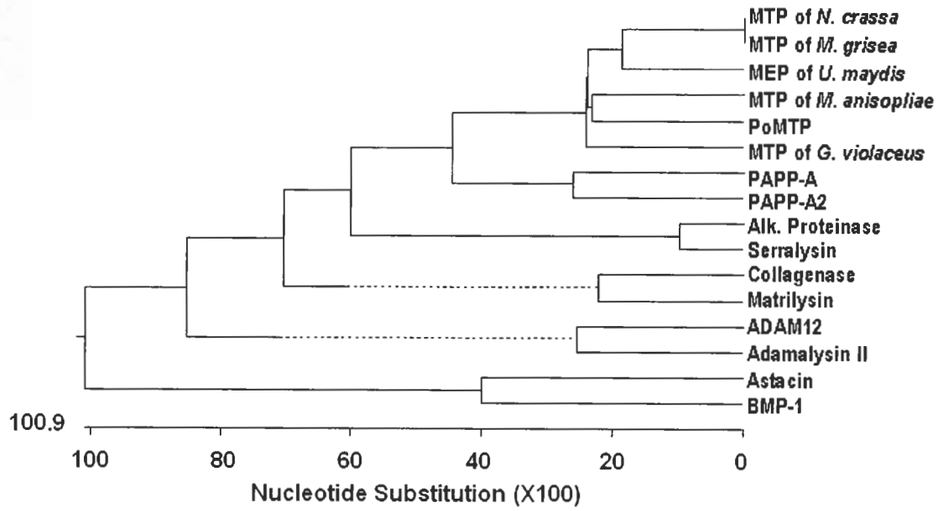


Fig. 3. Phylogenetic trees of metalloprotease gene inferred from cDNA sequences by MegAlign tree drawing software for windows (DNASTAR, Madison, WI) from aligned nucleotide sequence by CLUSTAL W program

3.2 Absolute expression of PoMTP metalloprotease by quantitative real-time PCR

What genes have the critical roles in a broad spectrum of mushroom development is the most attention point to many development scientists and mycologists. Numerous molecular genetic and cytological studies have been performed to unravel routes in basdiomycetes, but there is no accurate answer to this question. Therefore, many researches are now working to explain the molecular mechanisms of mushroom development.

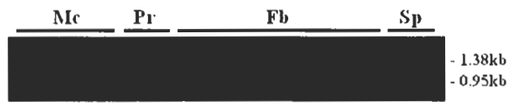
Metalloproteases are involved in numerous physiological functions, depending on their substrate specificity and the tissues and cellular compartments in which they are expressed. So, a putative metzincin family metalloprotease from the oyster mushroom *P. ostreatus*, called PoMTP was cloned.[43] To verify the developmental expression of the PoMTP metalloprotease, its mRNA level by using Northern blot analysis (Figure 4a) and quantitative RT-PCR at different developmental stages were analyzed. Total RNAs were isolated from mycelia, primordia, fruiting bodies and spores of the oyster mushroom. The expression of PoMTP metalloprotease mRNA was scarcely detected from mycelia and spores using both Northern blot analysis and quantitative RT-PCR. One major transcript was detected and its mRNA level appeared very highly at primordial and fruit body stages. The results from Northern blot analysis agreed with the RT-PCR results for developmental stages. As control genes, the mRNA levels of 18S rRNA and G3PDH genes were not changed significantly throughout the developmental stages.

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantify transcription levels of specific genes has always been central to any research into gene function.[56] So, functional genetics to test the gene functions on developmental stages was started by the quantitative gene expression assays. Quantitative RT-PCR is in common use for the quantitative gene expression assays. This method is the most sensitive method for the detection of low-abundance mRNA. However, it is a complex technique, there are substantial problems associated with its true sensitivity, reproducibility and specificity and, as a quantitative method, it suffers from the problems inherent in PCR. Recently, real-time PCR was introduced to overcome these limitations. And, with the use of appropriate standard curves, absolute copy numbers of mRNA can easily be calculated.[57]

To verify the absolute expression of the PoMTP metalloprotease, its mRNA levels at different developmental stages were analyzed by quantitative real-time PCR (Figure 4b, c). The cDNAs were synthesized from total

RNAs (1 or 3μg) that were treated to digest DNA contamination by DNase I, and were treated to digest RNA strain by RNase H. These cDNAs were used as template in a real-time PCR reaction. The amounts of PoMTP mRNA were calculated from standard curves of quantified PoMTP DNA fragments. The absolute amounts of PoMTP metalloprotease mRNA was 292.7 fg, 1590.3 fg, 1436.0 fg and 609.8 fg in mycelia, primordia, fruiting body and basidiospore, respectively. These results also agreed with the Northern and RT-PCR results for developmental stages. Thus, we can confirm that the great induction of PoMTP metalloprotease mRNA during mushroom fruiting process. This result indicates that the protease affects in the initiation and formation of fruiting bodies. It was reported that the elevated expression of the mitochondrial processing peptidase from mushroom *L. edodes* probably related with the increased energy demand for fruiting formation.[40] Higher expression of hydrophobin in the primordium than in the fruiting body from *L. edodes* revealed that it is more important in fruiting body initiation than in mature fruiting body maintenance, presumably for a higher rate of hyphal growth.

A



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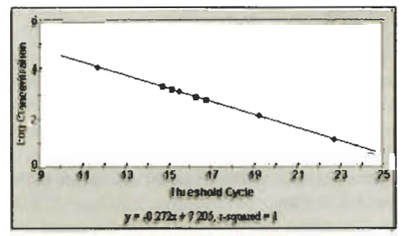

C

Stages	PoMTP
Mycelia	292.674 fg
Primordia	1590.341 fg
Fruiting bodies	1435.979 fg
Basidiospores	609.754 fg

Fig. 4. PoMTP metalloprotease mRNA levels during mushroom development

[A] The gene expression of PoMTP during the fruiting is analyzed by Northern blot analysis. Total RNA (5ug per lane) isolated from mycelia (Mc), primordial (Pr), fruiting bodies (Fb) and spores (Sp) at different stages of the *P. ostreatus* life cycle are subjected to electrophoresis in a formaldehyde-agarose gel, transferred to Hybond N+ membrane, and probed with ^{32}P-labeled PCR-amplified fragment. Lanes 1, 45 days cultured mycelia; Lane 2, mycelia under 9 days fruiting; Lane 3, primordia (11 days); Lane 4 to 7, initial (14 days), young (16 days), middle (18 days), mature fruiting bodies (20 days); Lane 8, spores. [B] To verify the expression of PoMTP mRNA, quantificative real-time RT-PCR is performed. The cDNAs synthesized with AMV reverse transcriptase and random hexamer or oligo d (T) primers are used as template in a PCR reaction with two specific primers. SMART CYCLER (Cepheid, SC1000-1) and SYBR Green II were used for amplification and detection. [c] The endpoint used in the real-time PCR quantification, Ct, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. The amounts of PoMTP mRNA are calculated from standard curves of quantified PoMTP DNA fragments.

Development in multicellular organisms is complexes and depends on a multitude of morphological, physical and genetic changes. Mushroom growth as animal and plant multicelluar organisms requires the formation of organized tissues from an initial mass of uncoordinated hyphae. Once primordia and fruiting bodies of mushroom are formed, it grows rapidly. At this time, an extensive change in cell interactions occurs for cell division and cell expansion. The function and substrates specificity of the PoMTP and the eucolycin subfamily metalloproteases were completely unknown at present. However, the fact that the PoMTP metalloprotease belongs to the metzincin family and the pattern of four cysteines in the zinc-binding domain is well conserved especially in the astacins subfamily are to be noted. Astacin subfamily metalloproteases have been shown to

function in a variety of biological events, including cell differentiation and morphogenesis during development through the regulation of growth factor activity and the modification of extracellular composition.[58] Furthermore, the PoMTP metalloprotease shares a significant homology with microbial collagenase (Accession no. JC4393, 45%) and animal pregnancy-associated plasma protein-A (PAPP-A, accession no. Q13219, 37%) in addition to the eucolysine members. It was reported that targets of ADAM protease in the reprolysins subfamily is extrcelluar matrix proteins, following by the alteration of cell migration.[59] The ADAMs are also responsible for activation of growth factors and cytokines. The PAPP-A metalloprotease is a growth regulatory factor which regulates insulin-like growth factor bioactivity during fetal development.[54] The possibility that the metzincin PoMTP metalloprotease may be involved via the regulation of growth factor activity in mushroom development cannot be excluded. Further studies on the function and gene regulation of the PoMTP metalloprotease will be helpful to a better understanding of mushroom development.

Acknowledgements

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