

Molecular cloning and sequencing of the gene encoding an extracellular aspartic proteinase from *Aspergillus fumigatus*

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Abstract

Oligonucleotide primers based on conserved regions of the aspergillopepsins (EC 3.4.23.18) were used to PCR amplify a 650 bp segment of the gene encoding the extracellular aspartic proteinase (PEP) from *Aspergillus fumigatus*. The segment was used as a probe for isolating and sequencing the gene from a genomic library of the fungus. Likewise the cDNA was amplified by reverse PCR, cloned and sequenced. The *pep* gene was found to consist of four exons encoding for 395 aa. The pre-proenzyme deduced has an N-terminal leader sequence of 70 aa preceding the sequence of the mature enzyme consisting of 325 aa with a calculated molecular mass of 34.4 kDa and an isoelectric point of 3.95. The N-terminal sequence of the mature enzyme matched the N-terminal aa sequence of PEP exactly. The nucleotide and the aa sequences of the pre-proenzyme were 70% and 71% homologous to the corresponding sequences of the aspergillopepsin from *A. niger* var. *awamori*. Southern analysis of digested genomic *A. fumigatus* DNA with a specific PCR probe suggested the presence of a single copy of the *pep* gene.

Keywords: *Aspergillus fumigatus*; Proteinase; DNA sequence; Aspartic protease (EC 3.4.23); Gene, *pep*; Aspergillopepsin (EC 3.4.23.18)

1. Introduction

Aspergillus fumigatus is, medically, the most important mould, causing invasive aspergillosis of the lung, a life threatening disease of the immunocompromised host. Because of its ability to infiltrate almost all tissues [1], extracellular proteinases of *Aspergillus fumigatus* have been suggested to be involved in penetration processes [2,3] and, therefore, important virulence factors. Three extracellular

proteinases of the fungus have been characterized in detail. Two of them, a subtilisin related serine proteinase (ALP) [3–5] and a metalloproteinase (MEP) [6,7] are most active at neutral to alkaline pH. The genes encoding ALP and MEP have been cloned and sequenced recently [8–10]. The third proteinase PEP is an aspartic proteinase and was the subject of earlier investigations [3,11]. In a previous paper [12] we reported that PEP is closely related to aspergillopepsin A from *Aspergillus niger* var. *awamori* [13].

In this report we describe the isolation and nucleotide sequence of both a genomic and a cDNA clone encoding PEP. As PEP is secreted during

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pulmonary aspergillosis and leads to a specific antibody response in patients [12], this might be a worthwhile basis for further investigations on the potential role of PEP in pathogenesis. In addition, the recombinant enzyme might serve as an immunogen for the development of a diagnostic antigen capture assay.

2. Materials and methods

2.1. Strains and cloning vectors

A. fumigatus strain CHUV-192-88 [4] was used for cloning and sequencing the *pep* gene. For all other experiments including PCR, cloning and sequencing of cDNA and southern blotting, *A. fumigatus* strain D141 [3] was used. For genomic DNA-cloning, *Escherichia coli* LE392 was used for the propagation of bacteriophage λ EMBL3 (Promega, Madison, WI). Plasmid subcloning experiments were performed with plasmid pMTL21 [14] in *E. coli* DH5 α using standard procedures [15]. For cloning of PCR-products *E. coli* INV α and plasmid pCRTM II (TA Cloning Kit; Invitrogen, San Diego, CA) were used.

2.2. Amplification of a specific DNA probe

Purified genomic DNA of *A. fumigatus* D141 served as a template to PCR amplify a DNA fragment of about 650 bp using two oligonucleotide primers (primer A and B, Table 1) based on conserved regions of the aspergillopepsin genes of *Aspergillus niger* var. *awamori* and *Aspergillus oryzae* [13]. The resulting product was subcloned, using the pCRII cloning kit.

2.3. Construction and screening of the genomic library

For the screening process, we used a previously constructed λ EMBL3 genomic library of *A. fumigatus* [8]. Approximately 50 000 recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe, Biorad). These filters were probed with ³²P-labelled DNA in a solution containing 7% SDS, 500 mM NaH₂PO₄ (pH 7.0) and 1 mM EDTA, at 65°C for 16 h. The membranes were exposed to X-ray films after two rinses in 2 $\times$ SSC, 0.1% SDS at 55°C. Positive plaques were purified and the DNA was isolated [17]. Agarose gel electrophoresis of restricted recombinant bacteriophage λ EMBL3 DNA and southern blotting were performed according to standard procedures [15].

2.4. DNA sequencing

Double-stranded DNA subcloned into plasmid pMTL21 or pCRII was sequenced using a Sequenase version 2.0 kit (USB, Cleveland, OH) as recommended by the supplier.

2.5. Isolation of RNA and synthesis of cDNA

For the isolation of RNA, a 3-day culture of *A. fumigatus* D141 was used. Total RNA was isolated according to a modified guanidinium thiocyanate method [18] using the RNA-CleanTM System (AGS GmbH, Heidelberg, Germany). cDNA synthesis was performed using RNase H⁻ reverse transcriptase (SuperscriptTM II, Gibco BRL, Gaithersburg, MD) according to standard procedures [15]. Specific synthesis of the cDNA was achieved by introducing the

Table 1
List of PCR primers

A	5' GAA GAC CAC GTA CTG GCT CTT 3'	Lys Ser Gln Tyr Val Val Phe
B	5' GCC TTT AGC TCC ATC AAC ACT G 3'	Ala Phe Ser Ser Ile Asn Thr
3' <i>pep</i>	5' CTA TGC CTG AGG GGC GAA 3'	Phe Ala Pro Gln Ala Stop
5' <i>pep</i>	5' ATG GTC GTC TTT AGC AAA GT 3'	Met Val Val Phe Ser Lys Val

A, B: conserved regions of the aspergillopepsins, similar in the amino acid sequences of *A. niger* var. *awamori* and *A. oryzae* proteinases. For the primers the nucleotide sequence of the *A. niger* var. *awamori* gene was used. 3' *pep*: C-terminal end of *A. fumigatus pep* gene; 5' *pep*: N-terminal end of *A. fumigatus pep* gene.

C-terminal antisense primer of the *pep* gene (Table 1, primer 3'*pep*) into the reaction mixture.

After reverse transcription, the cDNA-containing sample was directly introduced into a PCR reaction. PCR was performed using the same C-terminal primer as for reverse transcription and a sense primer (Table 1, primer 5'*pep*) based on the N-terminus of the *pep* gene.

3. Results and discussion

The acid proteinase secreted by *A. fumigatus* was inhibited by pepstatin and consequently is an aspartic proteinase [12]. Its N-terminal aa sequence was determined to be ?? Gly Ser Ala Val Thr Thr Pro Glu Gln Tyr Asp [12]. This sequence was 73% and 55% similar to the corresponding sequences of the aspartic proteinases secreted by *A. niger* var. *awamori* and *A. oryzae*, respectively [13,16]. As there are highly conserved regions in the proteinase genes of the two latter species, we constructed several oligonucleotide primers for PCR based on them. PCRs were performed with different primer combinations on *A. fumigatus* genomic DNA. However, only the combination of the two primers A and B (Table 1) led to a specific product of 650 bp near the C-terminus of the *pep* gene. This fragment was subsequently used as a probe for screening a genomic *A. fumigatus* library. Twelve hybridizing clones were identified and isolated. Restriction enzyme analysis of the purified λ phage DNA revealed a common 6 kb *Hind*III fragment that hybridized with the 650 bp PCR product. This fragment, of which a restriction map is shown in Fig. 1, was subcloned in pMTL21 for further analysis. The sequence hybridizing with the probe was localized on a 2.4 kb *Bgl*II fragment. A 1.5 kb part of the *Hind*III

fragment contained the promoter region and the *pep* gene which were sequenced. The aa sequence of the three possible reading frames deduced by the nucleotide sequence in Fig. 2 were compared with the aa sequence of *A. niger* var. *awamori* PEP and this suggested that the *A. fumigatus* *pep* gene consists of four exons (position 152–474, 526–803, 863–1111 and 1162–1496 in Fig. 2) and three introns (position 475–525, 804–862 and 1112–1161 in Fig. 2). The predicted intron/exon structure of the *pep* gene could be verified by sequencing *pep*-cDNA. For this purpose, the entire coding region of 1185 bp encoding for 395 aa of *A. fumigatus* *pep* gene was amplified in a PCR reaction and subsequently cloned in pCRII. Oligonucleotides based on the ends of the gene (5'*pep* and 3'*pep*; Table 1) were used as primers, whereas *pep*-cDNA generated by specific reverse transcription served as a template. Despite the fact that two different *A. fumigatus* strains were used for cDNA and for genomic cloning no exchanges could be observed upon comparison of the corresponding nucleotides. This observation indicates that the *pep* gene is highly conserved between the strains. Similar observations were made after cloning the cDNA and gene of the serine proteinase (ALP) from *A. fumigatus* [8].

In fact, the nucleotide sequences of the *pep* gene from *A. fumigatus*, the *pepA* gene from *A. niger* var. *awamori*, the *pepO* gene from *A. oryzae* and the *pep* gene from *A. satoi* are strikingly similar [13,16,19]. All four genes are composed of four exons and three introns with virtually identical length. Even the positions of the introns are strictly conserved between the species. The nucleotide and the aa sequences of the *pep* gene were 70–72% homologous to the corresponding sequences of the three other *Aspergillus* species.

Deduced from the other aspergillopepsins de-

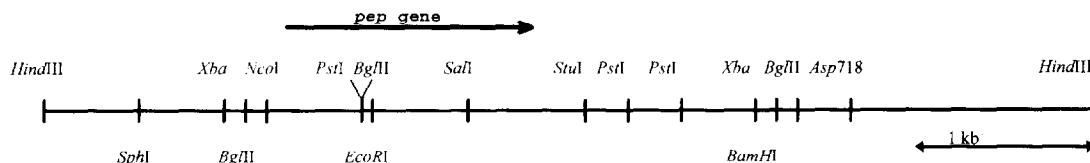


Fig. 1. Restriction map of the 6-kb *Hind*III fragment containing the *pep* gene obtained from positive recombinant λ EMBL3 bacteriophages. The *pep* gene consists of 1345 bp, introns included.

scribed so far and by N-terminal aa sequencing of PEP purified from culture supernatant, it appears that PEP is synthesized as a zymogen (pre-proenzyme)

with an N-terminal pre-proregion of 70 aa, followed by the mature enzyme of 325 aa with a calculated molecular mass of 34.4 kDa and an isoelectric point

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1      A AGGGAACAAA AAAAAAATA TATAA AGGAG GCATGTTTAC CAGCTGTGA CCGAATATCA
62    TCCTCATTCT CGTTCAGCAA GAGTTTGAGA TCGTCTCCTT TTTGTGCGTT GATACTTTTC CACGGTCCAG TAGTCCGTTG
142   TTTCATCAAG  ATG GTC GTC TTT AGC AAA GTC ACC GCT GTC GTC GTC GGT CTC TCG ACC ATT GTG TCT
-70      ↓      Met Val Val Phe Ser Lys Val Thr Ala Val Val Val Gly Leu Ser Thr Ile Val Ser
209   GCT GTC CCT GTG GTC CAG CCG CGC AAG GGC TTC ACT ATC AAC CAA GTG GCC AGA CCA GTG ACC AAC
-51   Ala Val Pro Val Val Gln Pro Arg Lys Gly Phe Thr Ile Asn Gln Val Ala Arg Pro Val Thr Asn
275   AAG AAG ACC GTC AAT CTT CCA GCT GTC TAT GCC AAT GCT TTG ACT AAG TAC GGG GGC ACT GTC CCC
-29   Lys Lys Thr Val Asn Leu Pro Ala Val Tyr Ala Asn Ala Leu Thr Lys Tyr Gly Gly Thr Val Pro
341   GAC AGT GTC AAG GCG GCT GCA AGC TCC GGC AGC GCT GTT ACT ACC CCC GAG CAA TAT GAC TCG GAA
-7   Asp Ser Val Lys Ala Ala Asp Ser Gly Ser Ala Val Thr Thr Pro Glu Gln Tyr Asp Ser Glu
407   TAC CTG ACC CCC GTC AAA GTC GGT GGA ACG ACC CTG AAC TTG GAC TTC GAC ACT GGC TCT GCA GAT
16   Tyr Leu Thr Pro Val Lys Val Gly Gly Thr Thr Leu Asn Leu Asp Phe Asp Thr Gly Ser Ala Asp
473   CT gtaagcgcac cttgtccctc tttcttgaat tgaattcact aattgttgtag  C TGG GTC TTC TCC TCC GAG
38   Le u Trp Val Phe Ser Ser Glu
545   CTT TCG GCT TCC CAG TCC AGC GGC CAT GCT ATC TAC AAG CCG TCC GCT AAT GCC CAA AAG CTG AAT
45   Leu Ser Ala Ser Gln Ser Ser Gly His Ala Ile Tyr Lys Pro Ser Ala Asn Ala Gln Lys Leu Asn
611   GGC TAC ACC TGG AAG ATC CAA TAT GGT GAT GGT AGC AGT GCC AGC GGT GAC GTC TAC AAG GAT ACC
67   Gly Tyr Thr Trp Lys Ile Gln Tyr Gly Asp Gly Ser Ser Ala Ser Gly Asp Val Tyr Lys Asp Thr
677   GTC ACT GTG GGT GGT GTC ACT GCT CAG AGC CAG GCT GTG GAG GCT GCC AGC CAT ATC AGC TCT CAA
89   Val Thr Val Gly Gly Val Thr Ala Gln Ser Gln Ala Val Glu Ala Ala Ser His Ile Ser Ser Gln
743   TTC GTG CAG GAT AAG GAC AAC GAT GGT CTG TTG GGT TTG GCA TTC AGC TCC ATC AAC ACT G gtga
111   Phe Val Gln Asp Lys Asp Asn Asp Gly Leu Leu Gly Leu Ala Phe Ser Ser Ile Asn Thr V
808   gtttca gtcaagatta ccagcttcca cctcagccca ctgactcgga ttgttacag TC AGT CCC CGC CCT CAG ACT
131   al Ser Pro Arg Pro Gln Thr
883   ACT TTC TTT GAC ACT GTC AAG TCC CAG TTG GAC TCT CCT CTC TTT GCT GTG ACC TTG AAG TAC CAT
138   Thr Phe Phe Asp Thr Val Lys Ser Gln Leu Asp Ser Pro Leu Phe Ala Val Thr Leu Lys Tyr His
949   GCT CCA GGC ACC TAC GAC TTT GGA TAC ATC GAC AAC TCC AAG TTC CAA GGG GAA CTC ACT TAT ACC
160   Ala Pro Gly Thr Tyr Asp Phe Gly Tyr Ile Asp Asn Ser Lys Phe Gln Gly Glu Leu Thr Tyr Thr
1015  GAC GTC GAC AGC TCC CAG GGT TTC TGG ATG TTC ACT GCT GAT GGC TAC GGT GTT GGC AAT GGT GCT
182   Asp Val Asp Ser Ser Gln Gly Phe Trp Met Phe Thr Ala Asp Gly Tyr Gly Val Gly Asn Gly Ala
1081  CCC AAC TCC AAC AGT ATC AGC GGC ATT GCT G gtatgttttc acagtgtctc ttcagaagga ccaagctgat
204   Pro Asn Ser Asn Ser Ile Ser Gly Ile Ala A
1152  ccacttttag  AC ACC GGC ACC ACC CTC CTC CTG CTT GAT GAC AGC GTT GTT GCC GAC TAC TAC CGC
214   sp Thr Gly Thr Thr Leu Leu Leu Leu Asp Asp Ser Val Val Ala Asp Tyr Tyr Arg
1218  CAG GTT TCC GGA GCC AAG AAC AGC AAC CAA TAC GGT GGT TAT GTC TTC CCC TGC TCC ACC AAA CTT
233   Gln Val Ser Gly Ala Lys Asn Ser Asn Gln Tyr Gly Gly Tyr Val Phe Pro Cys Ser Thr Lys Leu
1284  CCT TCT TTC ACT ACC GTC ATC GGA GGC TAC AAT GCC GTC GTT CCC GGT GAA TAC ATC AAC TAC GCC
255   Pro Ser Phe Thr Thr Val Ile Gly Gly Tyr Asn Ala Val Val Pro Gly Glu Tyr Ile Asn Tyr Ala
1350  CCC GTC ACT GAC GGC AGC TCT ACC TGC TAC GGC GGC ATC CAG AGC AAC TCT GGT TTG GGC TTT TCT
277   Pro Val Thr Asp Gly Ser Ser Thr Cys Tyr Gly Gly Ile Gln Ser Asn Ser Gly Leu Gly Phe Ser
1416  ATC TTC GGA GAT ATC TTC CTC AAG AGC CAG TAC GTC GTC TTC GAC TCC CAA GGC CCC AGA CTC GGC
299   Ile Phe Gly Asp Ile Phe Leu Lys Ser Gln Tyr Val Val Phe Asp Ser Gln Gly Pro Arg Leu Gly
1482  TTC GCC CCT CAG GCA TAG ATGAATA TGCACGCGGT TTTTGGTCTT TGAGGCCGGC CTGG
321   Phe Ala Pro Gln Ala ***

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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *A. fumigatus pep* gene. Introns are denoted by lower-case letters. A putative TATAA box is framed. Asp-32 and Asp-214 of the catalytic sites are double underlined. The putative cleavage site within the signal sequence for secretion is indicated by an arrow (↓). The first 10 amino acids of the mature proteinase are underlined. *** indicates the TAG stop codon. The *pep* sequence data was submitted to EMBL database, under accession No. X85092.

of 3.95. The putative cleavage site, Ala⁻⁵¹-Val⁻⁵⁰ [20] for the signal peptide leads to a proregion of 50 aa.

Southern analysis of digested genomic *A. fumigatus* DNA with the specific 650 bp PCR probe was performed using different restriction enzymes that had no cleavage sites within the gene (*Sma*I, *Msc*I, *Ssp*I and *Fsp*I). Consistent with the presence of a single copy of the gene, only one hybridization band per enzyme used was detected. The presence of a single copy gene has also been assumed for other aspergillopepsins [13,16].

A. fumigatus secretes two other proteinases when the fungus is cultivated at neutral pH in the presence of collagen as the sole nitrogen and carbon source: a serine or alkaline proteinase (ALP) and a metalloproteinase (MEP) which are closely related to the alkaline proteinase and the neutral proteinase I secreted by *A. oryzae*, respectively [21,22]. However *A. oryzae* secretes a second metalloproteinase or neutral proteinase II [23], which appears to be absent in *A. fumigatus*.

The role of ALP and MEP for the virulence of *A. fumigatus* remains obscure. Recently, gene disruption experiments have been performed [10,24,25] but strains of ALP- or MEP-deficient mutants or mutants deficient in both proteinases did not show significantly reduced virulence in murine models of aspergillosis compared with the wild type strain. Hence, the presence of the third proteinase, PEP, might be of importance for the pathogenicity of *alp/mep* mutants. However, PEP shows optimal activity at pH 2.8 and is inactive below pH 2.0 and above pH 6.5 [12]. Therefore, it is difficult to attribute a role in tissue invasion to this proteinase without postulating acidification of hyphal microenvironment by the fungus itself. Thus, the contribution of PEP to pathogenesis remains to be elucidated. However, specific antibody production of the host is known for all the three proteinases from *A. fumigatus* during pulmonary aspergillosis [3,7,12]. Therefore, PEP, ALP and MEP are potential targets for an antigen capture assay. Such an assay could be of diagnostic value and should involve the simultaneous monitoring of all three types of *A. fumigatus* proteinases secreted. As we are now familiar with all genes encoding, we intend to establish an assay on the basis of the recombinant enzymes.

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