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Characterization of an extracellular serine protease gene from the nematophagous fungus *Lecanicillium psalliotae*

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Abstract

The gene encoding a cuticle-degrading serine protease was cloned from three isolates of *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) by 3′ and 5′ RACE (rapid amplification of cDNA ends) method. The gene encodes for 382 amino acids and the protein shares conserved motifs with subtilisin N and peptidase S8. Comparison of translated cDNA sequences of three isolates revealed one amino acid polymorphism at position 230. The deduced protease sequence shared high degree of similarities to other cuticle-degrading proteases from other nematophagous fungi.

Introduction

Extracellular enzymes are important virulence factors in nematophagous and entomophagous fungi (Segers *et al.* 1994, Tunlid *et al.* 1994, Bonants *et al.* 1995, Joshi *et al.* 1995). Lopez-Llorca (1990) isolated a serine protease P32 from *Pochonia suchlasporia* (syn. *Verticillium suchlasporium*) and found it was involved in egg penetration of nematode. Subsequently, two proteases, VCP1 and PIP, were isolated from nematophagous fungi *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) and *Paecilomyces lilacinus*, respectively (Segers *et al.* 1994, Bonants *et al.* 1995). Similar extracellular proteases also had been found in entomophagous fungi (St Leger *et al.* 1992, Joshi *et al.* 1995). Moreover, collagenase and chitinase have been identified from nematophagous fungi *Arthrobotrys amerospora*, *Po. chlamydosporia* and *Po. suchlasporia* (Schenck *et al.* 1980, Tikhonov *et al.* 2002).

Lecanicillium psalliotae is a nematophagus fungus with commercial potential for the biocontrol of root knot and cyst nematodes. It produces an alkaline serine protease, Ver112, during infection of the saprophytic nematode *Panagrellus redivivus*. Ver112 had been purified from culture filtrates of *L. psalliotae*. The *N*-terminal amino acid sequence has been submitted to Swiss-Prot (accession number Q68GV9). In this report, we described the cloning of an alkaline serine protease from *L. psalliotae* by the 3′ and 5′ RACE method, the analysis of the primary amino acid sequence of protease Ver112 from three isolates, and comparison with other cuticle-degrading serine proteases isolated from different nematophagous and entomopathogenic fungi.

Materials and methods

Microorganisms and culture conditions

Three isolates (112, 602 and 608) of nematophagous fungus *Lecanicillium psalliotae* used in this study were originally isolated from field soil samples in Yunnan Province; strain 112 has been deposited in the China General Microbiological Culture Collection Center. Fungi were cultured in PD (potato/dextrose) medium at 26 °C with shaking at 200 rpm for 3 days.

Escherichia coli DH 5α was used in all DNA manipulations and grown in Luria–Bertani medium containing (per liter): 10 g tryptone, 10 g NaCl, 5 g yeast extract, and 16 g agar.

Genomic DNA and total RNA extraction

Mycelium were collected by filtration in a sterilized filter funnel and ground to a fine powder in liquid N_2. DNA was extracted according to the method of Zhang *et al.* (1996).

Total RNA extraction was done according to the manual of TRIzol Reagent (Invitrogen, America), and RNA was stored at −70 °C.

Amplification of 3′ and 5′ nucleotide sequence

A partial cDNA of Ver112 was obtained by 3′ RACE kit (Invitrogen, America) using a degenerate primer, SERP3-1 5′-ACNCARCARCARGG NGCNAC-3′, which was designed according to the *N*-terminal amino acid residues of the protease Ver112. The first strand cDNA and target cDNA were synthesized according to the manual of 3′ RACE system for rapid amplification of cDNA ends. 5′ RACE was conducted as described in the manual of 5′ RACE system for rapid amplification of cDNA ends using two gene-specific primers derived from the 3′ RACE product, R5-1 5′-AGTCTTGGACTCCGATGGTG-3′, and R5-2 5′-TGGGAGATGCGAGTAAGTC -3′.

Amplification of the Ver112 chromosomal gene and full-length cDNA

Two gene-specific primers, FP 5′-CTGATTATCAACAAGATGCGTC-3′ and RP 5′-TTACG TGGCGCCGTTGAAGGC-3′, were designed according to the PCR fragments of 3′ and 5′ RACE, genomic DNA and the first strand of cDNA was used as template, respectively. Target DNA and cDNA were amplified by a touchdown program (Kim *et al.* 2003).

The cDNA and genomic sequences were compared using the DNAman software package (Version 5.2.2, Lynnon Biosoft, Canada).

Cloning and sequencing

The PCR products were purified from a 1% agarose gel using a DNA fragment purification kit ver 2.0 (Takara, Japan) and subcloned into pGEM-T Vector (Promega, America). White colonies were randomly selected and purified using the plasmid DNA purification kit (Qiagen, German) and the plasmid DNA was sequenced using an ABI 3730 autosequencer (Perkin–Elmer, America) with four fluorescent dyes. The sequencing primers were T7 and SP6 universal primers (Takara, Japan). Sequence data were analyzed using DNAman software package. Sequence identity was compared with other cuticle-degrading protease gene using the GenBank database.

Sequence analysis

Database searches were performed using BlastX (http://www.ncbi.nlm.nih.gov/BLAST). Signal sequence prediction was performed using Signal P (http://www.cbs.dtu.dk/services/signalP) (Henrik *et al.* 1997). Multiple sequence alignments were performed using DNAman software package. Proteins were examined for conserved motifs using Pfam (http://pfam. wustl.edu/hmmsearch. shtml) (Garcia-Sanchez *et al.* 2004). N-linked glycosylation sites were predicted by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Cloning of the cuticle-degrading serine protease

Under the conditions described above, a 500 bp PCR product (Figure 1) was successfully amplified by 5′ RACE and sequencing indicated that the PCR fragment contained a putative start co-

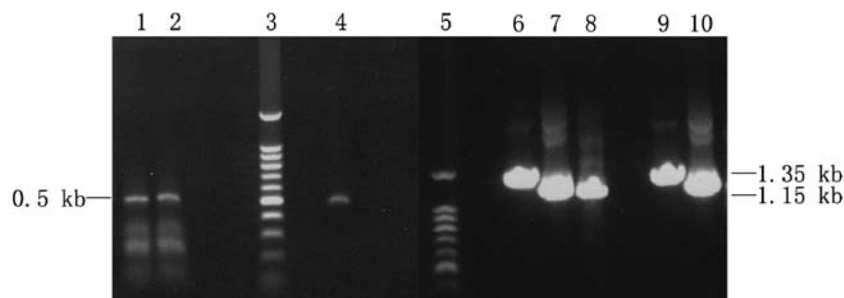

Fig. 1. Result of PCR amplification. Lanes 1, 2 and 4 – result of 5′ RACE amplification; lanes 3 and 5 – DNA marker (Ladder 100 bp, Promega, America); lanes 6 and 9 – PCR fragment amplified with genomic DNA as template; lanes 7, 8 and 10 – PCR fragment amplified with the first strand of cDNA as template.

don (ATG). One thousand one hundred and fifty and 1350 bp fragments (Figure 1) were amplified by using, respectively, the first strand of cDNA and genomic DNA as template from three isolates of *L. psalliotae*. These fragments were also cloned and sequenced. The combined nucleotide sequence for the partial DNA and cDNA were 1640 and 1440 bp, respectively.

Sequence analysis

The sequence of *Ver112* comprised an ORF, which contained three introns and four exons. It encoded a polypeptide of 382 amino acid residues with a M_r of 39.654, which shared conserved motifs with subtilisin N and peptidase S8. Comparison of Ver112 with other serine proteases from nematophagous fungi revealed that it was typical of fungal serine proteases, which possessed a pre-pro-peptide structure. It has a signal peptide (15 amino acids) consisting of the initial methionine, a core of seven hydrophobic residues, a helix-breaking residue (proline), and four hydrophobic residues before a signal peptidase cleavage site (Ala-Leu-Ala). Comparison of the deduced amino acid sequence with the *N*-terminal sequence of Ver112 revealed that the mature protein started at residue 103, and the final residue of the pro-peptide was an asparagine (N), position in 102. Each intron began with GT and ended with AG, which was a common feature of fungal introns and had been observed in the serine protease gene from *Acremonium chrysogenum* (Isogai *et al.* 1991). The mature protein consisted of 280 amino acids.

Comparison of the nucleotide sequences of Ver112 from three isolates of *L. psalliotae* revealed that they were very conservative, the nucleotide sequences from *L. psalliotae* 112 and 608 were identical, and there were four nucleotide residues different from *L. psalliotae* 602, two of them located at the second intron, and two other variable nucleotides located at different exons, which resulted in one amino acid polymorphism at position 230, arginine (A) changed to glycine (G). Like VCP1, Ver112 lacks any *N*-linked glycosylation site (Asn-X-Ser/Thr). These nucleotide sequences have been submitted to GenBank, under accession numbers AY 692148 (112 and 608) and AY870806 (602).

Comparison of Ver112 with other serine proteases isolated from nematophagous and entomopathogenic fungi

These cuticle-degrading proteases shared some similar biochemical properties of low molecular mass and being inhibited by PMSF (phenylmethylsulfonylfluoride) (Table 1). However, PII and Aozl isolated from nematode-trapping fungi *A. oligospora* had lower p*I* and higher molecular masses than other proteases from nematophagous and entomopathogenic fungi.

The databank search showed that Ver112 shared extensive similarities to fungal members of the subtilisin family of serine proteases (Figure 2). The deduced amino acid sequence of the Ver112 showed 39.6%, 41.7%, 62.8%, 75.7%, 57.0%, 61% and 58.2% identity, respectively, to Aozl (*Arthrobotrys oligospora*), PII (*A. oligospora*), PIP (*Pic. lilacinus*), Pr1 (*Beauveria bassiana*), PrA (*Metarhizium anisopliae*), Prk (*Tritirachium album*), and VCP1 (*Po. chlamydosporia*). The signal peptide and pro-region cleav-

Table 1. Partial characterization of cuticle-degrading serine proteases isolated from different nematophagous and entomopathogenic fungi.

Protease	Fungus	Molecular mass (kDa)	Inhibitor of protease	p*I*	Reference
PII	*Arthrobotrys oligospora*	35	PMSF[a]	4.6	Tunlid *et al.* (1994)
Aozl	*Arthrobotrys oligospora*	38	PMSF, SSI[b]	4.9	Zhao *et al.* (2004)
VCPl	*Pochonia chlamydosporia*	33	PMSF	10.2	Segers *et al.* (1994)
P32	*Pochonia suchlasporia*	32	PMSF, *p*CMB[c]	–	Lopez-Llorca LV (1990)
Ver112	*Lecanicillium psalliotae*	32	PMSF	–	GenBank (AAU01968)
PIP	*Paecilomyces lilacinus*	33	PMSF	10.2	Bonants *et al.* (1995)
PrA	*Metarhizium anisopliae*	25	PMSF	10.2	St Leger *et al.* (1992)
Pr1	*Beauveria bassiana*	32	PMSF	10.0	Joshi *et al.* (1995)

[a]PMSF, phenylmethylsulfonylfluoride.
[b]SSI, Streptomyces subtilisin inhibitor.
[c]*p*CMB, *p*-chloromercuric benzoic acid.

age sites of them were conserved and the first amino acid of mature proteases was alanine. They shared the conservation of the aspartic acid (Asp_{143})–histidine (His_{173})–serine (Ser_{328}) (in Ver112) catalytic triad. The two blocks of side-chains that form the sides of the substrate-binding S_1 pocket in subtilisin occur in regions of high similarity and consist of $Ser_{236}Leu_{237}Gly_{238}$ and $Ala_{262}Ala_{263}Gly_{264}$, respectively, in Ver112. Furthermore, the highly conserved Asn_{265} (in Ver112) is important in subtilisin for stabilization of the reaction intermediate formed during proteolysis (Kraut 1977).

Discussion

Extracellular serine proteases have been isolated, cloned and purified from several nematophagous and entomopathogenic fungi. From Table 1 and Figure 2, these cuticle-degrading serine proteases from different nematophagous and entomopathogenic fungi may be divided into two categories according to the difference of biochemical characterization and primary sequence. Class I is isolated from nematode-trapping fungi and has lower p*I*, and class II is isolated from nematode-parasitic or egg-parasitic fungi and has higher p*I*. However, whether the differences of biochemical characterization between classes II and I are important for the ability of the enzymes to degrade components of the nematode cuticle and eggshell, respectively, and whether the differences is connected to their mode of infection are currently not known.

The high degree of similarities between extracellular serine proteases from different nematophagous and entomopathogenic fungi suggest that they may derive from a common ancestral subtilisin-like protease gene. Cloning of Ver112 provides a good foundation for future investigation of infection mechanism and improvement the pathogenicity of nematophagous and entomopathogenic fungi.

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Fig. 2. Alignment of subtilase amino acid sequences from *Arthrobotrys oligospora* (PII and Aozl), *Paeciliomyces lilacinus* (PIP), *Beauveria bassiana* (Pr1), *Metarhizium anisopliae* (PrA), *Tritirachium album* (Prk), *Pochonia chlamydosporia* (VCP1) and *Lecanicillium psalliotae* (Ver112). The GenBank accession numbers are CAA63841, AAM93666, AAA91584, AAK70804, CAB64346, P06873, CAD20578 and AAU01968, respectively. Areas shaded in black are conserved regions (100% similarity), areas shaded in gray are high degree similarity (more than 50% similarity) and unshaded areas are regions of variability between the proteases. ▽ indicates Putative signal-sequence cleavage site; ▼ indicates Proregion cleavage site. ▲ indicates the aspartic acid (Asp_{143})–histidine (His_{173})–serine (Ser_{328}) (in Ver112) catalytic triad. The underlined region is the substrate-binding S_1 pocket in subtilisin.

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                                ▽
Aoz1       MLTNGLISLLAIAGLATNAFAGPIRKVSNAGAAG..AIADKYIVVLKKGLSDSAVSKHTNRI   60
PII        MLTNGLISLLAIAGLATNAFAGPIRKVSNAGAAG..AIADKYIVVLKKGLSDSAVQTYH.RI   59
PIP                                 ARAPLLTPRGASSSSTASTLSSSRTACPSPLSTRLSAL   38
Pr1        MR...LSIIAAALPL...AIAAP.VVEP....APLIEARG.QTIAGNYIVKLKDTATMSIMD   50
PrA       MFPSLLLLNLLPLAIA...APAKRAEPAPLLVPRGD.TIPDKYIVKYKETFDISAADSTIKEY   59
Prk        MRLSVLLSLLPLALG...APAVEQRSEAAPLIEARG.EMVANKYIVKFKEGSALSALDAAME   58
VCP1       MQLSVLLTLLPAVLA...APAIVEQRAEPAPLFTPKSS.IIAGKYIVKFKDGVARIAADEAT   58
Ver112     MR...LSIIAAVLPL...ALAAP.VAEPE..IAPLIEARGAQPIAGKYIVKLKDEAKFGIMN   53
Consensus

                                                                        ▼
Aoz1      SSFHSNVARDLTGARAHGVGRKFRFSSTGFNGYVGGFDKATLQEILNSPEVDYVEQDTVVTTY   123
PII       SSFHSNVARDLTGARAHGVGRKFRFSSTGFNGYVGGFDKATLQEILNSPEVDYVEQDTVVTTY   122
PIP       CPRRPTASTTT..................FSEASRNLNANDLKTLRDHPDVEYIEQDAIITIN   83
Pr1       .AASKVSK.PKFVYTDV............FPGYAASLSPEEVERLRHDPNVESIEQDAIVSIN   99
PrA       HAKAEKTYSHV..................FNGFAGALNATSIETLRNHPAVDFIENDATVRIS   104
Prk       KISGKPDHVYKNV................FSGFAATLDENMVRVLRAHPDVEYIEQDAVVTIN   105
VCP1      SALSAKADHVYSHL...............FNGFAGSLTKEELQTLRNHPDVDFIEKDAVMTAN   106
Ver112    .AKSKIPG.IERVYENV............LNGFSATLSNEELERLRRDPDVESIEQDAIFSIN   102
Consensus                                                 p v   e d

Aoz1      AE..QTDSTWGLDRISHEDYSAPYTYEYDETAAGAGTTVYVIDTGIRISHDEFQTVNGSSRAT   184
PII       AE..QTDSTWGLDRISHEDYSAPYTYEYDETAAGAGTTVYVIDTGIRISHDEFQTVNGSSRAT   183
PIP       AYTQQPGAPWGLGRISHRSKGS.TTYEYDTSG.GSGTCAYVIDTGVEASHPEFEG.....RAS   139
Pr1       AIVRQPGAPWGLGRISHRAKGD.TTYVYDSTA.GQGACVYVIDTGVEATHPEFEG.....RAK   155
PrA       AFVEQPGAPWGLSRISHRQRGG.SSYAYDDSA.GEGTCAYVIDTGVEASHPEFEG.....RAE   160
Prk       AAQTN..APWGLARISSTSPGT.STYYYDESA.GQGSCVYVIDTGIEASHPEFEG.....RAQ   159
VCP1      AIVEQQGAPWGLGRISNRQKGS.TTYRYDDSA.GNGACVYVLDTGIETTHPEFEG.....RAT   162
Ver112    AITQQQGATWGLTRISHRARGS.TAYAYDTSA.GAGACVYVIDTGVEDTHPDFEG.....RAK   158
Consensus a        wgl ris         y yd    g g   yv dtg    h  f       ra
                                                   ▲

Aoz1      WGFNSVDKTDSDGNGHGTHCAGTIAGKTYGVSKKAKVVAVKVLSAGGSGSTAGVVSGMNWVAE   247
PII       WGFNSVDKTDSDGNGHGTHCAGTIAGKTYGVSKKAKVVAVKVLSAGGSGSTAGVVSGMNWVAE   246
PIP       QIKSFISGQNTDGNGHGTHCAGTIGSKTYGVAKKTKIYGVKVLDNSGSGSYSGIISGMDFAVQ   202
Pr1       QVKTFVSG.SKDGHGHGTHCAGTIGSKTYGVAKKVSIFGVKVLEDSGSGSLSGVIAGMDYVAQ   217
PrA       FIRSFVAGENSDRNGHGTHVAGTIGSKKYGVAKKTKILGIKVLSDQGSGDYSGILAGMDFAIQ   223
Prk       MVKTYYYS.SRDGNGHGTHCAGTVGSRTYGVAKKTQLFGVKVLDDNGSGQYSTIIAGMDFVAS   221
VCP1      WLKSFIDGENNDGHGHGTHCAGTVGSKTYGVAKKAKLLAVKVLDNGGSGSYAGVIAGMEFVSQ   225
Ver112    QIKSYAST.ARDGHGHGTHCAGTIGSKTWGVAKKVSIFGVKVLDDSGSGSLSNIVAGMDFVAS   220
Consensus            d  ghgth agt      gv kk      kvl   gsg       gm
                         ▲

Aoz1      N.....ATPNFSVASMSLGGSKSTALNAAVDCIFNAGITIVVAAGNENQDAKNVSPASAPNAI   305
PII       N.....ATPNFSVASMSLGGSKSAALNTAVDAIFNAGITIVVAAGNENQDAKNVSPASAPNAI   304
PIP       DSKSR.SCPKGVVANMSLGGGKAQSVNDGAAAMIRAGVFLAVAAGNDNANAANYSPASEPTVC   264
Pr1       DRRTRSECTKGAIASMSLGGGYSAAVNKAAANLQASGVFVAVAAGNDNRDAANTSPASEPSVC   280
PrA       DSRTR.GCPKGVVANMSLGGGYSAAINQAAAKMIQSNVFLAVAAGNDAKDASQTSPASEPSVC   285
Prk       DKNNR.NCPKGVVASLSLGGGYSSSVNSAAARLQSSGVMVAVAAGNNNADARNYSPASEPSVC   283
VCP1      DYKTR.GCPNGAIASMSLGGPFSASVNQAAAAMVSSGVFLSVAAGNDGADAARYSPASEPSAC   287
Ver112    DRQSR.NCPRRTVASMSLGGGYSAALNQAAARLQSSGVFVAVAAGNDNRDAANTSPASEPTVC   282
Consensus              a  slgg      n              vaagn    a   spas p

Aoz1      TVGAIDSSNKIASLSNWGTLIDVFAPGVGVLSSWATSDKETKTISGTSMACPHVAGLAAYYIS   368
PII       TVGAIDSSNKIASFSNWGTLIDVFAPGVGVLSSWATSDKETKTISGTSMACPHVAGLAAYYIS   367
PIP       TVGATTSSDARSSFSNYGNLVDIFAPGSNILSTWIGG..TTNTISGTSMATPHIVGLGAYLAG   325
Pr1       TVGATDSSDRRSSFSNYGKVLDIFAPGTGILSTWING..GTNTISGTSMATPHIAGLGAYLWV   341
PrA       TVGATDSSDRLSSFSNYGAAVDILAPGSDILSTWIGG..ITKSISGTSMATPHIVGLGAYLSS   346
Prk       TVGASDRYDRRSSFSNYGSVLDIFGPGTSILSTWIGG..STRSISGTSMATPHVAGLAAYLMT   344
VCP1      TVGATTSTDARSSFSNFGKLVDIFAPGSAILSTWING..GTRSISGTSMATPHVAGLAAYLNA   348
Ver112    TVGATDSNDVRSTFSNYGRVVDIFAPGTSITSTWIGG..RTNTISGTSMATPHIAGLAAYLFG   343
Consensus tvga          sn g   d   pg    s w      t  isgtsma ph  gl ay
                                                       ▲

Aoz1      ASEGGADPATITDKITSSRRQWSGHREHPWLPKQDRLQRICLSTHSPKTNHQVTIVA         425
PII       ASEGGADPATITDKITSS..AVSGQVTG...NIRGSPNKIAYNGYA                    408
PIP       LE.GFPGAQALCKRIQTL..STKNVLTG...IPSGTVNYLAFNGNPSG                  367
Pr1       LG.KGT.AGNLCKVIQDL..STKNVLTG...VPSGTVNYLAFNGAT                    380
PrA       LE.GFPGAQALCERIRSL..AIRNTISG...VPGGTVNLLAFNGNPSG                  388
Prk       LG.KTT.AASACRYIADT..ANKGDLSN...IPFGTVNLLAYNNYQA                   384
VCP1      LQ.GVVSPAALCKKIQDT..AIKNALTG...VPASTVNFLAYNGA                     387
Ver112    LE.GGS.AGAMCGRIQTL..STKNVLTS...IPSGTVNYLAFNGAT                    382
Consensus               i
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