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Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens

Dianpeng Zhang[1], Davide Spadaro[1,2]*, Silvia Valente[1], Angelo Garibaldi[1] and Maria Lodovica Gullino[1]

[1] Centre of Competence for the Innovation in the Agro-environmental Sector, University of Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

[2] DiVaPRA – Plant Pathology, University of Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

*Corresponding author. Tel.: +39-011-6708942; fax: +39-011-6709307.

E-mail address: davide.spadaro@unito.it (D. Spadaro)

Abstract

An alkaline protease gene was amplified from genomic DNA and cDNA of the antagonistic yeast-like fungus *Aureobasidium pullulans* PL5, a biocontrol agent effective against *Monilinia laxa* on stone fruit and *Botrytis cinerea* and *Penicillium expansum* on pome fruits. An open reading frame of 1,248 bp encoding a 415-amino acid (aa) protein with a calculated molecular weight (M_r) of 42.9 kDa and an isoelectric point (pI) of 4.5 was characterized. The *cDNAALP5* gene had an 18-amino acid signal peptide, one *N*-gylcosylation, one histidine active site, and one serine active site. The *ALP5* gene with a M_r of 1,351 bp contained two introns. One intron was of 54 bp, while the other was of 50 bp. Protein BLAST and phylogenetic tree analysis of the deduced amino sequences from the *cDNAALP5* gene showed that the encoded protein had 100% homology to a protease enzyme (ALP2) of a sea strain of *A. pullulans*, suggesting that the protein ALP5 was an alkaline serine protease. Expression of *ALP5* in *Escherichia coli* BL21 (DE3), followed by identification with Western-blotting, purification with Ni-NTA and analysis of enzymatic activity, yielded an homogeneous recombinant ALP5 which hydrolysed the substrate casein and inhibited the mycelial growth of the pathogens. At its optimal pH of 10.0 and reaction temperature of 50°C, the recombinant protease exhibited the highest activity towards the substrate casein, though the highest stability was at lower temperatures and pH between 7.0 and 9.0. This study provided the direct evidence that extracellular proteases secreted by the antagonist *A. pullulans* PL5 played a role in the biocontrol activities against some postharvest pathogens of apple and peach.

Keywords: *Aureobasidium pullulans*, biological control, *Botrytis cinerea*, *Monilinia laxa*, *Penicillium expansum*, postharvest.

1. Introduction

Monilinia spp., *Botrytis cinerea* and *Penicillium expansum* are among the most important postharvest pathogens on fruit and vegetables. Three species of *Monilinia* can cause severe losses on stone fruits, but *M. laxa* is the most dangerous in European countries (Pellegrino et al., 2009); *B. cinerea* could cause grey mould on pome and stone fruits, and *P. expansum* can cause blue mould decay, which is one of the most destructive disease of pears and apples and it is accompanied by the production of patulin, a mycotoxin with immunosuppressive effects on humans (Moake et al., 2005; Spadaro et al., 2008).

Biological control is an effective alternative to fungicidal treatment in controlling postharvest diseases of fruits (Droby et al., 2009; Spadaro and Gullino, 2004). Knowledge about the modes of action of biocontrol agents (BCAs) is essential for developing appropriate commercial formulations and application methods to maximize the potential use of biological control (Janisiewicz and Korsten, 2002). For antagonistic yeasts or yeast-like fungi, the modes of action include nutrient competition and site exclusion (Bencheqroun et al., 2007; Saravanakumar et al., 2008), induction of host resistance (El-Ghaouth et al., 1998; Ippolito et al., 2000) and mycoparasitism (Wisniewski et al., 1991). Recently more attention has been paid to studying the production of lytic enzymes by BCAs due to their roles in breaking down the cell walls of pathogens and inhibiting the spore germinations (Masih and Paul, 2002; Smits et al., 2001).

The cell wall-degrading enzymes, mostly chitinases, glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents (Harman et al., 2004). Those enzymes attach the cell wall of phytopathogenic fungi, causing cell lysis and subsequent death (Castoria et al., 1997; Tseng et al., 2008). Although the mechanism of mycoparasitism is not completely known (Andrews et al., 1994), this process has been assumed to involve the expression of extracellular cell wall-degrading

82 enzymes (Chan and Tian, 2005). So far, extensive research has been done on chitinases and
83 glucanases from microbial antagonists. Many chitinase genes have been cloned and characterized
84 from antagonistic *Trichoderma* spp. (Draborg et al., 1996; Garcia et al., 1994; Limon et al., 1995;
85 Matroudi et al., 2008), yeast (Saravanakumar et al., 2009) and bacteria (Gupta et al., 1995). Also some
86 glucanase genes from fungal (Cohen-Kupiec et al., 1999; El-Katatny, 2008), yeast (Chamberms et al.,
87 1993; Grevesse et al., 2003; Jijakli and Lepoivre, 1998; Zhang et al., 2011) and bacterial (Cheng et al.,
88 2009) biocontrol agents were cloned and biochemically studied. However, information on protease
89 from microorganisms is very limited and moreover, up to date, there are no reports on cloning and
90 characterizing protease genes from biocontrol agents.
91 The yeast-like fungus *Aureobasidium pullulans* De Bary (Arnaud) has been successfully applied to
92 control *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* on apple, sweet cherry, grapes, strawberry
93 and peach (Bencheqroun et al., 2007; Ippolito et al., 2000; Lima et al., 1997; Schena et al., 2003).
94 Modes of action elucidated for *A. pullulans* include nutrient competition (Bencheqroun *et al*., 2007),
95 induction of host resistance (Ippolito et al., 2000) and production of glucanase, chitinase and protease
96 (Zhang et al., 2010a).
97 Protease refers to a group of enzymes whose catalytic function is to hydrolyse (breakdown) peptide
98 bonds of proteins. Proteases are divided into four major groups according to the character of their
99 catalytic active site and conditions of action: serine proteinases, cysteine (thiol) proteinases, aspartic
100 proteinases, and metalloproteinases (Barrett et al., 2003). Attachment of a protease to a certain group
101 depends on the structure of catalytic site and the amino acid (as one of the constituents) essential for
102 its activity, and hence, proteases differ in their ability to hydrolyse various peptide bonds (Hedstrom
103 2002).

Besides their particular role in degrading cell wall of phytopathogenic fungi, proteolytic enzymes occupy the most relevant position among industrial enzymes and are very important in digestion as they breakdown the protein foods to liberate the amino acids needed by the body (Feijoo-Siota and Villa, 2011). Moreover, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation (Hooper 2002; Silva and Malcata, 2005). In addition, the enzymatic hydrolysis of proteins by using proteases is still one of the most important means to produce bioactive peptides (Kristinsson and Rasco, 2000). Therefore, it is very important to seek for new protease-producing microbial sources and to develop some new proteases. So far, the most commonly used proteases are obtained from *Bacillus* sp., lactic bacteria, and marine yeasts (He et al., 2006; Ma et al., 2007; Minervini et al., 2003; Okamoto et al., 1997). However, no report on protease-related genes obtained from terrestrial yeasts was published.

The antagonistic yeast-like fungus *A. pullulans* strain PL5 isolated from the carposphere of fruits showed a high efficacy in controlling *M. laxa* on stone fruits and *B. cinerea* as well as *P. expansum* on pome fruits under semi-commercial conditions (Zhang et al., 2010a, 2010b). In our previous study, the strain PL5 exhibited a high capability of producing protease activity which effectively hydrolysed the substrate casein. Therefore, in this paper, the protease gene from *A. pullulans* strain PL5 was cloned, characterized and its antifungal activity and role in the biocontrol were elucidated. This aspect could greatly enhance postharvest biocontrol of fungal pathogens, by improving the selection of antagonists with a protease activity.

2. Materials and methods

2.1 Microorganisms, fruit, vectors and molecular kits

A. pullulans strain PL5 was isolated from the carposphere of plum cv. Angeleno, harvested in organic orchards located in Piedmont, Northern Italy, and identified by using molecular tools (Zhang et al., 2010a). The strain PL5 was maintained on nutrient yeast dextrose agar (nutrient broth 8 g/L, yeast extract 5 g/L, glucose 10 g/L and agar 20 g/L; NYDA) slants and grown in liquid medium YPD (20 g/L dextro-glucose, 20 g/L peptone casein, 10 g/L yeast extract) at 25°C for 48 h on a rotary shaker (ASAL, Italy) at 200 rpm. Several strains of *B. cinerea*, *P. expansum* and *M. laxa* were isolated from rotted apples, identified by observing their morphology and by sequencing their ITS and 5.8 rDNA regions, and selected for their virulence by inoculation in artificially wounded apples (*M. laxa*, in peaches) and maintained on Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) at 4°C (Zhang et al., 2010a). The conidia were harvested after the fungal strains were incubated on PDA in Petri dishes at 25°C for 7 days. The required concentrations of the pathogen conidia were determined by a Bürker chamber (Knittel, Germany).

The oligonucleotides and pGEM-T vector in this study were obtained from Promega (Madison, WI, USA). *Eschericha coli* strains including DH5α and BL21 as hosts for plasmids were purchased from Invitrogen (Eugene, OR, USA). Bacterial cultures were grown according to the manufacturer's instructions and standard methods. The kits for DNA and RNA manipulations were purchased from Qiagen (Hilden, Germany). The kit "Gene Walking Made Easy" and the materials for enzyme assays were purchased from Sigma-Aldrich (Italy).

2.2 Pathogen mycelium inhibition by *A. pullulans* PL5 and its crude secreted enzymes

The interactions between the antagonist and the pathogen hyphae were assessed in Petri dishes containing PDA for *B. cinerea* and *P. expansum* or peach juice agar for *M. laxa*. The plates were divided into three sections, 30 mm wide each, along the diameter. Pathogen mycelial plugs (5 mm in diameter) were corked from a 4 day old PDA culture plate and fixed upon one point on the agar surface. After 48h at 25±2°C, 50 µL aliquots of the antagonist cell suspension containing 10^8 cells/mL were streaked onto PDA and peach juice agar at 30 mm from the pathogen plug (Spadaro et al., 2002). The dual cultures were washed with deionized water for about 2 min after 48h co-culture at 25±2°C. The interactions of the antagonist and pathogen were directly observed under light microscope (Axioskop 40, Germany). The experiments were repeated twice.

Purification of hydrolase proteins was carried out according to Tseng et al. (2008). Ammonium sulphate (Merck, Germany) was added to the antagonist *A. pullulans* PL5 culture supernatant and stirred at 4°C for overnight to give up to 75% saturation. Following centrifugation at 18,000 g for 30 min at 4°C, the precipitated protein pellets were resuspended in 3 mL deionized water. A 0.1% protease inhibitor cocktail (Sigma, St Louis, MO, USA) was added, and samples were dialyzed (dialysis membrane molecular weight cut off =12,000 kDa) against a 100-fold volume of deionized water at 4°C for 24 h and then stored at -20°C for further studies.

To assess the activity of the purified enzyme against the pathogens, *B. cinerea* was selected for its proper time of growth, not as fast as *P. expansum*, and not as slow as *M. laxa*. The pathogen was incubated on PDA in Petri dish at 25°C for 4 days, and then two small wells were made near the pathogen mycelia by removing the medium with a cork. A 100 µL purified enzyme solution was added into one well and 100 µL uncultured medium was added into the other well, serving as a control (CK). After incubation for two days, the inhibition of the pathogen mycelia was investigated.

2.3 Determination of protease activity produced by *A. pullulans* PL5

Proteolytic activity produced by *A. pullulans* PL5 was assayed against azocasein as described by Inamura et al. (1985) with small modifications. Briefly, a linear rate of increase was obtained up to an A_{440} of approximately 0.2. One unit of protease activity was reported as an amount which gave an absorbance of 0.001 at 440 nm.

2.4 Isolation of DNA and RNA from *A. pullulans* strain PL5

Two ml of YPD culture of the strain PL5, grown at 25°C for 48 h, were centrifuged at 5,000 g for 10 min. DNA was extracted from the pellets with the DNeasy® extraction kit and RNA was extracted with the RNeasy® extraction kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purification and concentrations were checked by the ratio of 260/280 nm and the absorbance values at 260 nm with the spectrophotometer (Nanodrop 2000, Italy). Purified DNA and RNA were stored in TE buffer (10 mM Tris-HCl; 0.1 mM EDTA; pH 8.0) at -20°C for further studies.

2.5 Cloning the protease genes from the genomic DNA of *A. pullulans* strain PL5

Degenerate primers for amplifying the partial sequences of protein genes were designed according to the conserved protein sequences (GHGTHVAGT and TATNTISGTSMA) of alkaline proteases already published (Fig. 1). The forward degenerate primer was designed as 5'-GNCANGGNCANCANGTNGCNGGNAC-3' and the reverse primer as 5'-CATRCTNGTNCCRCTDATNGTRTTNG-3'. The PCR reaction system (20 µL) was prepared by mixing 1 µL of DNA template (50 ng), 200 mM of each deoxynucleotide triphosphate (dNTPs), 2 µL

of 10 X buffer (Taq DNA Polymerase, Qiagen, Hilden, Germany), 0.7 mM each degenerate primer, and 1.0 U Taq DNA Polymerase. The conditions of PCR program were as followings: 95°C, 3 min; 32 cycles: 94°C, 15 s; from 55 to 35°C (-0.5°C as gradients), 45 s; 72°C, 30 s; 72°C, 10 min; 4°C. The PCR products were subjected to agarose (2.0%, w/v) gel electrophoresis in TBE buffer stained with SYBR-safe® (Invitrogen, Eugene, OR, USA), followed by excising from the gel and purifying by QIAquick gel extraction kits (Qiagen, Hilden, Germany). The purified fragments were ligated into pGEM-T cloning vector before transformation into chemically competent cells of *E. coli* strain DH5α. Transformants were grown at 37°C on Luria Broth (LB) agar containing ampicillin (100 µg/mL), IPTG (50 mM) and X-gal (80 µg/mL) for blue / white screening of recombinant colonies. Transformed *E. coli* strains were confirmed for the presence of insert using universal primers (M13F and M13R).

To amplify and identify 5' and 3' flanking regions of the alkaline protease gene from the genomic DNA of *A. pullulans* strain PL5, special primers and restriction digestion enzymes were designed according to the obtained sequences and the chemical kit "Gene Walking Made Easy" (UVS1, Sigma, St Louis, MO, USA). The specific process was performed according to the supplier's instructions. The fragments from Vectorette *Cla* I and *EcoR* I library of *A. pullulans* strain PL5 were purified and ligated to pGEM-T cloning vector, followed by sequencing as described above. The obtained sequences were then subjected to BLAST and ORF Finder program at NCBI to assemble the whole sequence of the targeted gene.

2.6 Cloning the protease gene from the cDNA of *A. pullulans* strain PL5

In order to amplify the gene encoding alkaline protease by PCR, the forward primer (Pf) was designed as 5'-ATGTGGAAGAAGAGTGTTGC-3', and reverse primer (Pr) as 5'-TAACGACCG

CTGTTGTTGTAAAC-3' according to the whole sequence of the protease gene obtained from the genomic DNA. The first cDNA was synthesized using Reverse Transcript kits (Qiagen, Hilden, Germany) according to the manufacturer instructions. The PCR reaction system (50 µL) was composed by 5.0 µL 10X buffer, 4.0 µL (2.5 mM) dNTPs, 1.0 µL (50 mM) Pf, 1.0 µL (50 mM) Pr, 1.0 U Taq DNA Polymerase, 2.0 µL (10.0 ng ml^{-1}) cDNA and 36.0 µL H_2O. The conditions of PCR program were as follows: 94°C, 3 min; 35 cycles: 94°C, 30 s; 58°C, 45 s; 72°C, 90 s; 72°C, 10 min; 4°C. To sequence the gene obtained from cDNA, the PCR fragments were ligated into pGEM-T vector and transformed into *E. coli* strain DH5α, as described above.

2.7 DNA sequence and computer analysis

BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for the nucleotide sequence analysis, deduction of the amino acid sequence and database searchers. Multiple sequence alignments of DNA and amino acid sequence were performed using the programs of DNA-MAN 6.0 and Clustal W (http://www.ebi.ac.uk/Tools/es/Cgi-bin/clustalw2). The phylogenetic tree was generated using ClustalX-2.09 and MEGA-4.1 by neighbour-joining method.

2.8 Construction of plasmids and expression of the *ALP5* gene in *E. coli* BL21

To further characterize the alkaline protease encoded by the *ALP5* gene, the gene was transformed into *E. coli* BL21 (DE3) and induced with IPTG. To amplify the *ALP5* fragment without signal peptide or terminator, the primers PF-*Eco*R I 5'-G/AATTCGCTGCTCCCGCCATTGAC -3' (bases underlined encode *Eco*R I restriction site) and PR-*Hin*d III 5'-A/AGCTTGCGACCGCTGTTGTTGTAAAC-3' (bases underlined encode *Hin*d III restriction site)

were synthesized. The amplicons without stop codons or signal peptides were ligated into pET-23a(+) with restriction sites of *Eco*R I and *Hin*d III to generate the construct of pET-23a(+)-*ALP5*. The expression construct was then inserted into *E. coli* BL21 (DE3). The transformants harbouring the plasmid pET-23a (+) served as controls. The transformants were screened on Luria-Bertani (LB) broth containing 100 μg/mL ampicillin and incubated on a rotary shaker (150 rpm) at 37°C until OD_{600nm} reached 0.4-0.8, followed by induction with IPTG (final concentration 1.0 mM). The bacterial strains were then grown at 37°C for 5-6 h. Diagrammatic representation of the expression of *ALP5* in *E. coli* BL21 is shown in Fig. 2.

2.9 SDS-PAGE, western-blotting and purification of the recombinant protease

To characterize the recombinant proteases produced by the transformants of pET-23a(+)-*ALP5*, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sambrook and Russell (2001). The bacterial strains were grown and induced with IPTG as described above. The recombinant proteases (6×His-tagged fusion proteins) were then purified by affinity chromatography with the kit of Ni-NTA (No. 102, Qiagen GmbH) according to the supplier's instructions. For SDS-PAGE analysis, the concentration of stacking gel was 7% and the concentration of separating gel was 12%. To further confirm the specific protein expressed by the *ALP5* gene, western-blotting with monoclonal anti-polyhistidine as primary antibody and A1293-AlkPhos APA Mouse Fab ads HIgG as secondary antibody was carried out by the methods described by Sambrook and Russell (2001).

2.10 Effects of temperature and pH on the activity and stability of the recombinant protease

ALP5

The effect of temperature on the activity of recombinant alkaline serine protease was determined by incubating the recombinant enzyme between 20-60°C using the standard assay conditions used for the determination of the protease activity. In a similar way, the effect of pH values on the recombinant protease activity was investigated by incubating the recombinant enzyme at different pH values (4.0-12.0). The relative activity at different pH values and temperatures was calculated when the recombinant ALP5 activity obtained at the pH of 10.0 and the temperature of 50°C was used as a reference (relative activity was 100%).

The effect of temperature on the stability was examined by pre-incubating the enzyme at different temperatures (0-60°C) for 24 h. The residual activity was measured immediately as described above. The sample pre-incubated at 4°C was used as a reference (relative activity was 100%). The effect of pH value on the stability was tested by 24 h pre-incubation of the recombinant enzyme in 0.05 M potassium phosphate buffer (pH 4.0-12.0). The remaining activities of the recombinant enzyme were measured immediately after this treatment with the standard method as described above. The relative activity was calculated when the recombinant enzyme activity obtained at pH 7.0 pre-incubation was used as a reference (relativity activity was 100%). Three replicates for each treatment were prepared.

2.11 Antifungal activity of the recombinant protease ALP5 against the postharvest pathogens

The antifungal activity of the recombinant protease ALP5 in controlling the growth of *B. cinerea, P. expansum and M. laxa* was assessed in PDB (Potato Dextrose Broth, Merck) under sterile conditions as described by Lorito et al. (1993), with small modifications. The recombinant proteases were obtained and purified as described before. The protein concentration of the purified enzyme solution

was determined according to Bradford (1976) by using bovine serum albumin (A1933, Sigma) as a standard. Aliquots (300 µL) of each pathogen conidial suspension (1×10^6 conidia/mL) in Ringer solution were transferred to tubes containing 2.4 mL PDB. Then 300 µL of the purified recombinant enzyme solution (80 µg/mL) was added to the tubes and reached a final concentration of 8 µg/mL PDB, serving as enzyme treatment (Pathogen+ Enzyme). Aliquots (300 µl) of inactivated enzymes, by boiling at 100°C for 10 min, or sterile distilled water instead of the enzyme solution were added to the tubes and served as controls (Pathogen+ Inactivated enzyme, and Pathogen+ Water). After 18 h incubation of the 45° sloping tubes at 25°C on a rotary shaker (200 rpm), 100 conidia per replicate were observed microscopically and their germination rate (%) and germ tube length (µm) were measured. Three replications of three tubes were prepared for each treatment and the experiment was repeated twice.

2.12 Statistical analysis

All the experiments were performed at least twice. Data from all the experiments were analyzed using analysis of variance (ANOVA) and the SPSS version 12.0 (SPSS, 1989-2003). The means were separated at 5% significance level, by using Duncan's Multiple Range Test (DMRT).

Results

3.1 Pathogen mycelium inhibition by *A. pullulans* PL5 and its crude secreted enzymes

After 7 days co-culture in PDA plates at 25±2°C, direct interaction *in vitro* was observed (Fig. 3 a, b, c). *A. pullulans* PL5 significantly inhibited the hyphae elongation of the pathogens. However, no attachment of fungal hyphae was observed.

After 2 days co-culture in the presence of purified crude enzymes produced by *A. pullulans* PL5 in PDA plates at 25±2°C, an inhibition of the pathogen mycelia was observed. However, when incubation was without crude enzymes (CK), the mycelium of *A. pullulans* PL5 grew normally (Fig. 3 d).

3.2 Production of alkaline protease by *A. pullulans* PL5 and its activity

As shown in Fig. 4, *A. pullulans* PL5 produced alkaline protease with high activities when grown in LBMS medium with *M. laxa*, *B. cinerea* or *P. expansum* cell walls as sole carbon sources. When *A. pullulans* PL5 was cultured in the LBMS medium, at 0 h incubation, no alkaline protease was detected, while at 24 h incubation, a protease activity was detected. At 72 h, the maximum level of protease activity was observed. At 96 h incubation, the activity began decreasing, after its maximum level. Similar pattern of alkaline protease activity from 0 h to 96 h incubation was observed when *A. pullulans* PL5 was grown in minimal salt medium with *M. laxa*, *B. cinerea* or *P. expansum* cell walls as sole carbon sources, and the maximum activities in the three different LBMS medium were 116, 121 and 129 U/mg protein, respectively.

3.3 Cloning alkaline protease genes from genomic DNA and cDNA of *A. pullulans* PL5

To clone the partial sequences of the gene encoding extracellular alkaline protease in *A. pullulans* PL5, degenerate primers were designed according to the conserved amino acid sequences of alkaline proteases from different species of the yeast (Fig. 1). PCR amplification produced a reliable fragment of about 600 bp (data not shown). Analysis of the sequence by BLAST program suggested that the fragment showed a high identity (87%) to the alkaline serine protease gene *ALP2*, suggesting that the

putative alkaline protease gene was isolated. New primers were designed according to the sequence of the obtained fragment (600 bp) of the putative alkaline protease gene in combination with the kit "Gene Walking Made Easy". The fragments obtained were sequenced, assembled, and analysed with BLAST and ORF Finder programs. Finally, the whole gene of 1,351 bp (GenBank accession number HQ113463) encoding the alkaline protease, named as *ALP5*, was cloned from the genomic DNA of the antagonistic yeast strain PL5 (Fig. 5 and 6).

After PCR with the cDNA of the strain PL5 as template and the primers specific to *ALP5* gene, a specific fragment of 1,248 bp was produced (Fig. 5 and 6). The *cDNAALP5* sequence presented 100% identity to the corresponding coding sequence of the genomic DNA. The organization of the gene is illustrated in Fig. 6. Comparison of *ALP5* gene with *cDNAALP5* revealed that there are two introns inside *ALP5* gene. One intron was of 54 bp, while the other was of 50 bp in size. The two introns divided the open reading frame of *cDNAALP5* into three parts. The size, percentage of (G+C) and sequence characteristics of the introns were displayed in Table 1. The flanking sequences of the second intron are consistent with the typical eukaryotic splice sites, including an invariant GT at 5 prime and an invariant AG at 3 prime of the introns, while the first intron had GA at its 5 prime and GT at its 3 prime that are non-typical eukaryotic splice sites (Table 1).

3.4 Protease ALP5 of *A. pullulans* PL5 and phylogenetic analysis

As seen in Fig. 6, an open reading frame of 1,248 bp encoding a 415-amino acid (aa) protein with a calculated molecular weight (M_r) of 42.9 kDa and an isoelectric point (pI) of 4.5 was characterized. The protease ALP5 had a 18 amino acid signal peptide (positions from 1 to 18), one *N*-gylcosylation, one histidine active site, and one serine active site, suggesting that the protease ALP5 was an alkaline serine protease.

To reveal the relationship of the protease ALP5 to proteases from other microorganisms, protein BLAST and phylogenetic analysis were performed. As seen in Fig. 7, ALP5 and selected proteases clustered into three groups. Among these proteases, ALP5 is close to proteases from *Aspergillus* spp. and moreover, ALP5 deduced amino acid sequence shared 100% homology to the protease ALP2 (Genbank accession number: ABW86845.1) of the sea yeast, *A. pullulans* strain HN2-3, which has been known as an alkaline serine protease, confirming that the protein ALP5 was alkaline serine protease.

3.5 SDS-PAGE, western-blotting and purification of the recombinant proteases

As seen in Fig. 8a, SDS-PAGE showed that one specific recombinant protein at the size of about 43 kDa was produced from the positive transformants harbouring the gene *ALP5* as compared to controls without containing the gene *ALP5*. The size of the recombinant proteins was the same as calculated from the deduced amino acids of the protease gene *ALP5*. Western-blotting analysis of the proteins from the positive transformants with his-tag monoclonal antibody showed that one specific protein band of the same size obtained by SDS-PAGE was identified (Fig. 8b), indicating that the recombinant protein was fused with His-tag. The recombinant proteases were purified with Ni-NTA affinity chromatography and were subjected to SDS-PAGE analysis. Results showed that one specific band with the molecular weight of about 43 kDa was identified (Fig. 8c), suggesting that the protein was the recombinant ALP5. In addition, the results of Fig. 8a and Fig. 8c revealed that the recombinant protein had an apparent molecular mass of 43 kDa.

3.6 Effects of temperature and pH values on the recombinant ALP5 activities

The effect of temperature on the recombinant ALP5 activity was investigated when the enzyme reactions were performed at temperatures from 20°C to 60°C. As seen in Fig. 9a, the highest activity of the recombinant ALP5 was observed when the enzyme reaction was performed at 50°C. During the range of 20-50°C, the activity increased with an increase of the reaction temperature. But when the enzyme reaction temperature exceeded 50°C, the activity started decreasing, indicating that the optimal temperature of the recombinant ALP5 activity was 50°C. Similarly, the optimal pH of the activity was also determined. As seen in Fig. 9b, the highest activity was detected when the reaction system was at pH 10.0. At pH 4.0, the recombinant protein showed the lowest activity as compared to those at other pH values.

3.7 Effects of temperature and pH values on the recombinant protease ALP5 stability

The recombinant protease ALP5 showed the highest residual activity when pre-incubated at pH 7.0 and 4°C (Fig. 10). Like other enzymes, the recombinant protease ALP5 showed a good stability when pre-incubated at lower temperatures (Temperature≤ 10°C) (Fig. 10a). Moreover, when pre-incubated at 20-50°C, the recombinant ALP5 still showed a high residual activity in hydrolysing the substrate (Fig. 10 a). However, when pre-incubated at 60°C, the residual activity dramatically decreased. These results suggested that the recombinant enzyme had a good thermal stability. As shown in Fig. 10b, when the recombinant ALP5 was pre-incubated at the acid pH values (pH≤ 5.0) or at the extremely strong alkaline pH values (pH≥ 11.0), residual activities were lower as compared to that at pH 7.0-8.0, indicating that the recombinant ALP5 showed a poor stability at acidic or at extremely alkaline conditions.

3.8 Antifungal activity of the recombinant protease in controlling the growth of pathogens

By co-culturing in liquid medium (PDB), the activity of the recombinant enzyme APL5 in controlling the conidial germination and germ tube length of *B. cinerea*, *P. expansum* and *M. laxa* was investigated (Table 2). No significant differences in germination percentage were observed on *B. cinerea* or *P. expansum* treated by the recombinant protease, however, the germination percentage of the pathogen *M. laxa* was significantly reduced from 98% to 50% in presence of the recombinant ALP5. This result suggested that the responses to the lytic activity of the enzymes are various with different pathogens. Among all treatments by the recombinant protease, the germ tube length of the pathogen conidia was significantly lower as compared with those of controls. In the presence of the recombinant protease ALP5, the germ tube lengths of *B. cinerea*, *P. expansum* and *M. laxa* were 68, 80 and 60 µm, respectively, while the germ tube length of their controls were 213, 186 and 125 µm, respectively. These results indicated that the recombinant protease ALP5 is highly active in reducing the pathogen spore germination and germ tube elongation.

4. Discussion

The strain PL5 of *A. pullulans* secreted several enzymes with hydrolytic activity (Zhang et al., 2010a). Protease was among them and of high activity. In the present investigation, the role of the protease enzyme produced by the antagonist *A. pullulans* PL5 in controlling pathogens was studied. Moreover, an alkaline serine protease gene was successfully cloned from the antagonist and expressed in *E. coli* BL21. Understanding the modes of action is essential for developing appropriate commercial formulations and application methods to maximize the potential use of microbial biocontrol agents (Janisiewicz and Korsten, 2002). Production of lytic enzymes, especially glucanase, chitinase and

protease, has been proposed as an important mode of action of biocontrol agents against pathogens (Castoria et al., 1997; Chan and Tian, 2005). Bar-Shimon et al., (2004) studied the role of exo-b-1,3-glucanase (*CoEXG1*) in the biocontrol activity of *Candida oleophila* using the gene-knockouts and over-producing transformants, and found that, compared to the wild type of yeasts, the biocontrol activity of CoEXG1-over-producing yeasts was not significantly increased but the activity of *CoEXG1*-knockouts yeasts was significantly decreased, suggesting that the production of exo-glucanase is involved in the biocontrol of pathogens. This study demonstrated that the antagonist *A. pullulans* PL5 produced the high activities of extracellular proteases which significantly inhibited the mycelial growth of pathogens. In addition, it was also demonstrated that the recombinant protease PL5 expressed in *E. coli* BL21 inhibited the pathogen mycelial growth *in vitro*. These results provided the direct evidence that production of protease plays an important role in the activity of *A. pullulans* PL5 against pathogens. On the other hand, the elucidation of some gene functions is useful for the registration of BCAs since the more information on BCAS is supplied, the easier their applications are accepted by their consumers. Therefore, cloning and characterization of the protease genes from the antagonistic yeast, *A. pullulans* PL5 is important to the registration and application of the yeast. This discovery could greatly enhance biocontrol of postharvest diseases, by improving the future selection of new antagonists characterized by a protease activity against the fungal pathogens.

Microbial proteases are classed into various groups, based on their activity under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme. Among them, the alkaline serine proteases, which are active in a neutral to alkaline pH range and have a serine center, are the most important group of enzymes exploited commercially (Gupta et al., 2002). Although proteases from bacteria (Gupta et al., 2002), and fungi (Macchione et al., 2008; Morita et

al., 1994) have been extensively investigated and used in different fields, only a few studies were performed on the proteases and the protease-related genes from the yeasts. In this study, the protease gene *ALP5* was cloned and characterized from the strain PL5 of *A. pullulans*, and the protease encoded by the gene *ALP5* belongs to alkaline serine protease family.

The protease from the terrestrial yeast, *A. pullulans* PL5 shows an extremely high homology to that from the sea yeast, *A. pullulans* strain HN2-3, suggesting that yeast enzyme genes are relatively conserved among strains of the same species. Although the protease gene is not easy to be expressed in prokaryotes, such as *E. coli*, as compared to eukaryotes, such as *Pichia pastoria* and *Saccharomyces cerevisiae*, we successfully cloned and expressed the protease gene *ALP5* in *E. coli* BL21 (DE3), without the signal peptide, in order to reduce the toxic effects (lytic activity) of the active recombinant proteases on the growth of the bacterial host. The recombinant ALP5 expressed from *E. coli* showed lower activity than the native protease secreted by *A. pullulans* PL5, which maybe resulted from the fusion of his-tag with the recombinant protease and the renaturation of extracted proteins from conclusions. Previous studies (Moser et al., 1994) revealed that the refolded recombinant protease had a lower level of proteolytic activity compared with that of the native protease and proposed that the decrease in activity was most probably caused by refolding problems related to leader sequences present in the proteases.

The activity and stability are the most important attributes of enzymes. In this study, the optimal temperature and pH values of the recombinant protease ALP5 were determined. In addition, the effects of temperatures and pH values on the stability of the recombinant enzyme were investigated. It was found that the recombinant enzyme performed the highest activity in hydrolysing substrate when the reaction was at 50°C and pH of 10.0. These results observed on the terrestrial yeast, *A. pullulans*

458 strain PL5 were very similar to those on the sea yeast, *A. pulluans* strain HN2.3 (Ni et al., 2009) and
459 on the bacterium *Bacillus* sp. 103 (Joo et al., 2004), suggesting that most of alkaline protease from
460 different microorganisms shared similar optimal temperature and pH values. Actually, the optimum
461 temperature at 40-60°C and the high optimum pH of the enzyme activity is typical characteristics of
462 the most alkaline proteases (Gupta et al., 2002; Mehrotra et al., 1999; Ni et al., 2009).
463 In conclusion, the yeast *A. pullulans* strain PL5 produced high activity of proteases, which played an
464 important role in controlling fungal pathogens，and the protease gene *APL5* of *A. pullulans* strain PL5
465 was cloned and successfully expressed in *E. coli* BL21. ALP5 was identified as an alkaline serine
466 protease, with an important role in the biocontrol of postharvest pathogens of apples and peaches.
467 Future studies involve the use of protease gene-knockouts or gene-over-expressing transformants, to
468 better understand the role of the protease in the biocontrol activity.
469

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476

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625 **Tables**

626 **Table 1**

627 Size, percentage (G+C) and sequence characteristics of the introns of *ALP5* gene. The splice sites cleaving

628 the gene was marked with "/" and the flanking nucleotides of the introns were highlighted with a box.

Intron	Flanking sequences of splice-sites	Size(bp)	G+C(%)
1	AGGTgagtcttggtcaactataatatctagtcatcaaccatactaattacaaacaggtCGCTG	54	46.3%
2	TGGAgtaagattcttttctttcattatgtcagccacatactaacaacgccatagTCCAGT	50	46.0%

Table 2

Antifungal activity of the recombinant protease ALP5 in controlling the growth of *B. cinerea*, *P. expansum* and *M. laxa in vitro*. The pathogen conidia at 1×10^5 conidia/mL were co-cultured with the recombinant protease at 8 μg/mL in PDB containing 100 μg/mL ampicillin, serving as the enzyme treatment (Pathogen+ Enzyme), and inactivated enzymes or sterile distilled water instead of the enzyme solution served as controls (Pathogen+ Inactivated enzyme, and Pathogen+ Water). Three replications of three tubes were prepared for each treatment and the experiment was repeated twice.

Treatments	*B. cinerea*		*P. expansum*		*M. laxa*	
	GTL (μm) **	GP (%) **	GTL (μm) **	GP (%)**	GTL (μm) **	GP (%) **
Pathogen+enzyme	68 ± 9.0 a	97 ± 0.6 a	80 ± 9.0 a	97 ± 0.6 a	60 ± 6.6 a	50 ± 2.8 a
Pathogen+inactivated enzyme	210 ± 15.6 b	99 ± 2.0 a	188 ± 16.5 b	100 ± 0.0 a	118 ± 12.4 b	96 ± 1.0 b
Pathogen+water (Control)	213 ± 10.0 b	99 ± 0.8 a	186 ± 18.8 b	100 ± 0.0 a	125 ± 12.6 b	98 ± 0.6 b

** GTL: Germ tube length; GP: Germination percentage.

The results are the mean of two independent experiments. "±" stands for standard error of the means. Values of each column followed by different letters show significant difference ($P<0.05$) according to analysis by Duncan's Multiple Test (SPSS 13.0).

Figures

Figure 1

Alignment of the deduced amino acid sequence of five alkaline serine protease genes retrieved from NCBI database: *Aureobasidium pullulans* alkaline serine protease (ABW86845.1); *Aureobasidium pullulans* alkaline serine protease ALP2 (ABP82774); *Neosartorya fischeri* NRRL181 alkaline serine protease Alp1 (XP_001266852.1); *Aspergillus fumigatus* Af293 alkaline serine protease Alp1 (XP_751651.1); *Aspergillus fumigatus* uncleaved alkaline protease (ALP) (CAA77666.1); Multiple sequence alignment of proteins was performed by using the DNAMAN 6.0. Identical residues are highlighted by black boxes. The potential conserved region (GHGTHVAGT and TATNTISGTSMA) marked with asterisks were used for designing the degenerate primers for amplifying the partial sequence of alkaline serine protease gene from *A. pullulans* strain PL5.

Figure 2

Diagrammatic representation of the construction of plasmid (pET23a-*ALP5*) and expression of *ALP5* in *E. coli* BL21.

Figure 3

Interaction of *A. pullulans* PL5 and three postharvest pathogens *in vitro*, and inhibition of *B. cinerea* growth by purified extracellular crude enzymes produced by *A. pullulans* PL5: a) interaction of *A. pullulans* PL5 and *B. cinerea*; b) interaction of *A. pullulans* PL5 and *M. laxa*; c) interaction of *A. pullulans* PL5 and *P. expansum*; d) inhibition of *B. cinerea* hyphal growth by purified extracellular crude enzymes

produced by *A. pullulans* PL5.

Figure 4

Extracellular protease activities (EC. 3.4.21.63) of *A. pullulans* PL5 grown in LBMS medium supplemented with 2 mg ml^{-1} CWP (cell wall preparation of each pathogen: *M. laxa*, *B. cinerea*, or *P. expansum*) as sole carbon source for 96 h at 25°C. Bars represented standard deviations of the means.

Figure 5

Amplification of the protease genes from cDNA and genomic DNA of *A. pullulans* PL5 by PCR. Line M is DNA molecular standards (Cat. No. 239085, Qiagen); Lane1 is the PCR product with cDNA of *A. pullulans* PL5 as template; Line2, the PCR product with genomic DNA as template; Line3, Blank control.

Figure 6

Nucleotide sequence of the alkaline serine protease *ALP5* gene of *A. pullulans* PL5and deduced amino acid sequence of the gene product. The start codon is in bold and the signal peptide was highlighted with the inverted arrows. *N*-glycosylation site, histidine active site and serine active site were underlined with the single line, respectively. The stop codon is marked with the asterisk. Two introns were highlighted with lower case letters in grey color, respectively. The gene was designated as *ALP5* and the sequence has been submitted to NCBI (accession number HQ113460).

Figure 7

Phylogenetic analysis, based on the deduced amino acid sequences, of ALP5 and closely related proteases,

performed using the program MEGA 4.1. The numbers at node indicate the bootstrap percentages of 1000 resamples. The proteases retrieved from NCBI database are: *Aureobasidium pullulans* alkaline serine protease (ABW86845.1); *Neosartorya fischeri* NRRL181 alkaline serine protease Alp1 (XP_001266852.1); *Aspergillus fumigatus* Af293 alkaline serine protease Alp1 (XP_751651.1); *Aspergillus fumigatus* serine proteinase (AAB07672.1); *Aspergillus fumigatus* uncleaved alkaline protease (ALP) (CAA77666.1); *Aspergillus clavatus* (ACX47962.1) alkaline protease; *Aspergillus clavatus* NRRL1 alkaline serine protease Alp1 (XP_001272038.1); *Trichoderma hamatum* (AAP15044.1) alkaline proteinase; Aspergillus oryzae RIB40 hypothetical protein (XP_001820144.1); *Aspergillus oryzae* preproalkaline protease (CAA38527.2); *Hypocrea virens* extracellular serine protease (AAO63588.1); *Aspergillus fumigatus* (CAA75804.1) alkaline protease; *Aspergillus fumigatus* (CAA75806.1) alkaline protease; *Aspergillus versicolor* extracellular alkaline serine protease (ADE74975.1); *Aspergillus* sp. MK285 alkaline protease (AAT85628.1); *Aspergillus viridinutans* alkaline protease (AAT85626.1); *Aspergillus viridinutans* alkaline protease (AAT85627.1); *Verticillium albo-atrum* VaMs.102 alkaline proteinase (XP_003004413.1).

Figure 8

a) SDS-PAGE analysis of expression of *ALP5* in *E. coli* BL21 (DE3): line 1and 2, total protein extracts from IPTG-induced transformants harboring the plasmid pET23a-ALP5; line 3, protein extracts from *E. coli* cell containing empty vector pET23a; line M, protein molecular weight markers; b) Western-blotting confirmation of recombinant protein with monoclonal anti-polyhistidine as first antibody and A1293-AlkPhos APA Mouse Fab ads HIgG as second antibody: line 4, protein extracts from IPTG -induced transformants harboring the plasmid pET23a-ALP5; c) Line 5 and 6, recombinant alkaline

703 protease purified to homogeneity by affinity chromatography**.**

704

705 **Figure 9**

706 Effects of temperature (a) and pH (b) on the activity of recombinant alkaline protease assay. The activity
707 was measured and determined as described in the section 2. 10. Three replicates of each treatment were
708 prepared. Data are presented as means ± SD, n=3.

709

710 **Figure 10**

711 Effects of temperature (a) and pH (b) on the activity of recombinant alkaline protease assay. The effect of
712 temperature on the stability was examined by pre-incubating the enzyme at different temperatures (0-60°C)
713 for 24 h. The residual activity was measured and determined as described in the section 2. 10. Three
714 replicates of each treatment were prepared. Data are presented as means ± SD, n=3.