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

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23   **Cloning, characterization, expression and antifungal activity of an alkaline serine protease of**

24   *Aureobasidium pullulans* **PL5 involved in the biological control of postharvest pathogens**

25

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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).

104   Besides their particular role in degrading cell wall of phytopathogenic fungi, proteolytic enzymes

105   occupy the most relevant position among industrial enzymes and are very important in digestion as

106   they breakdown the protein foods to liberate the amino acids needed by the body (Feijoo-Siota and

107   Villa, 2011). Moreover, proteolytic enzymes have been used for a long time in various forms of

108   therapy. Their use in medicine is gaining more and more attention as several clinical studies are

109   indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune

110   regulation (Hooper 2002; Silva and Malcata, 2005). In addition, the enzymatic hydrolysis of proteins

111   by using proteases is still one of the most important means to produce bioactive peptides (Kristinsson

112   and Rasco, 2000). Therefore, it is very important to seek for new protease-producing microbial

113   sources and to develop some new proteases. So far, the most commonly used proteases are obtained

114   from *Bacillus* sp., lactic bacteria, and marine yeasts (He et al., 2006; Ma et al., 2007; Minervini et al.,

115   2003; Okamoto et al., 1997). However, no report on protease-related genes obtained from terrestrial

116   yeasts was published.

117   The antagonistic yeast-like fungus *A. pullulans* strain PL5 isolated from the carposphere of fruits

118   showed a high efficacy in controlling *M. laxa* on stone fruits and *B. cinerea* as well as *P. expansum* on

119   pome fruits under semi-commercial conditions (Zhang et al., 2010a, 2010b). In our previous study, the

120   strain PL5 exhibited a high capability of producing protease activity which effectively hydrolysed the

121   substrate casein. Therefore, in this paper, the protease gene from *A. pullulans* strain PL5 was cloned,

122   characterized and its antifungal activity and role in the biocontrol were elucidated. This aspect could

123   greatly enhance postharvest biocontrol of fungal pathogens, by improving the selection of antagonists

124   with a protease activity.

125

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

148   The interactions between the antagonist and the pathogen hyphae were assessed in Petri dishes

149   containing PDA for *B. cinerea* and *P. expansum* or peach juice agar for *M. laxa*. The plates were

150   divided into three sections, 30 mm wide each, along the diameter. Pathogen mycelial plugs (5 mm in

151   diameter) were corked from a 4 day old PDA culture plate and fixed upon one point on the agar

152   surface. After 48h at $25\pm2°C$, 50 µL aliquots of the antagonist cell suspension containing 10^8 cells/mL

153   were streaked onto PDA and peach juice agar at 30 mm from the pathogen plug (Spadaro et al., 2002).

154   The dual cultures were washed with deionized water for about 2 min after 48h co-culture at $25\pm2°C$.

155   The interactions of the antagonist and pathogen were directly observed under light microscope

156   (Axioskop 40, Germany). The experiments were repeated twice.

157   Purification of hydrolase proteins was carried out according to Tseng et al. (2008). Ammonium

158   sulphate (Merck, Germany) was added to the antagonist *A. pullulans* PL5 culture supernatant and

159   stirred at 4°C for overnight to give up to 75% saturation. Following centrifugation at 18,000 g for 30

160   min at 4°C, the precipitated protein pellets were resuspended in 3 mL deionized water. A 0.1%

161   protease inhibitor cocktail (Sigma, St Louis, MO, USA) was added, and samples were dialyzed

162   (dialysis membrane molecular weight cut off =12,000 kDa) against a 100-fold volume of deionized

163   water at 4°C for 24 h and then stored at -20°C for further studies.

164   To assess the activity of the purified enzyme against the pathogens, *B. cinerea* was selected for its

165   proper time of growth, not as fast as *P. expansum,* and not as slow as *M. laxa*. The pathogen was

166   incubated on PDA in Petri dish at 25°C for 4 days, and then two small wells were made near the

167   pathogen mycelia by removing the medium with a cork. A 100 µL purified enzyme solution was

168   added into one well and 100 µL uncultured medium was added into the other well, serving as a

169   control (CK). After incubation for two days, the inhibition of the pathogen mycelia was investigated.

170

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.

176

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.

184

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

9

192 of 10 X buffer (Taq DNA Polymerase, Qiagen, Hilden, Germany), 0.7 mM each degenerate primer,

193 and 1.0 U Taq DNA Polymerase. The conditions of PCR program were as followings: 95°C, 3 min; 32

194 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

195 PCR products were subjected to agarose (2.0%, w/v) gel electrophoresis in TBE buffer stained with

196 SYBR-safe® (Invitrogen, Eugene, OR, USA), followed by excising from the gel and purifying by

197 QIAquick gel extraction kits (Qiagen, Hilden, Germany). The purified fragments were ligated into

198 pGEM-T cloning vector before transformation into chemically competent cells of *E. coli* strain DH5α.

199 Transformants were grown at 37°C on Luria Broth (LB) agar containing ampicillin (100 µg/mL),

200 IPTG (50 mM) and X-gal (80 µg/mL) for blue / white screening of recombinant colonies.

201 Transformed *E. coli* strains were confirmed for the presence of insert using universal primers (M13F

202 and M13R).

203   To amplify and identify 5' and 3' flanking regions of the alkaline protease gene from the genomic

204 DNA of *A. pullulans* strain PL5, special primers and restriction digestion enzymes were designed

205 according to the obtained sequences and the chemical kit "Gene Walking Made Easy" (UVS1, Sigma,

206 St Louis, MO, USA). The specific process was performed according to the supplier's instructions. The

207 fragments from Vectorette *Cla* I and *EcoR* I library of *A. pullulans* strain PL5 were purified and

208 ligated to pGEM-T cloning vector, followed by sequencing as described above. The obtained

209 sequences were then subjected to BLAST and ORF Finder program at NCBI to assemble the whole

210 sequence of the targeted gene.

211

212 **2.6 Cloning the protease gene from the cDNA of *A. pullulans* strain PL5**

213   In order to amplify the gene encoding alkaline protease by PCR, the forward primer (Pf) was

214 designed as 5'-ATGTGGAAGAAGAGTGTTGC-3', and reverse primer (Pr) as 5'-TAACGACCG

215 CTGTTGTTGTAAAC-3' according to the whole sequence of the protease gene obtained from the

216 genomic DNA. The first cDNA was synthesized using Reverse Transcript kits (Qiagen, Hilden,

217 Germany) according to the manufacturer instructions. The PCR reaction system (50 µL) was

218 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

219 U Taq DNA Polymerase, 2.0 µL (10.0 ng ml^{-1}) cDNA and 36.0 µL H$_2$O. The conditions of PCR

220 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;

221 4°C. To sequence the gene obtained from cDNA, the PCR fragments were ligated into pGEM-T

222 vector and transformed into *E. coli* strain DH5α, as described above.

223

224 **2.7 DNA sequence and computer analysis**

225 BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI)

226 were used for the nucleotide sequence analysis, deduction of the amino acid sequence and database

227 searchers. Multiple sequence alignments of DNA and amino acid sequence were performed using the

228 programs of DNA-MAN 6.0 and Clustal W (http://www.ebi.ac.uk/Tools/es/Cgi-bin/clustalw2). The

229 phylogenetic tree was generated using ClustalX-2.09 and MEGA-4.1 by neighbour-joining method.

230

231 **2.8 Construction of plasmids and expression of the *ALP5* gene in *E. coli* BL21**

232 To further characterize the alkaline protease encoded by the *ALP5* gene, the gene was transformed

233 into *E. coli* BL21 (DE3) and induced with IPTG. To amplify the *ALP5* fragment without signal

234 peptide or terminator, the primers PF-*Eco*R I 5'-G/AATTCGCTGCTCCCGCCATTGAC -3' (bases

235 underlined encode *Eco*R I restriction site) and PR-*Hin*d III

236 5'-A/AGCTTGCGACCGCTGTTGTTGTAAAC-3' (bases underlined encode *Hin*d III restriction site)

237 were synthesized. The amplicons without stop codons or signal peptides were ligated into pET-23a(+)

238 with restriction sites of *Eco*R I and *Hin*d III to generate the construct of pET-23a(+)-*ALP5*. The

239 expression construct was then inserted into *E. coli* BL21 (DE3). The transformants harbouring the

240 plasmid pET-23a (+) served as controls. The transformants were screened on Luria-Bertani (LB) broth

241 containing 100 µg/mL ampicillin and incubated on a rotary shaker (150 rpm) at 37°C until OD_{600nm}

242 reached 0.4-0.8, followed by induction with IPTG (final concentration 1.0 mM). The bacterial strains

243 were then grown at 37°C for 5-6 h. Diagrammatic representation of the expression of *ALP5* in *E. coli*

244 BL21 is shown in Fig. 2.

245

246 **2.9 SDS-PAGE, western-blotting and purification of the recombinant protease**

247   To characterize the recombinant proteases produced by the transformants of pET-23a(+)-*ALP5*,

248 sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as

249 described by Sambrook and Russell (2001). The bacterial strains were grown and induced with IPTG

250 as described above. The recombinant proteases (6×His-tagged fusion proteins) were then purified by

251 affinity chromatography with the kit of Ni-NTA (No. 102, Qiagen GmbH) according to the supplier's

252 instructions. For SDS-PAGE analysis, the concentration of stacking gel was 7% and the concentration

253 of separating gel was 12%. To further confirm the specific protein expressed by the *ALP5* gene,

254 western-blotting with monoclonal anti-polyhistidine as primary antibody and A1293-AlkPhos APA

255 Mouse Fab ads HIgG as secondary antibody was carried out by the methods described by Sambrook

256 and Russell (2001).

257

258 **2.10 Effects of temperature and pH on the activity and stability of the recombinant protease**

259   **ALP5**

260   The effect of temperature on the activity of recombinant alkaline serine protease was determined by

261   incubating the recombinant enzyme between 20-60°C using the standard assay conditions used for the

262   determination of the protease activity. In a similar way, the effect of pH values on the recombinant

263   protease activity was investigated by incubating the recombinant enzyme at different pH values

264   (4.0-12.0). The relative activity at different pH values and temperatures was calculated when the

265   recombinant ALP5 activity obtained at the pH of 10.0 and the temperature of 50°C was used as a

266   reference (relative activity was 100%).

267   The effect of temperature on the stability was examined by pre-incubating the enzyme at different

268   temperatures (0-60°C) for 24 h. The residual activity was measured immediately as described above.

269   The sample pre-incubated at 4°C was used as a reference (relative activity was 100%). The effect of

270   pH value on the stability was tested by 24 h pre-incubation of the recombinant enzyme in 0.05 M

271   potassium phosphate buffer (pH 4.0-12.0). The remaining activities of the recombinant enzyme were

272   measured immediately after this treatment with the standard method as described above. The relative

273   activity was calculated when the recombinant enzyme activity obtained at pH 7.0 pre-incubation was

274   used as a reference (relativity activity was 100%). Three replicates for each treatment were prepared.

275

276   **2.11 Antifungal activity of the recombinant protease ALP5 against the postharvest pathogens**

277   The antifungal activity of the recombinant protease ALP5 in controlling the growth of *B. cinerea, P.*

278   *expansum and M. laxa* was assessed in PDB (Potato Dextrose Broth, Merck) under sterile conditions

279   as described by Lorito et al. (1993), with small modifications. The recombinant proteases were

280   obtained and purified as described before. The protein concentration of the purified enzyme solution

281   was determined according to Bradford (1976) by using bovine serum albumin (A1933, Sigma) as a

282   standard. Aliquots (300 µL) of each pathogen conidial suspension (1×10^6 conidia/mL) in Ringer

283   solution were transferred to tubes containing 2.4 mL PDB. Then 300 µL of the purified recombinant

284   enzyme solution (80 µg/mL) was added to the tubes and reached a final concentration of 8 µg/mL

285   PDB, serving as enzyme treatment (Pathogen+ Enzyme). Aliquots (300 µl) of inactivated enzymes, by

286   boiling at 100°C for 10 min, or sterile distilled water instead of the enzyme solution were added to the

287   tubes and served as controls (Pathogen+ Inactivated enzyme, and Pathogen+ Water). After 18 h

288   incubation of the 45° sloping tubes at 25°C on a rotary shaker (200 rpm), 100 conidia per replicate

289   were observed microscopically and their germination rate (%) and germ tube length (µm) were

290   measured. Three replications of three tubes were prepared for each treatment and the experiment was

291   repeated twice.

292

293   **2.12 Statistical analysis**

294   All the experiments were performed at least twice. Data from all the experiments were analyzed

295   using analysis of variance (ANOVA) and the SPSS version 12.0 (SPSS, 1989-2003). The means were

296   separated at 5% significance level, by using Duncan's Multiple Range Test (DMRT).

297

298   **Results**

299   **3.1 Pathogen mycelium inhibition by *A. pullulans* PL5 and its crude secreted enzymes**

300   After 7 days co-culture in PDA plates at 25±2°C, direct interaction *in vitro* was observed (Fig. 3 a,

301   b, c). *A. pullulans* PL5 significantly inhibited the hyphae elongation of the pathogens. However, no

302   attachment of fungal hyphae was observed.

303   After 2 days co-culture in the presence of purified crude enzymes produced by *A. pullulans* PL5 in

304   PDA plates at 25±2°C, an inhibition of the pathogen mycelia was observed. However, when

305   incubation was without crude enzymes (CK), the mycelium of *A. pullulans* PL5 grew normally (Fig. 3

306   d).

307

308   **3.2 Production of alkaline protease by *A. pullulans* PL5 and its activity**

309   As shown in Fig. 4, *A. pullulans* PL5 produced alkaline protease with high activities when grown in

310   LBMS medium with *M. laxa*, *B. cinerea* or *P. expansum* cell walls as sole carbon sources. When *A.*

311   *pullulans* PL5 was cultured in the LBMS medium, at 0 h incubation, no alkaline protease was

312   detected, while at 24 h incubation, a protease activity was detected. At 72 h, the maximum level of

313   protease activity was observed. At 96 h incubation, the activity began decreasing, after its maximum

314   level. Similar pattern of alkaline protease activity from 0 h to 96 h incubation was observed when *A.*

315   *pullulans* PL5 was grown in minimal salt medium with *M. laxa*, *B. cinerea* or *P. expansum* cell walls

316   as sole carbon sources, and the maximum activities in the three different LBMS medium were 116,

317   121 and 129 U/mg protein, respectively.

318

319   **3.3 Cloning alkaline protease genes from genomic DNA and cDNA of *A. pullulans* PL5**

320   To clone the partial sequences of the gene encoding extracellular alkaline protease in *A. pullulans*

321   PL5, degenerate primers were designed according to the conserved amino acid sequences of alkaline

322   proteases from different species of the yeast (Fig. 1). PCR amplification produced a reliable fragment

323   of about 600 bp (data not shown). Analysis of the sequence by BLAST program suggested that the

324   fragment showed a high identity (87%) to the alkaline serine protease gene *ALP2*, suggesting that the

325 putative alkaline protease gene was isolated. New primers were designed according to the sequence of

326 the obtained fragment (600 bp) of the putative alkaline protease gene in combination with the kit

327 "Gene Walking Made Easy". The fragments obtained were sequenced, assembled, and analysed with

328 BLAST and ORF Finder programs. Finally, the whole gene of 1,351 bp (GenBank accession number

329 HQ113463) encoding the alkaline protease, named as *ALP5*, was cloned from the genomic DNA of

330 the antagonistic yeast strain PL5 (Fig. 5 and 6).

331   After PCR with the cDNA of the strain PL5 as template and the primers specific to *ALP5* gene, a

332 specific fragment of 1,248 bp was produced (Fig. 5 and 6). The *cDNAALP5* sequence presented 100%

333 identity to the corresponding coding sequence of the genomic DNA. The organization of the gene is

334 illustrated in Fig. 6. Comparison of *ALP5* gene with *cDNAALP5* revealed that there are two introns

335 inside *ALP5* gene. One intron was of 54 bp, while the other was of 50 bp in size. The two introns

336 divided the open reading frame of *cDNAALP5* into three parts. The size, percentage of (G+C) and

337 sequence characteristics of the introns were displayed in Table 1. The flanking sequences of the

338 second intron are consistent with the typical eukaryotic splice sites, including an invariant GT at 5

339 prime and an invariant AG at 3 prime of the introns, while the first intron had GA at its 5 prime and

340 GT at its 3 prime that are non-typical eukaryotic splice sites (Table 1).

341

342 **3.4 Protease ALP5 of *A. pullulans* PL5 and phylogenetic analysis**

343   As seen in Fig. 6, an open reading frame of 1,248 bp encoding a 415-amino acid (aa) protein with a

344 calculated molecular weight (M_r) of 42.9 kDa and an isoelectric point (pI) of 4.5 was characterized.

345 The protease ALP5 had a 18 amino acid signal peptide (positions from 1 to 18), one *N*-gylcosylation,

346 one histidine active site, and one serine active site, suggesting that the protease ALP5 was an alkaline

347 serine protease.

348   To reveal the relationship of the protease ALP5 to proteases from other microorganisms, protein

349   BLAST and phylogenetic analysis were performed. As seen in Fig. 7, ALP5 and selected proteases

350   clustered into three groups. Among these proteases, ALP5 is close to proteases from *Aspergillus* spp.

351   and moreover, ALP5 deduced amino acid sequence shared 100% homology to the protease ALP2

352   (Genbank accession number: ABW86845.1) of the sea yeast, *A. pullulans* strain HN2-3, which has

353   been known as an alkaline serine protease, confirming that the protein ALP5 was alkaline serine

354   protease.

355

356   **3.5 SDS-PAGE, western-blotting and purification of the recombinant proteases**

357   As seen in Fig. 8a, SDS-PAGE showed that one specific recombinant protein at the size of about 43

358   kDa was produced from the positive transformants harbouring the gene *ALP5* as compared to controls

359   without containing the gene *ALP5*. The size of the recombinant proteins was the same as calculated

360   from the deduced amino acids of the protease gene *ALP5*. Western-blotting analysis of the proteins

361   from the positive transformants with his-tag monoclonal antibody showed that one specific protein

362   band of the same size obtained by SDS-PAGE was identified (Fig. 8b), indicating that the

363   recombinant protein was fused with His-tag. The recombinant proteases were purified with Ni-NTA

364   affinity chromatography and were subjected to SDS-PAGE analysis. Results showed that one specific

365   band with the molecular weight of about 43 kDa was identified (Fig. 8c), suggesting that the protein

366   was the recombinant ALP5. In addition, the results of Fig. 8a and Fig. 8c revealed that the

367   recombinant protein had an apparent molecular mass of 43 kDa.

368

369   **3.6 Effects of temperature and pH values on the recombinant ALP5 activities**

370   The effect of temperature on the recombinant ALP5 activity was investigated when the enzyme

371   reactions were performed at temperatures from 20°C to 60°C. As seen in Fig. 9a, the highest activity

372   of the recombinant ALP5 was observed when the enzyme reaction was performed at 50°C. During the

373   range of 20-50°C, the activity increased with an increase of the reaction temperature. But when the

374   enzyme reaction temperature exceeded 50°C, the activity started decreasing, indicating that the

375   optimal temperature of the recombinant ALP5 activity was 50°C. Similarly, the optimal pH of the

376   activity was also determined. As seen in Fig. 9b, the highest activity was detected when the reaction

377   system was at pH 10.0. At pH 4.0, the recombinant protein showed the lowest activity as compared to

378   those at other pH values.

379

380   **3.7 Effects of temperature and pH values on the recombinant protease ALP5 stability**

381   The recombinant protease ALP5 showed the highest residual activity when pre-incubated at pH 7.0

382   and 4°C (Fig. 10). Like other enzymes, the recombinant protease ALP5 showed a good stability when

383   pre-incubated at lower temperatures (Temperature≤ 10°C) (Fig. 10a). Moreover, when pre-incubated

384   at 20-50°C, the recombinant ALP5 still showed a high residual activity in hydrolysing the substrate

385   (Fig. 10 a). However, when pre-incubated at 60°C, the residual activity dramatically decreased. These

386   results suggested that the recombinant enzyme had a good thermal stability. As shown in Fig. 10b,

387   when the recombinant ALP5 was pre-incubated at the acid pH values (pH≤ 5.0) or at the extremely

388   strong alkaline pH values (pH≥ 11.0), residual activities were lower as compared to that at pH 7.0-8.0,

389   indicating that the recombinant ALP5 showed a poor stability at acidic or at extremely alkaline

390   conditions.

391

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

414 protease, has been proposed as an important mode of action of biocontrol agents against pathogens

415 (Castoria et al., 1997; Chan and Tian, 2005). Bar-Shimon et al., (2004) studied the role of

416 exo-b-1,3-glucanase (*CoEXG1*) in the biocontrol activity of *Candida oleophila* using the gene-knockouts

417 and over-producing transformants, and found that, compared to the wild type of yeasts, the biocontrol

418 activity of CoEXG1-over-producing yeasts was not significantly increased but the activity of

419 *CoEXG1*-knockouts yeasts was significantly decreased, suggesting that the production of exo-glucanase is

420 involved in the biocontrol of pathogens. This study demonstrated that the antagonist *A. pullulans* PL5

421 produced the high activities of extracellular proteases which significantly inhibited the mycelial

422 growth of pathogens. In addition, it was also demonstrated that the recombinant protease PL5

423 expressed in *E. coli* BL21 inhibited the pathogen mycelial growth *in vitro*. These results provided the

424 direct evidence that production of protease plays an important role in the activity of *A. pullulans* PL5

425 against pathogens. On the other hand, the elucidation of some gene functions is useful for the

426 registration of BCAs since the more information on BCAS is supplied, the easier their applications are

427 accepted by their consumers. Therefore, cloning and characterization of the protease genes from the

428 antagonistic yeast, *A. pullulans* PL5 is important to the registration and application of the yeast. This

429 discovery could greatly enhance biocontrol of postharvest diseases, by improving the future selection

430 of new antagonists characterized by a protease activity against the fungal pathogens.

431 Microbial proteases are classed into various groups, based on their activity under acidic, neutral,

432 or alkaline conditions and on the characteristics of the active site group of the enzyme. Among them,

433 the alkaline serine proteases, which are active in a neutral to alkaline pH range and have a serine

434 center, are the most important group of enzymes exploited commercially (Gupta et al., 2002).

435 Although proteases from bacteria (Gupta et al., 2002), and fungi (Macchione et al., 2008; Morita et

436   al., 1994) have been extensively investigated and used in different fields, only a few studies were

437   performed on the proteases and the protease-related genes from the yeasts. In this study, the protease

438   gene *ALP5* was cloned and characterized from the strain PL5 of *A. pullulans*, and the protease

439   encoded by the gene *ALP5* belongs to alkaline serine protease family.

440   The protease from the terrestrial yeast, *A. pullulans* PL5 shows an extremely high homology to that

441   from the sea yeast, *A. pullulans* strain HN2-3, suggesting that yeast enzyme genes are relatively

442   conserved among strains of the same species. Although the protease gene is not easy to be expressed

443   in prokaryotes, such as *E. coli*, as compared to eukaryotes, such as *Pichia pastoria* and

444   *Saccharomyces cerevisiae*, we successfully cloned and expressed the protease gene *ALP5* in *E. coli*

445   BL21 (DE3), without the signal peptide, in order to reduce the toxic effects (lytic activity) of the active

446   recombinant proteases on the growth of the bacterial host. The recombinant ALP5 expressed from *E. coli*

447   showed lower activity than the native protease secreted by *A. pullulans* PL5, which maybe resulted

448   from the fusion of his-tag with the recombinant protease and the renaturation of extracted proteins

449   from conclusions. Previous studies (Moser et al., 1994) revealed that the refolded recombinant

450   protease had a lower level of proteolytic activity compared with that of the native protease and

451   proposed that the decrease in activity was most probably caused by refolding problems related to

452   leader sequences present in the proteases.

453   The activity and stability are the most important attributes of enzymes. In this study, the optimal

454   temperature and pH values of the recombinant protease ALP5 were determined. In addition, the

455   effects of temperatures and pH values on the stability of the recombinant enzyme were investigated. It

456   was found that the recombinant enzyme performed the highest activity in hydrolysing substrate when

457   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

476

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624

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%

629 **Table 2**

630 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

631 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+

632 Enzyme), and inactivated enzymes or sterile distilled water instead of the enzyme solution served as controls (Pathogen+ Inactivated enzyme, and Pathogen+

633 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

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

635 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

636 significant difference ($P<0.05$) according to analysis by Duncan's Multiple Test (SPSS 13.0).

31

Figures

Figure 1

640 Alignment of the deduced amino acid sequence of five alkaline serine protease genes retrieved from NCBI

641 database: *Aureobasidium pullulans* alkaline serine protease (ABW86845.1); *Aureobasidium pullulans*

642 alkaline serine protease ALP2 (ABP82774); *Neosartorya fischeri* NRRL181 alkaline serine protease Alp1

643 (XP_001266852.1); *Aspergillus fumigatus* Af293 alkaline serine protease Alp1 (XP_751651.1);

644 *Aspergillus fumigatus* uncleaved alkaline protease (ALP) (CAA77666.1); Multiple sequence alignment of

645 proteins was performed by using the DNAMAN 6.0. Identical residues are highlighted by black boxes. The

646 potential conserved region (GHGTHVAGT and TATNTISGTSMA) marked with asterisks were used for

647 designing the degenerate primers for amplifying the partial sequence of alkaline serine protease gene from

648 *A. pullulans* strain PL5.

649

650 **Figure 2**

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

652 *coli* BL21.

653

654 **Figure 3**

655 Interaction of *A. pullulans* PL5 and three postharvest pathogens *in vitro*, and inhibition of *B. cinerea*

656 growth by purified extracellular crude enzymes produced by *A. pullulans* PL5: a) interaction of *A.*

657 *pullulans* PL5 and *B. cinerea*; b) interaction of *A. pullulans* PL5 and *M. laxa*; c) interaction of *A. pullulans*

658 PL5 and *P. expansum*; d) inhibition of *B. cinerea* hyphal growth by purified extracellular crude enzymes

659　produced by *A. pullulans* PL5.

660

661　**Figure 4**

662　Extracellular protease activities (EC. 3.4.21.63) of *A. pullulans* PL5 grown in LBMS medium

663　supplemented with 2 mg ml^{-1} CWP (cell wall preparation of each pathogen: *M. laxa*, *B. cinerea*, or *P.*

664　*expansum*) as sole carbon source for 96 h at 25°C. Bars represented standard deviations of the means.

665

666　**Figure 5**

667　Amplification of the protease genes from cDNA and genomic DNA of *A. pullulans* PL5 by PCR. Line M is

668　DNA molecular standards (Cat. No. 239085, Qiagen); Lane1 is the PCR product with cDNA of *A.*

669　*pullulans* PL5 as template; Line2, the PCR product with genomic DNA as template; Line3, Blank control.

670

671　**Figure 6**

672　Nucleotide sequence of the alkaline serine protease *ALP5* gene of *A. pullulans* PL5and deduced amino acid

673　sequence of the gene product. The start codon is in bold and the signal peptide was highlighted with the

674　inverted arrows. *N*-glycosylation site, histidine active site and serine active site were underlined with the

675　single line, respectively. The stop codon is marked with the asterisk. Two introns were highlighted with

676　lower case letters in grey color, respectively. The gene was designated as *ALP5* and the sequence has been

677　submitted to NCBI (accession number HQ113460).

678

679　**Figure 7**

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

681 <mark>performed using the program MEGA 4.1. The numbers at node indicate the bootstrap percentages of 1000</mark>

682 <mark>resamples.</mark> The proteases retrieved from NCBI database are: *Aureobasidium pullulans* alkaline serine

683 protease (ABW86845.1); *Neosartorya fischeri* NRRL181 alkaline serine protease Alp1

684 (XP_001266852.1); *Aspergillus fumigatus* Af293 alkaline serine protease Alp1 (XP_751651.1);

685 *Aspergillus fumigatus* serine proteinase (AAB07672.1); *Aspergillus fumigatus* uncleaved alkaline protease

686 (ALP) (CAA77666.1); *Aspergillus clavatus* (ACX47962.1) alkaline protease; *Aspergillus clavatus* NRRL1

687 alkaline serine protease Alp1 (XP_001272038.1); *Trichoderma hamatum* (AAP15044.1) alkaline

688 proteinase; Aspergillus oryzae RIB40 hypothetical protein (XP_001820144.1); *Aspergillus oryzae*

689 preproalkaline protease (CAA38527.2); *Hypocrea virens* extracellular serine protease (AAO63588.1);

690 *Aspergillus fumigatus* (CAA75804.1) alkaline protease; *Aspergillus fumigatus* (CAA75806.1) alkaline

691 protease; *Aspergillus versicolor* extracellular alkaline serine protease (ADE74975.1); *Aspergillus* sp.

692 MK285 alkaline protease (AAT85628.1); *Aspergillus viridinutans* alkaline protease (AAT85626.1);

693 *Aspergillus viridinutans* alkaline protease (AAT85627.1); *Verticillium albo-atrum* VaMs.102 alkaline

694 proteinase (XP_003004413.1).

695

696 **Figure 8**

697 a) SDS-PAGE analysis of expression of *ALP5* in *E. coli* BL21 (DE3): line 1and 2, total protein extracts

698 from IPTG-induced transformants harboring the plasmid pET23a-ALP5; line 3, protein extracts from *E.*

699 *coli* cell containing empty vector pET23a; line M, protein molecular weight markers; b) Western-blotting

700 confirmation of recombinant protein with monoclonal anti-polyhistidine as first antibody and

701 A1293-AlkPhos APA Mouse Fab ads HIgG as second antibody: line 4, protein extracts from IPTG

702 -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.