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Biocontrol activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in *Pichia pastoris* against four postharvest pathogens on apple

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ABSTRACT

The yeast-like fungus *Aureobasidium pullulans* PL5 is a microbial antagonist against postharvest pathogens of fruits. The strain is able to produce hydrolases, including glucanases, chitinases and proteases. The alkaline serine protease gene *ALP5* from *A. pullulans* was cloned, inserted into the vector pPIC9 to construct pPIC9/*ALP5*, and then expressed in *Pichia pastoris* strain KM71. ALP5 had a molecular mass of 42.9 kDa after 5 days growth with 1% methanol induction at 28 °C. The recombinant protease expressed in *P. pastoris* showed its highest activity under alkaline conditions (at pH 10) and a temperature of 50 °C. The antifungal activity of the recombinant protease was investigated against *Penicillium expansum*, *Botrytis cinerea*, *Monilinia fructicola* and *Alternaria alternata in vitro* and on apple. The recombinant protease reduced significantly the spore germination and the germ tube length of the tested pathogens in PDB medium. The highest level of protease efficacy was observed against *M. fructicola* and *B. cinerea*, whereas a lower efficacy was observed against *P. expansum* and *A. alternata* indicating a possible effect of the pathogen cell wall composition on the proteolytic activity of the recombinant protease. The presence of protease was able to cause the swelling of the hyphae of *B. cinerea*, under an optical microscope. The recombinant protease expressed in *P. pastoris* was more active against the pathogens *in vitro* than the same enzyme expressed in *E. coli* in previous studies. The efficacy of ALP5 was also evaluated against the pathogens *in vivo* on cv Golden Delicious apples. The protease was more efficient in controlling *M. fructicola*, *B. cinerea* and *P. expansum* than *A. alternata*. However, the extent of the activity was dependent on the enzyme concentration and the length of fruit storage. This study demonstrated the capacity of the alkaline serine protease to keep its enzymatic activity for some days in the unfavorable environment of the fruit wounds. The alkaline serine protease could be developed as a postharvest treatment with antimicrobial activity for fruit undergoing a short storage period.

1. Introduction

Penicillium expansum, *Botrytis cinerea*, *Monilinia fructicola* and *Alternaria alternata* are among the most severe postharvest pathogens on apples in production areas where the most advanced storage technologies are available (Khamis et al., 2012; Martini et al., 2013; Snowdon, 1990). To control postharvest diseases of fruits, few synthetic fungicides are admitted (Singh and Sharma, 2007; Zhu, 2006). However, pathogen resistance to fungicides (Holmes and Eckert, 1999), and the willingness to use safer and eco-friendly treatments, have generated interest in the development of alternative non-chemical methods to reduce postharvest losses (Lopez-Reyes et al., 2010; Nunes, 2012). Biological control using microbial antagonists has emerged as one of the most promising alternatives to fungicides, either alone or as part of an integrated pest management (Janisiewicz and Korsten, 2002). A clear understanding about the mode of action of biocontrol agents is important for a successful implementation of postharvest biocontrol technology (Droby et al., 2009; Zhang et al., 2011).

Among the different biocontrol agents, yeasts are promising and gaining popularity (Jamalizadeh et al., 2011; Janisiewicz et al., 2010; Spadaro et al., 2008). In particular, the yeast-like fungus *Aureobasidium pullulans* De Bary (Arnaud), has been shown to be effective against *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* on various fruit, including apple, grapes, sweet cherry, strawberry and peach (Bencheqroun et al., 2007; Ippolito et al., 2000; Lima et al., 1997; Schena et al., 2003). Moreover, the strain PL5 of *A. pullulans* showed high efficacy in the control of *B. cinerea* and *P. expansum* on apples, as well as *Monilinia laxa* on plums and peaches (Zhang et al., 2010a).

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Several mechanisms have been reported to play a significant role in the biocontrol activity of *A. pullulans* strains, including the induction of defense responses (Ippolito et al., 2000) and competition for nutrients (Bencheqroun et al., 2007). Recently, it was demonstrated that the strain PL5 secretes β-1,3-glucanase, exo-chitinase and endo-chitinase, in addition to the secretion of alkaline serine protease (Zhang et al., 2010a, 2012).

In mycoparasitism, fungal proteases may be significantly involved in antagonistic activity, because they may play a significant role in fungal cell wall lysis, which is composed of chitin and glucan polymers embedded in, and covalently linked to, a protein matrix (Wessels, 1986). The inner layer of fungal cell walls is primarily composed of glucans and chitin arranged as interwoven microfibrils, while the outer electron dense layer is mainly composed of covalently bound mannosylated proteins (Klis et al., 2002). Proteases catalyze the cleavage of peptide bonds in proteins. In recent years, there has been an increasing interest in the study of proteolytic enzymes, because they constitute one of the most important groups of industrial enzymes due to their commercial value and potential application in several fields, including food science and technology, pharmaceutical industries and detergent manufactories (Feijoo-Siota and Villa, 2011).

The protease gene *ALP5* of *A. pullulans* strain PL5 was previously cloned and expressed in *Escherichia coli* BL21 (Zhang et al., 2012), showing a low enzymatic activity. Prokaryotic expression systems could present some drawbacks, including incorrect protein processing, folding and posttranslational modification, lower heterologous protein expression levels, and lower activity. *Pichia pastoris* has recently emerged as an important yeast host for heterologous protein expression (Cregg et al., 1993; Macauley-Patrick et al., 2005). As a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression systems, such as protein processing and folding, and posttranslational modifications, while being as easy to manipulate as *E. coli* (Balamurugan et al., 2007). In the yeast expression system, the secreted heterologous protein is the vast majority in the medium, and, if there are glycosylation sites, glycosylation may occur at these sites. For this reason, yeast genes could be better expressed in eukaryotic expression systems, such as *P. pastoris*.

Therefore, the objectives of this research were to clone the protease gene *ALP5* from *A. pullulans* strain PL5 and to express it in *P. pastoris* to evaluate its activity. A second objective was to demonstrate the antifungal activity of the recombinant protease in controlling different postharvest pathogens *in vitro* and *in vivo* on fruits, and to prove its involvement in the biocontrol activity of the yeast-like fungus PL5.

2. Materials and methods

2.1. Microorganisms, plasmids and molecular kits

A. pullulans strain PL5 was isolated from the carposphere of cv Angeleno plum and selected for its efficacy (Zhang et al., 2010b). It was identified through microscopic observation of cell and colony morphology, and by sequencing of the ribosomal region ITS (Genbank accession number: FJ919775).

Strains of *B. cinerea*, *P. expansum*, *M. fructicola* and *A. alternata* were isolated from rotten apples or peaches (Pellegrino et al., 2009; Saravanakumar et al., 2008), then selected throughout this work for their virulence by inoculation in artificially wounded cv Golden Delicious apples. Each strain was maintained at 4 °C on PDA (potato dextrose agar, Merck, Germany) slants.

The *E. coli* strain DH5α used in this study as host for plasmids, was obtained from Invitrogen (Life Technologies, Carlsbad, USA). The oligonucleotides, pGEM-T vector and the *E. coli* strain JM109 were purchased from Promega (Madison, USA). *P. pastoris* KM71 strain (Invitrogen) was used as host for transformations with the plasmid pPIC9 (Invitrogen).

2.2. Total RNA isolation and first-strand cDNA synthesis

The strain PL5 was grown in liquid medium YPD (20 g D-glucose, 20 g peptone casein, and 10 g yeast extract per liter) at 25 °C. After 48 h shaking at 200 rpm on a rotatory shaker (ASAL, Italy), the culture was centrifuged for 10 min at 5000 ×*g*. RNA was extracted from the pellet with RNeasy® extraction kit (Qiagen, Hilden, Germany), then the first-strand cDNA was synthesized using a reverse transcript kit according to the manufacturer's instructions (Qiagen). RNA concentration and purity were checked by a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, USA).

2.3. Cloning of ALP5-encoding cDNA gene

In order to amplify the cDNA of the *ALP5* gene encoding the alkaline serine protease by PCR, we designed the forward primer (Pf-*ALP5*) as 5′-ACTGAATTCATGTGGAAGAAGAGTGTTGC-3′ and reverse primer (Pr-*ALP5*) as 5′-AATGAATTCTAACGACCGCTGTTGTTGTAAAC-3′; (bases underlined encode an *Eco*RI restriction site) according to the sequence of the protease gene obtained from the genomic DNA (GenBank accession number HQ113460.1). PCR conditions were as follows: an initial step at 94 °C for 3 min, and 35 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 90 s. The purified PCR amplicons were ligated into pGEM-T-Easy cloning vector, then transformed into chemically competent cells of *E. coli* strain DH5α.

2.4. Construction of the expression vector pPIC9–ALP5

The product was subsequently digested with *EcoRI* and ligated into the pPIC9 vector, which was previously digested with the same restriction enzyme. The consequent plasmid pPIC9–*ALP5* was transformed into *E. coli* JM109 (Promega). The recombinant plasmid was isolated from the positive transformants using Qiaprep Spin Miniprep Kit (Qiagen). The presence and correct orientation of the insert sequence was confirmed by DNA sequencing at BMR Genomics (Padova, Italy).

2.5. Transformation of P. pastoris

The pPIC9–*ALP5* construct was linearized by a *Stu I* enzyme for efficient integration into the *P. pastoris* genome, then it was transformed into the *P. pastoris* strain KM71. The empty vector (pPIC9) was also transformed in *P. pastoris* for negative control tests following the manufacturer's instructions. After transformation with plasmid pPIC9–*ALP5*, His + transformants of *P. pastoris* KM71 were purified on minimal medium plates without histidine to ensure pure clonal isolates, then the genomic DNA of the transformants was isolated and PCR amplification and sequencing were done to confirm whether the protease cDNA was integrated into the genomic DNA of *P. pastoris*. Primers used for PCR, 5′AOX1 (5′-GACTGGTTCCAATTGACAGC-3′) and 3′AOX1 (5′-GCAAATGGCATTCTGACATCC-3′) were provided by the manufacturer. The PCR screening of the positive recombinants produced a 1.7 kb fragment, while the control yeast transformed with pPIC9 produced a 492 bp product (data not shown), which confirmed the integration of the insert into the *P. pastoris* genome.

2.6. Expression and purification of A. pullulans strain PL5 protease

Transformed *P. pastoris* isolates were cultured in 100 mL of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 1% glycerol) for approximately 24 h at 28 °C with constant shaking till OD 600 nm reached about 2–6. Cells were centrifuged and the cell mass was resuspended in 20 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) to induce expression of the recombinant proteins. The culture was supplemented daily with 100% methanol to a final concentration of 1% to