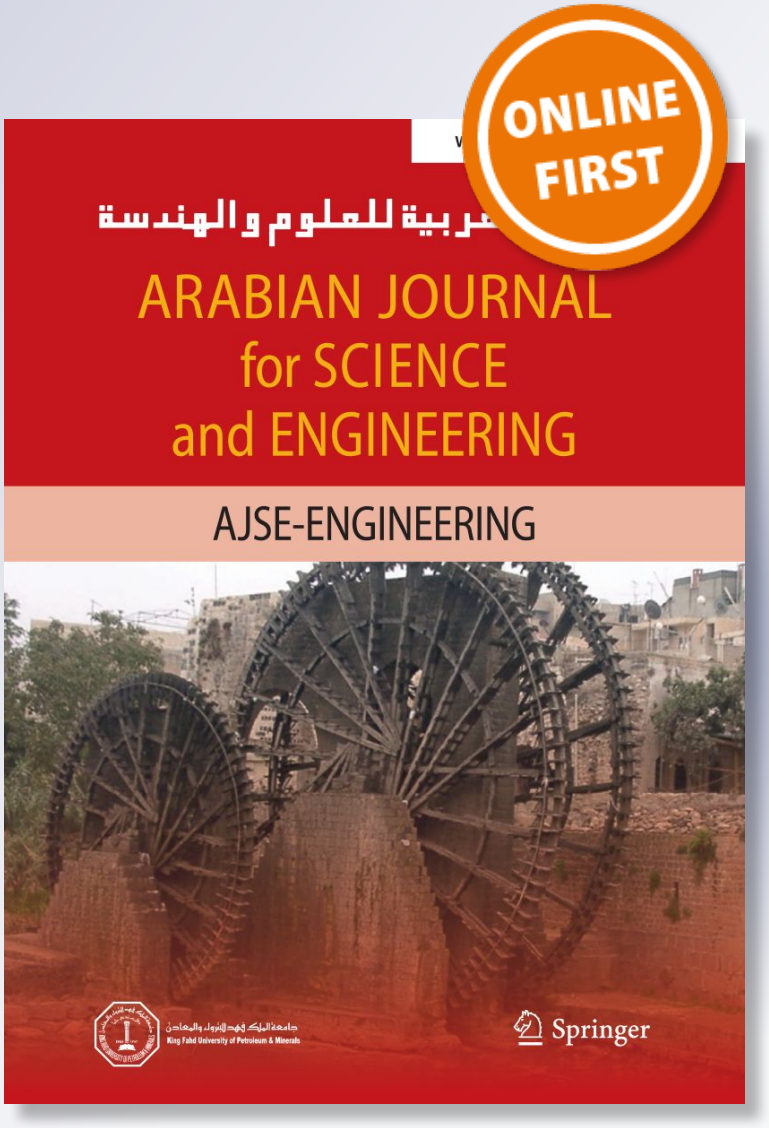
Reduction of Extracellular Proteases Increased Activity and Stability of Heterologous Protein in $${ Aspergillus}$$ A s p e r g i l l u s $${ niger}$$ n i g e r

Nurhaida Kamaruddin, Reginald Storms, Nor Muhammad Mahadi, Rosli Md. Illias, et al.

Arabian Journal for Science and Engineering

ISSN 2193-567X

Arab J Sci Eng
DOI 10.1007/s13369-017-2914-3



Springer

Arab J Sci Eng
DOI 10.1007/s13369-017-2914-3

RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Reduction of Extracellular Proteases Increased Activity and Stability of Heterologous Protein in *Aspergillus niger*

Nurhaida Kamaruddin[1,2] · Reginald Storms[3] · Nor Muhammad Mahadi[4] · Rosli Md. Illias[5] · Farah Diba Abu Bakar[1] · Abdul Munir Abdul Murad[1]

Received: 7 August 2017 / Accepted: 25 October 2017

Abstract The heterologous protein production in *Aspergillus niger* is often limited by the activity of the host extracellular proteases. To improve heterologous production, a transcription factor controlling expression of several extracellular protease-encoding genes, *prtT*, was deleted in *A. niger* PY11 and the mutant (AnΔ*prtT*) characterised. Extracellular proteolytic activity of AnΔ*prtT* was reduced as compared to the wild type, a result that was confirmed by RT-PCR analyses that showed reduced expression levels of several protease gene transcripts. To compare the efficiency of the mutant and parental (PY11) strains as hosts for heterologous protein production, the cutinase gene from *Glomerella cingulate*, under control of the glucoamylase A promoter, was integrated into each genome. The cutinase activity of PY11 and AnΔ*prtT* harbouring the *G. cingulata* cutinase gene was increased 20- and 36-fold higher, respectively, than the untransformed parental strains, suggesting that the ability of the mutant to produce heterologous protein is better than the wild type. Cutinase activity in culture filtrates prepared using both strains was stable at 4 °C for extended periods; however, during incubation at 25°C the heterologous cutinase in culture filtrates prepared using AnΔ*prtT* retained 80% activity after a two-week incubation versus the less than 3% activity that was retained in culture filtrates prepared using PY11. This indicates that the reduction of extracellular proteases greatly improves the stability of heterologous proteins produced by *A. niger*.

Keywords *Aspergillus niger* · Extracellular proteases · Transcription factor · Cutinase

1 Introduction

The filamentous fungus *Aspergillus niger* is regarded as a safe microorganism and widely used in industry for the production of organic acids, enzymes and for basic genetic research [1,2]. The use of *A. niger* as a host for the production of homologous and heterologous proteins relies on its ability to secrete a broad range of hydrolytic enzymes in large quantities into the growth medium; however, native proteases often cause protein degradation either during secretion or storage [3]. Several studies showed that this problem can be reduced by the deletion of protease genes [4,5].

Aspergilli produce a broad spectrum of proteases in a species-specific manner [6], but acid proteases predominate in *A. niger*. Four extracellular acidic proteases, two aspartyl proteases, PepA and PepB, and two serine carboxypeptidases, PepF and PepG, have been characterised [7–11]. Further, an extracellular subtilisin-type serine protease, PepD, was cloned based on conserved amino acid sequences within subtilisins [12]. Three protease genes that are homologous to yeast vacuolar proteases have also been cloned from *A. niger*, a pepsin-type arpartyl endoprotease,

✉ Abdul Munir Abdul Murad
munir@ukm.edu.my

1 School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

2 Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjung Malim, Perak, Malaysia

3 Department of Biology, Concordia University, 7141 Sherbrooke Street West, Montreal, QC H4B 1R6, Canada

4 Malaysia Genome Institute, Jalan Bangi, 43000 Kajang, Selangor, Malaysia

5 Department of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia

Published online: 08 November 2017

pepE, that is the homologue of the vacuolar *pep*4 gene product in yeast, a subtilisin-type serine endoprotease, *pepC*, and a serine carboxypeptidase, *cpy* [13,14]. Furthermore, four genes (*pepAa, pepAb, pepAc* and *pepAd*) encoding pepsin-like aspartic proteases have been identified using a comparative genomics approach [4]. Each protein possesses different characteristics and may have distinct functions. The secretion levels of recombinant laccase in the *pepAa*, *pepAb* and *pepAd* disruption mutants were increased by about 21, 42 and 30%, respectively, in *A. niger* [4].

Protease-specific transcription factor, *prtT*, was first cloned and characterised from *A. niger* [15]. *prtT* controls expression of several major protease-encoding genes. Disruption of *prtT* in *A. niger* resulted in reduced expression of four proteases (*pepA, pepB, pepD* and *pepF*). In *A. oryzae*, the alkaline serine protease (AlpA) and the neutral metalloprotease M36 (Np1) were expressed at lower levels in the *prtT* mutant as compared to the wild type [15]. Similarly, deletion of *A. fumigatus prtT* resulted in the loss of secreted protease activity and decreased transcription of six secreted protease genes [16,17]. Whole-genome sequencing of *A. niger* suggested that the number of proteases encoded is approximately 200. Of these, 32 were predicted to have a signal peptide or have strong similarity to other secreted proteases in other organisms [18]. Given this large complexity of proteases and their probable regulation, serial deletion of individual genes would not only be laborious and time-consuming, but probably only a partial solution to the problem. We hypothesised that deletion of the protease-specific transcription factor *prtT* might be a simpler way of minimising proteolytic activity in the culture filtrate and improve the stability of the heterologous proteins during storage.

Previously, disruption of *prtT* gene from *A. niger* strain AB1.13 has been reported by Punt et al. [15]. However, the stability of heterologous protein produced from the mutant has not yet been shown. Here we describe the disruption of *prtT* in *A. niger* strain PY11, characterise growth and extracellular protease activity of the mutant as well as examine the effect of the mutation on the stability of a heterologous protein produced from the strain.

2 Materials and Methods

2.1 Strains, Plasmids and Culture Conditions

A. niger strain PY11 (*cspA1*$^-$ pyrG6$^-$ Δ glaA::HIS, Δ*bglA::HISG*) is a β-glucosidase A and glucoamylase A deficient strain. PY11 was derived from strain N593 (*cspA1*$^-$ pyrG6$^-$) [19] by targeted gene replacement of the *glaA* and *bglA* genes as described previously [20]. A mutant strain (An*Δprt T*) of *A. niger* PY11 was constructed in this study. Mycelia were grown from conidia in minimal media (MM)

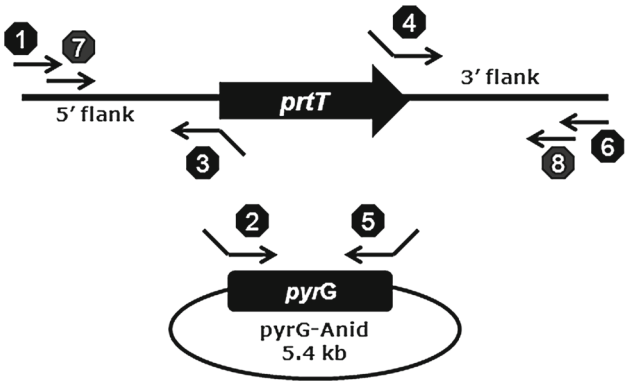


Fig. 1 Construction of the *prtT* disruption cassette. Fusion PCR consisted of two rounds of PCR. In the first PCR, primers 1 and 3 were used to amplify DNA at the 5′ region of *prtT*; primers 4 and 6 were used for the 3′ region; and primers 2 and 5 were used to amplify the selectable marker (*pyrG* from the pyrG-Anid plasmid). Complementary regions generated at the 3′-end of the *prtT* upstream fragment and the 5′-end of *pyrG* as well as the 5′-end of the *prtT* downstream fragment and 3′-end of *pyrG* allowed overlapping during the second round of PCR

as described by Kafer [21] and supplemented with various carbon sources. Concentrations of 10 mM uracil and uridine were added to the MM when needed. Strains for protoplast generation and DNA isolation were grown in complete media (CM) at 30°C and agitated (200 rpm in an incubator shaker) [21]. *Escherichia coli* DH5α [22] was the host used for maintenance and production of plasmid DNAs. Plasmids ANIp2 and Anid-pyrG [20] were used as expression vector and as the source of *Aspergillus nidulans pyrG* used for gene replacement. Casein–gelatin media contained MM supplemented with 1% (w/v) glucose, 0.5% (w/v) casamino acids, 1% (w/v) gelatin, 1% (w/v) casein and 0.01% (w/v) Triton X-100. Starch plates contained MM supplemented with 1% (w/v) glucose and 1% (w/v) starch. Optimised media for protease expression contained MM supplemented with 1% (w/v) glucose and 3% (w/v) wheat bran. Expression media (MMJ) comprised of MM with maltose 15% (w/v) as carbon the source [23]. Conidial suspensions were made in saline-Tween^{TM} 80 0.05% (v/v) and vigorously shaken to break conidial chains and reduce conidial aggregation.

2.2 Construction and Verification of the *A. niger prt*T Disruption Mutant

The *prtT* gene disruption cassette was generated using PCR. The fusion PCR strategy is outlined in Fig. 1, and the oligonucleotide sequences are listed in Table 1. Oligonucleotides for amplification of *prtT* flanking regions were designed based on the *A. niger* genome sequence available at the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Aspni5/Aspni5.home.html). The first round of PCR involved amplification of the flanking sequences of the *prtT* gene using

Table 1 Oligonucleotide primers used in this study

Name	Description	Sequence (5′–3′)
Primer 1	Forward primer to amplify 2.3-kb 5′ region of *prtT* for construction of disruption cassette	5-CGGAGAAGTTGCCTCTATCGTTGGC-3
Primer 3	Reverse primer to amplify 2.3-kb 5′ region of *prtT* for construction of disruption cassette	5-ATGTATGCTATACGAAGTTATGGCGCGCC ATCCTTAATTAACTCGCCAAGCTCTTGTGC TCCCAT-3
Primer 4	Forward primer to amplify 2.0-kb 3′ region of *prtT* for construction of disruption cassette	5-GCATACATTATACGAAGTTATACGCGTGC GGCCGCCACCGCGGTGATGGGATACTCAA CGGAATGC-3
Primer 6	Reverse primer to amplify 2.0-kb 3′ region of *prtT* for construction of disruption cassette	5-CTCCCAGGAACAGGCTACACAGCCG-3
Primer 2	Forward primer to amplify 3.5-kb *pyrG* marker from pyrG-Anid plasmid for construction of disruption cassette	5-AGATCAAATACGAAACGCCTTCTTCATGG GAGCACAAGAGCTTGGCGAGTTAATTAAG GATGGCG-3
Primer 5	Reverse primer to amplify 3.5-kb *pyrG* marker from pyrG-Anid plasmid for construction of disruption cassette	5-CGATACGCTGCTAGCCCAGTCACAGGCAT TCCGTTGAGTATCCCATAAGCTGGAGCTC CACCGCGG-3
Primer 7	Forward primer to amplify 7.8 kb of complete *prtT* disruption cassette	5-TGTCGAGCGTCATGGCTAATAACGTTA-3
Primer 8	Reverse primer to amplify 7.8 kb of complete *prtT* disruption cassette	5-GCCAAAAGATTACTGAAATCTATACCTGC A-3
Out-Ds-F	Forward primer to amplify 2.2-kb fragment to determine the correct integration of *prtT* disruption cassette at the *prtT* locus	5-CCCTTCTGAAGTGTGTATTGAGC-3
Out-Ds-R	Reverse primer to amplify 2.2-kb fragment to determine the correct integration of *prtT* disruption cassette at the *prtT* locus	5-GAACTACCCCATCTACCAAGACACC-3
prtT-F	Forward primer to amplify 1.8-kb fragment of *prtT* in RT-PCR for expression analysis	5-GAAACGCCTTCTTCATGGGAG-3
prtT-R	Reverse primer to amplify 1.8-kb fragment of *prtT* in RT-PCR for expression analysis	5-GAGTCGATAGCGCCCTTGG-3
gpd-F	Forward primer to amplify 1.3-kb fragment of *gpdA* in RT-PCR for loading control	5-CCATCCTCTCTTCATCACTTCCCTC-3
gpd-R	Reverse primer to amplify 1.3-kb fragment of *gpdA* in RT-PCR for loading control	5-TCGGCAATCAGTCTCACTCACAG-3
pepA-F	Forward primer to amplify 642-bp fragment of *pepA* in RT-PCR for expression analysis	5-GGCTACACTTGGGACATCTCCTACG-3
pepA-R	Reverse primer to amplify 642-bp fragment of *pepA* in RT-PCR for expression analysis	5-AGTCGAGATGGGAGCGTAGTTG-3
pepB-F	Forward primer to amplify 900-bp fragment of *pepB* in RT-PCR for expression analysis	5-TCTGGATCCATGAAGTTCTCTACCATCCT-3
pepB-R	Reverse primer to amplify 900-bp fragment of *pepB* in RT-PCR for expression analysis	5-GATGAATTCGACGTAGGTGACAGTGACG C-3
pepD-F	Forward primer to amplify 1.1-kb fragment of *pepD* in RT-PCR for expression analysis	5-TTCAGTGCCACAGAACAGGTCC-3
pepD-R	Reverse primer to amplify 1.1-kb fragment of *pepD* in RT-PCR for expression analysis	5-CCACATTGGTGACAGCATTCC-3
pepF-F	Forward primer to amplify 856-bp fragment of *pepF* in RT-PCR for expression analysis	5-ATGCTGTTTCGCAGTCTGTTGTC-3
pepF-R	Reverse primer to amplify 856-bp fragment of *pepF* in RT-PCR for expression analysis	5-CACATTGCTCATGGATGCTCTCTAG-3

Accession nZ4umber of genomic sequence of *A. niger* from JGI (http://genome.jgi-psf.org/Aspni5/Aspni5.home.html); *prtT* (57330), *pepA* (201655), *pepB* (52082), *pepD* (203039), *pepF* (56161)

PY11 genomic DNA as the template and primers 1 and 3, and 4 and 6. In a separate reaction and the selectable marker, *pyrG* was amplified using plasmid pyrG-Anid as the template and primers 2 and 5. The amplified *pyrG* gene was flanked by 34-bp direct repeats of *loxP* for marker recycling. A PCR amplification mixture was set up in a 0.2-mL PCR tube containing 20 μL reaction mixture consisting of 0.5 μL of each primer (20 μM), 1 μL of genomic DNA (about 100 ng in each reaction mixture), 10 μL of 2X *Phusion*™ FlashHigh-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) and 8 μL of double distilled water. PCR was performed in a Tpersonal Thermal Cycler (Biometra, Göttingen, Germany) programmed as follows: 94°C for 2 min (1 cycle); followed by 94°C for 1 min, 55°C for 30 s and 72°C for 15 s/1-kb PCR product (30 cycles); finally 72°C for 1 min (1 cycle). The PCR products were separated by electrophoresis on 1% (w/v) agarose gels and further purified from the gel with MEGA-spin*TM* Agarose Gel Extraction kit (Intron Biotechnology, Gyeonggi-do, Korea). In a second round of PCR, all three purified PCR products of the first round PCR were combined and a fusion product amplified using nested primers 7 and 8 in Phusion™ FlashHigh-Fidelity PCR Master Mix (Finnzymes). In this "fusion" PCR, the upstream and downstream regions of *prtT* were linked to the *pyrG* marker via the complementary overlapping regions that overlap.

For transformation, 1.1 μg of purified disruption cassette was used. Transformation of PY11 was performed as described by Storms et al. [20]. Transformants were isolated by selection on MM agar without uracil and uridine. Integration of the disruption cassette at the correct locus in the transformants was verified by PCR. The forward primer (Out-Ds-F) for verification was designed to complement the *pyrG* sequences in the cassette while the reverse primer (Out-Ds-R) was designed to complement the locus outside the amplified 3′-region of *prtT*. DNA was extracted from PY11 and An*ΔprtT* for Southern blot analyses, 20 μg aliquots digested with *Bam*HI and *Eco*RV and run on 1% (w/v) agarose gels. The restricted DNA was transferred to Nylon Hybond + membranes and hybridised with appropriate probes.

2.3 Extraction of Genomic DNA

Conidia (1×10^6) were added to 250-mL CM media, incubated at 30°C for 16 h (shaking at 250 rpm), filtered and frozen with liquid nitrogen prior to genomic DNA extraction as described by Oh et al. [24]. Briefly, the ground frozen mycelia were transferred into 10 mL extraction buffer before cold 20% polyvinylpyrrolidone (PVP) was added at a final concentration of 6% (v/v). Following an addition of SDS (2% w/v), the mixture was incubated at 65°C for 10 min. Subsequently, 0.1 volumes of 5 M potassium acetate was added and the mixture was incubated on ice for 30 min followed by centrifugation at 13,000 rpm (Sigma 3–18 K, UK), 4°C for 10 min. The supernatant was transferred into a fresh tube, and 5 μL of RNase A (10 mg/mL) was added. The mixture was incubated at 37°C for 30 min. Further DNA precipitation and purification steps were carried out using standard methods as described by Sambrook and Russell [22].

2.4 RNA Extraction and RT-PCR Analysis

Total RNA was extracted from cells grown in MM medium (inoculated with overnight precultures in CM) containing 0.5% (w/v) BSA and 0.5% (w/v) gelatin (induced medium) or dextrose (non-induced medium) for 8 h at 30°C. Total RNA was isolated from each strain using the TRIZOL™ Reagent (Life Technologies, California, USA) following the filamentous fungus protocol. For RT-PCR analysis, cDNA was synthesised from each DNase-treated RNA preparation using Superscript™ III First Strand Synthesis System (Life Technologies, Carlsbad, California, USA). PCR was carried out using *A. niger* specific primers for *prtT*, glyceraldehyde-3-phosphate dehydrogenase (*gpd*) as a loading control and selected protease genes (about 20 ng for each reaction mixture).

2.5 Growth Analysis

Conidia (1×10^5) were used to inoculate 50 mL MM with 1% (w/v) glucose and incubated at 30°C (shaking at 200 rpm) for 6 days. Every 24 h, samples were drawn from each flask and mycelia harvested by filtration. The biomass was washed three times with deionised water and then dried in an oven at 60°C for 24 h.

2.6 Analysis of Fungal Enzymatic Activity

Proteolytic and starch-degrading activities were assessed on solid media by spotting 10^5 conidia on casein–gelatin and starch agar plates, respectively. The colonies were grown for 7 days, and the growth was monitored by measuring the radius of the fungal colonies. The diameter of the halo zones was measured.

2.7 Extracellular Protease Assay

Extracellular proteolytic activity in culture filtrates was determined as described by van den Hombergh et al. [25] with the following modifications. A total of 250 μL culture filtrate was incubated with 67 μL of 3% (w/v) BSA (fraction V) (Sigma–Aldrich, Missouri, MO, USA) in 0.1 mM sodium acetate buffer (pH 4.0) at 30°C. Buffers and substrate solutions contained 5 mM sodium azide to prevent microbial growth. After 30 min, reactions were stopped with 500 μL of 10% (w/v) trichloroacetic acid (TCA). After holding at

0°C for 30 min, precipitated proteins were removed by centrifugation at 10, 000×g for 5 min. Subsequently, 250 μL of the TCA-soluble fraction was used for colorimetric detection of protease activity by adding 625 μL of 0.5 M sodium carbonate followed by 125 μL Folin and Ciocalteu's reagent (diluted 1: 3 with distilled water). The mixture was incubated at 37°C for 30 min for colour development. Of the 1-mL mixture, 200 μL was placed in 96-well micro-titre plate and the optical density was measured at 655 nm. Tyrosine was used as the standard. One unit of enzyme activity was defined as the amount of enzyme that produced a colour equivalent to 1.0 μmole of tyrosine per min at pH 4.0 at 37°C from BSA (colour by Folin and Ciocalteu's reagent). Extracellular protease activities were expressed in units per mL of culture filtrate. All experiments were carried in triplicate.

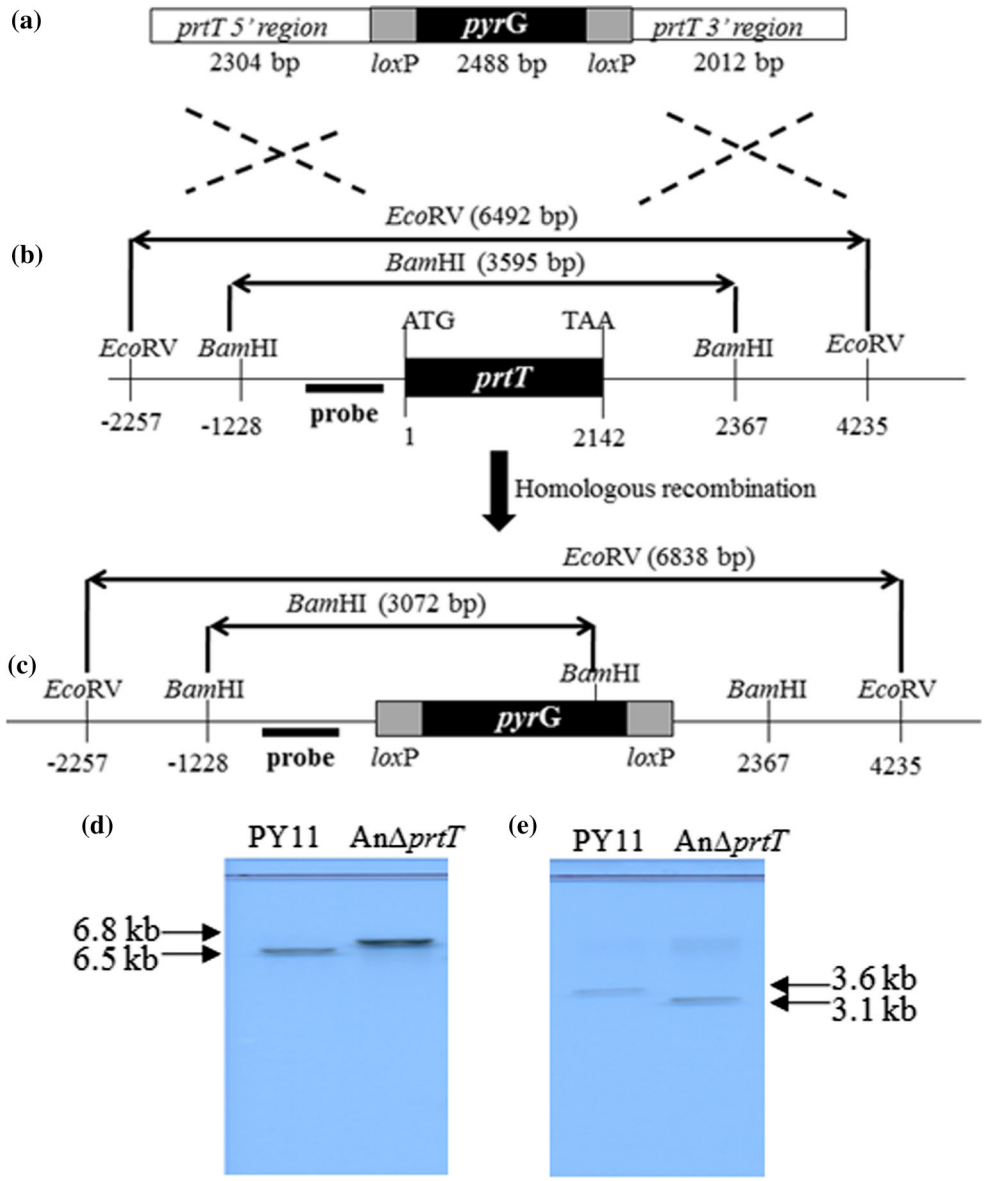


Fig. 2 Schematic representation of *prtT* replacement. **a** *prtT* disruption cassette. **b** Predicted restriction map of the *prtT* locus in *A. niger* PY11. The dotted line between **a** and **b** represents crossing-over between two homologous regions during disruption of *prtT*. **c** Predicted restriction map of the deleted *prtT* allele. **d** Southern blots using genomic DNA digested with *Eco*RV. **e** Southern blot using genomic DNA digested with *Bam*HI. Both (d) and (e) were probed with a 1.1-kb *prtT* 5′-fragment. Lane 1: PY11; Lane 2: An$\Delta prtT$. In the wild type, 6.5- and 3.6-kb DNA fragments were detected in genomic DNA digested with *Eco*RV and *Bam*HI, respectively. In An$\Delta prtT$, the expected 6.8- and 3.1-kb bands were identified

2.8 Transformation of Reporter Gene Expression Cassette

The efficiency of An$\Delta prtT$ and PY11 as hosts for heterologous protein production was tested by transforming both strains with plasmid ANIp2-cut which carries the *Glomerella cingulata* cutinase gene. The ANIp2 backbone vector contains the highly inducible promoter of glucoamylase (*Gla*Pr) to drive expression of the gene of interest, the glucoamylase terminator (*Gla*Tt), *pyrG* as a selectable marker and an ampicillin resistance gene. Transcription of the glucoamylase A promoter is highly induced by maltose. Transformants were selected for their abilities to grow on a MM agar without uridine. The integration of ANIp2-cut vector carrying the cutinase gene in the genome of the transformants was verified by PCR. Culture filtrates for cutinase assays were prepared by inoculating 50-mL MMJ medium in 150-mm-diameter Petri dishes. Cultures were harvested at day 4, and production of the recombinant cutinases was visualised by SDS-PAGE electrophoresis. Concentrations of total proteins were measured using the Bradford assay [26], with BSA as the standard. Immuno-detection of the cutinase was performed using antibodies raised against the *G. cingulata* cutinase that was produced in our laboratory by Mohd Khairul Ikhsan dan Mohd Rohaizad (personal communication).

2.9 Cutinase Assay

Cutinase activity was determined using p-nitrophenyl laurate (pNPL) (Sigma-Aldrich) as a substrate by the method described by Kumar et al. [27]. The assay was carried out by adding 1 µL of culture filtrate to 2.5 µL of 20 µM pNPL and the mixture topped up to 100 µL with 0.5 M Tris-HCl buffer (pH 8.0) prior to incubation for 10 min at room temperature. The reaction was stopped by freezing at − 20 °C for 8 min. The p-nitrophenol produced was quantified by reading the absorbance at 405 nm. One unit of cutinase activity was defined as the amount of enzyme required to convert 1 µmol of pNPL to p-nitrophenol per minute, under the specified conditions. All experiments were carried out three times.

2.10 Stability of the Heterologous Cutinase

To compare the stability of the heterologous cutinase during storage, culture filtrates of PY11 and An$\Delta prtT$ were

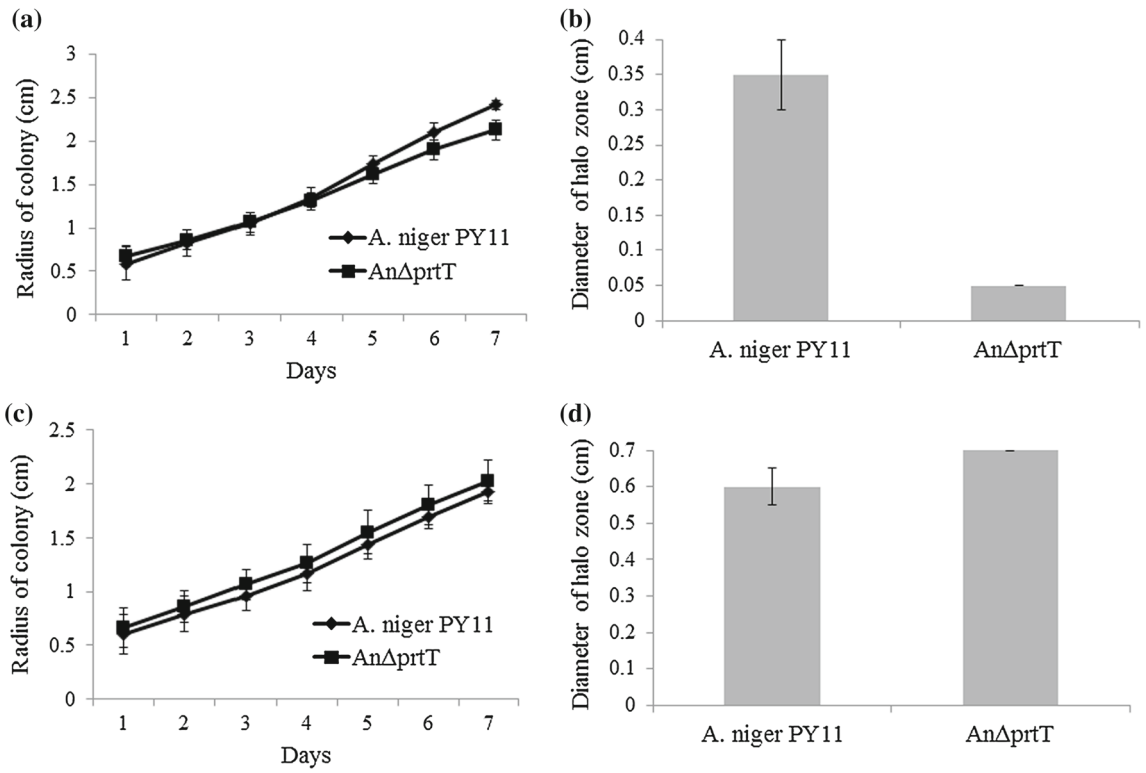


Fig. 3 Proteolytic and starch-degrading activities of An$\Delta prtT$ as compared to PY11. **a** Colonial growth of PY11 and An$\Delta prtT$ on MM agar containing 1% (w/v) casein and 1% (w/v) gelatin. **b** Proteolytic halo (on day 7) of PY11 and An$\Delta prtT$ on MM agar containing casein (1%) and gelatin (1%). **c** Colonial growth of PY11 and An$\Delta prtT$ on MM agar containing starch (1%). **d** Starch degradation (on day 7) of PY11 and An$\Delta prtT$ on MM agar containing starch (1%). The results are expressed as the mean ± standard deviation

filter-sterilised using 0.22 μm filters (Sartorius AG, Göttingen, Germany). Portions (20 μL) in 0.2-mL PCR tubes were incubated at 4 or 25°C, and the residual cutinase activity was determined every week for a total of 6 weeks when cutinase stability was followed at 4°C and every 2 days for a total of 14 days when cutinase stability was followed at 4°C.

3 Results

3.1 Deletion of *prtT*

To delete *prtT* in *A. niger* PY11, a disruption cassette was constructed. In three separate PCR syntheses, the *pyrG* gene as well as the upstream and downstream regions of *prtT* was amplified, yielding the predicted 3.5-, 2.3- and 2.0-kb fragments, respectively. Fusion PCR gave the complete 7.8-kb disruption cassette which was used to transform *A. niger* PY11. Transformation efficiency of 538.2 transformants per μg DNA was obtained. A total of eight uridine prototrophs were purified and screened by PCR for positive insertion mutants. PCR analysis identified one transformant, designated as An$\Delta prtT$, in which the disruption cassette had apparently replaced the native *prtT* locus (data not shown). Genomic DNA isolated from An$\Delta prtT$ was digested with both *Bam*HI and*Eco*RV. The digested DNA was probed with the 1.1-kb fragment of the 5′ region of *prtT*. Hybridisation with this probe highlighted a 6.8-kb *Eco*RV and 3.1-kb *Bam*HI DNA fragment, thereby confirming that deletion cassette had replaced the wild type *prtT* locus (Fig. 2).

Protease plate assays conducted on the An$\Delta prtT$ mutant confirmed that this strain exhibits the mutant phenotype, characterised by loss of halo production on MM containing casein and gelatin plates even though the mutant has similar colonial growth to the parent strain (Fig. 3a, b). Starch degradation was not affected in An$\Delta prtT$, suggesting that it is not impaired in the general secretion of proteins such as amylases (Fig. 3c, d). Conidia production by An$\Delta prtT$ on minimal medium containing BSA as the sole nitrogen source was poor (data not shown), suggesting that the ability of An$\Delta prtT$ to degrade proteins was significantly impared. Hyphal growth of PY11 and An$\Delta prtT$ with nitrate as the nitrogen source was comparable, indicating that disruption of *prtT* did not affect fungal hyphal growth (Fig. 4a). In contrast, the proteinase activity measured by BSA degradation by culture filtrates prepared using PY11 increased linearly throughout six-day growth, whereas the protease activity of An$\Delta prtT$ remained very low (Fig. 4b).

3.2 Expression of *A. niger* Extracellular Protease Genes

Deletion of *prtT* in *A. niger* AB1.13 by Punt et al. [15] showed that at least four extracellular proteases including

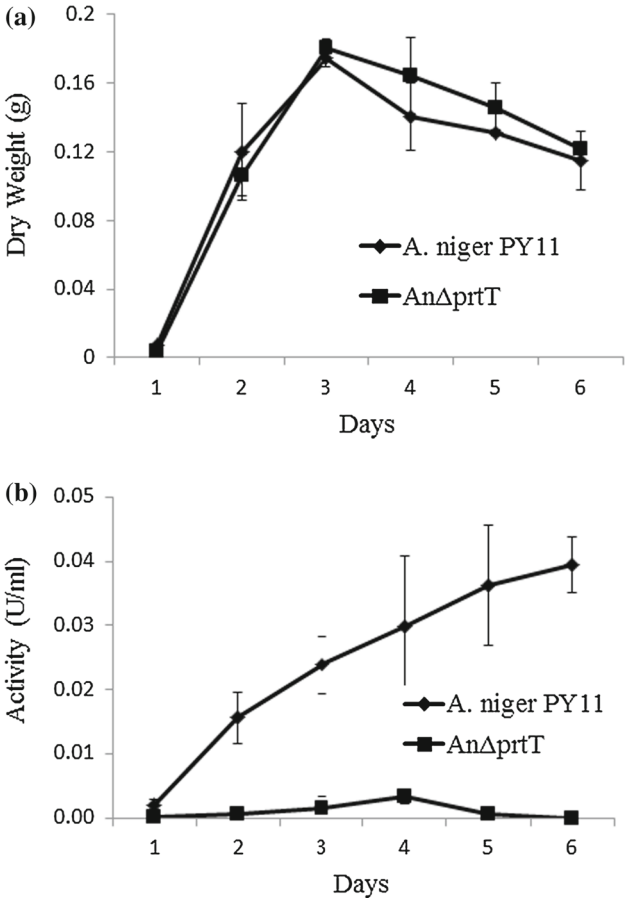


Fig. 4 Growth and extracellular proteolytic activity of An$\Delta prtT$ and PY11. **a** Growth of An$\Delta prtT$ in comparison with PY11. Strains were grown for 6 days at 30 °C in shaken MM supplemented with 1% (w/v) glucose. The biomass was filtered, washed, dried and weighed. **b** BSA degradation by An$\Delta prtT$ and PY11. Strains were grown for 6 days at 30 °C in shaken MM supplemented with 1% (w/v) glucose and 3% (w/v) wheat bran. Supernatants were incubated with BSA, and the release of soluble peptides was monitored by colorimetric detection (at 655 nm) using Folin and Ciocalteu's reagent. The results are representative of two independent experiments and are expressed as the mean ± standard deviations

aspergillopepsin A, *pepA* (201655), aspergillopepsin B, *pepB* (52082), subtilisin-like serine protease, *pepD* (203039), and serine-type carboxypeptidase, *pepF* (56161), are under the control of this transcription factor in *A. niger*. As compared to strain AB1.13, in strain PY11, reduced expression of *pepA*, *pepB* and *pepF* in An$\Delta prtT$ under both protease inducing and non-inducing conditions was apparent (Fig. 5a–e). These results show that *prtT* is important in modulating mRNA expression from the genes for these extracellular proteases. The *pepD* gene product could not be detected in either strain under any conditions, suggesting that it is not expressed at significant levels under the conditions tested. Primers used to amplify *pep*D were confirmed to be working in the control reaction containing *A. niger* genomic DNA (data not shown).

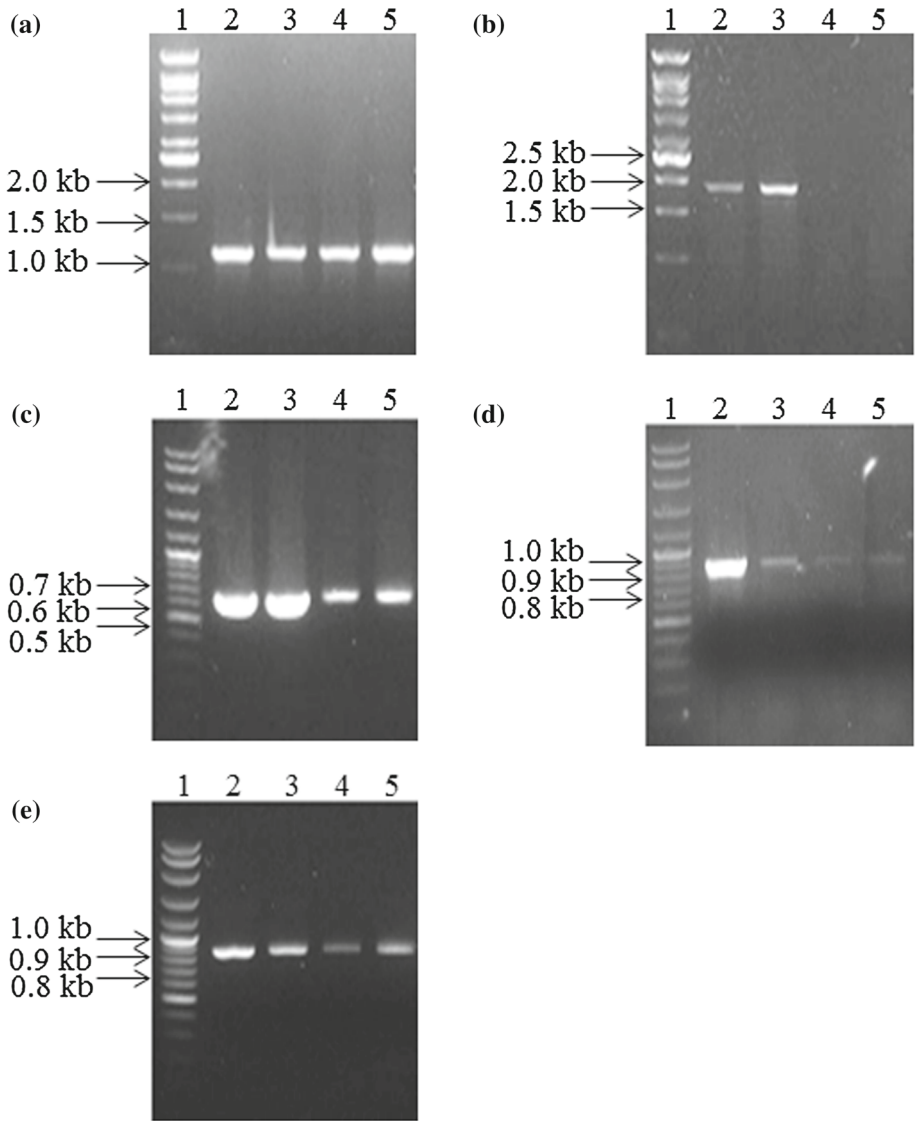


Fig. 5 RT-PCR profiles of relative expression of extracellular protease genes in PY11 and An$\Delta prtT$. Strains were cultured in MM containing 0.5% (w/v) BSA and 0.5% (w/v) gelatin (protease inducing media) and MM containing dextrose (non-inducing media). Expression was determined by RT-PCR using primers given in Table 1. **a** Expression of *A. niger gpdA* (1.3-kb amplicon), a housekeeping gene used as loading control. **b** Transcripts of *prtT* (1.8-kb amplicon) in An$\Delta prtT$ were not detected. **c**–**e** Expression of *pepA* (642-bp PCR product), *pepB* (900-bp PCR product) and *pepF* (856-bp PCR product), respectively. Lane 1: 1-kb DNA ladder for (**a**) and (**b**), 100-bp DNA ladder for (**c**)–(**e**); Lane 2: un-induced PY11; Lane 3: induced PY11; Lane 4: un-induced An$\Delta prtT$ mutant; Lane 5: induced An$\Delta prtT$ mutant

3.3 Effect of *prtT* Deletion on Heterologous Cutinase Production

To assess the suitability of An$\Delta prtT$ as hosts for improved heterologous protein production, *G. cingulata* cutinase expression by ANIp2-cut transformants of An$\Delta prtT$ and its parent strain PY11 was compared by SDS-PAGE and Western blot analyses. Both An$\Delta prtT$ and PY11 were able to express a distinct band corresponding to the 21-kDa cutinase (Fig. 6a, b). Cutinase activity was also detected in culture filtrates of both strains, indicating that the recombinant cutinase was secreted from *A. niger*. In contrast, neither *G. cingulata* cutinase protein nor its activity was detected in control culture filtrates from PY11 and An$\Delta prtT$ that did not harbour ANIp2-cut. For each strain, two independent cutinase-producing transformants were analysed. The activity of the heterologous cutinase produced by An$\Delta prtT$-cut1 and An$\Delta prtT$-cut2 transformants was 36.3 and 36.7 U/mL, respectively, which was higher than the PY11-cut1 and PY11-cut2 transformants (21.2 and 20.4 U/mL) (Fig. 7a, b). This indicates that the deletion of *prtT* did not limit the ability of An$\Delta prtT$ to efficiently produce the heterologous *G. cingulata* cutinase.

The stability of *G. cingulata* cutinase in culture filtrates of PY11 and An$\Delta prtT$ was compared by incubating the culture filtrates at 4 °C for six weeks and at 25 °C for two weeks. The degradation of the heterologous cutinase at 4 °C was determined weekly by measuring the residual activity of the target protein. The recombinant cutinases of both strains were stable at 4 °C with minimum proteolytic degradation over a six-week incubation (Fig. 8a). At 25 °C, the residual activity was measured every second day. An$\Delta prtT$-cut1 and An$\Delta prtT$-cut2 retained about 80% of their initial cutinase activities over the entire 14-day incubation period, whereas

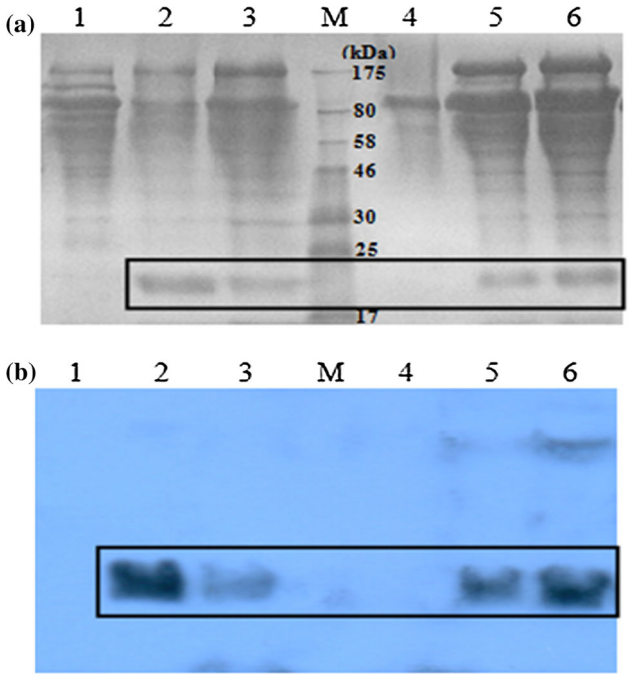


Fig. 6 SDS-PAGE (**a**) and Western blot (**b**) profiles of proteins secreted by ANIp2-cut transformants of PY11 and An$\Delta prtT$ as compared to their respective hosts. Lane 1: crude extracts of untransformed PY11, as a control; Lane 2: crude extracts of PY11-cut1; Lane 3: crude extracts of PY11-cut2; Lane 4: crude extracts of untransformed An$\Delta prtT$, as a control; Lane 5: crude extracts of An$\Delta prtT$-cut1; Lane 6: crude extracts of An$\Delta prtT$-cut2. Lane M contains protein marker (NEB, England)

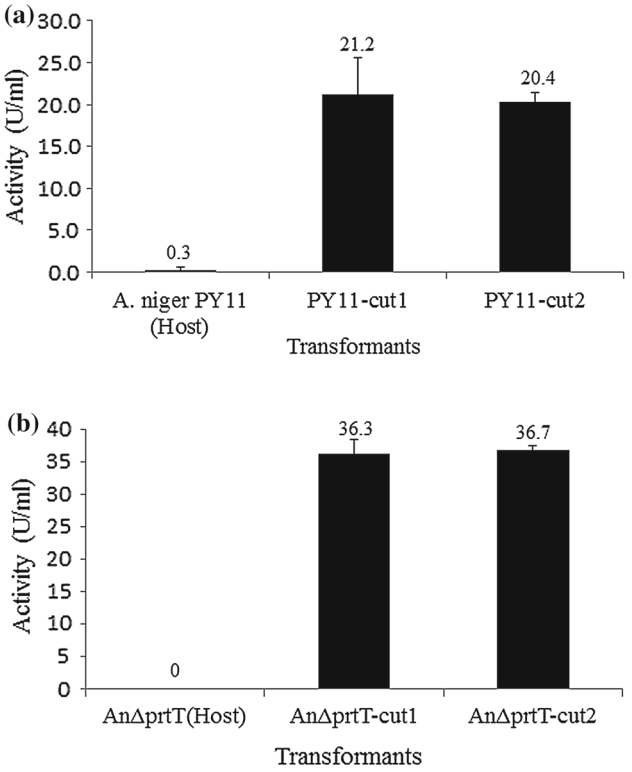


Fig. 7 Heterologous cutinase activities of PY11- and An$\Delta prtT$-producing cutinase transformants as compared to their respective hosts. **a** Heterologous cutinase activity of PY11-producing cutinase. **b** Heterologous cutinase activity of An$\Delta prtT$-producing cutinase. The results are expressed as the mean ± standard deviation

PY11-cut1 and PY11-cut2 lost more than 50% of their initial activities after six days of incubation and retained negligible activity after 14 days (Fig. 8b). We conclude that the decrease in extracellular proteolytic activity of An$\Delta prtT$ retards degradation of the heterologous protein in the culture filtrate.

4 Discussion

As heterologous proteins produced from *A. niger* are prone to proteolytic degradation, understanding regulation of secreted proteases is crucial in developing improved expression hosts. In this work, we describe the deletion of the *A. niger prtT* gene and analyse the effects of extracellular protease limitation on the activity of a heterologous protein produced by the mutant strain. Disruption of *prtT* results in significantly reduced secreted protease activity that is characterised by loss of halo production on MM containing casein and gelatin. BSA degradation is also starkly reduced even after induction with 3% (w/v) wheat bran. These results accord with the *prtT* mutant phenotypes in *A. niger*, *A. oryzae* and *A. fumigatus* [15,17] where *prtT* is a central activator of secreted protease expression in *A. fumigatus, A. niger* and *A. oryzae*.

We measured the transcript levels of four signal peptide-containing proteases including *pepA*, *pepB*, *pepD* and *pepF* in both PY11 and An$\Delta prtT$, but the *pepD* transcript was not detected in either strain under any conditions. *pepD* encodes a serine alkaline protease which is a homologue of *A. fumigatus ALP1* and *A. oryzae Alp1* [15,17], both of which are regulated by *prtT*. Expression of *pepA*, *pepB* and *pepF* was reduced in An$\Delta prtT$. According to van den Hombergh et al. [28], the acid proteases PEPA and PEPB constitute 84 and 6% of the extracellular proteolytic activity of *A. niger*, respectively. This clearly demonstrates that *prtT* is a major transcription factor. Deletion of *pepA* resulted in reduced degradation of bovine prochymosin and increased expression of thaumatin in *A. awamori* [7,29], a secreted serine carboxypeptidase isolated from *A. saitoi* and *A. fumigatus* is a homologue of *A. niger pepF* [17,30], and its expression level was also reduced in the *A. fumigatus prtT* mutant.

Regulation of *prtT*-dependent proteases varies among *Aspergilli*. Four proteases are under *prtT* control in *A. niger*, three of which (*pepA*, *pepB* and *pepF*) have acid pH optima [31]. Yet disruption of *prtT* in *A. oryzae* results in the absence of AlpA (an alkaline protease) and Np1 (a metalloprotease) that are homologues of Alp1 and Mep in *A. fumigatus*, respec-

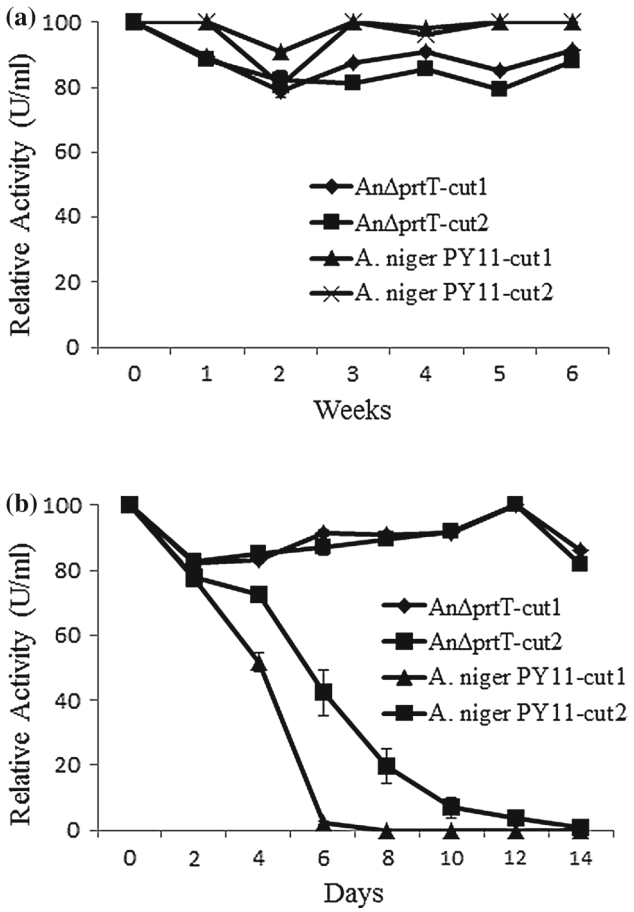


Fig. 8 Stability of heterologous cutinase in the culture filtrates of PY11- and An$\Delta prtT$-producing cutinase. **a** Relative activity profile of heterologous cutinase incubated at 4 °C for 6 weeks. **b** Relative activity profile of heterologous cutinase incubated at 25 °C for 2 weeks. Per cent relative activity based on an initial activity of 100%

tively [15–17]. ALP and MEP are responsible for most of the proteolytic activity detected in non-acid producing aspergilli, such as *A. fumigatus, A. nidulans* and *A. oryzae* [32–34]. Yet other protease genes such as sedolisin, *sedB*, and dipeptidyl-peptidase, *dppV*, are not regulated by *prtT* in *A. fumigatus* [16].

Development of protease-deficient mutants is a useful tool in reducing the degradation of heterologous proteins in *Aspergillus*. In *A. oryzae*, deletion of ten protease genes improved the production of bovine chymosin and human lysozyme [35]. However, in most filamentous fungi, multiple gene disruptions are often tedious and time-consuming due to high frequency of illegitimate integration of the transformed DNA, resulting in low gene targeting efficiencies. Thus, a large number of transformants need to be screened for the desired mutant [36,37]. For successive gene disruptions in filamentous fungi, one might need larger homologous flanking sequences of the target gene to enhance gene targeting, multiple auxotrophic markers in a single host and a highly efficient gene targeting system [37,38].

We show here, however, that deletion of *prtT* is a simpler and more effective way of reducing the effect of proteases on heterologous proteins. This is due to the role of *prtT* as a regulator that regulates the expression of major protease genes and its deletion would result in a reduction of extracellular proteases. The loss of major proteases in the culture filtrate of Δ*prt*T mutant may lead to less proteolytic degradation of heterologous protein that ultimately contributes to the stability of the heterologous cutinase.

Besides using protease-deficient mutants, reduction of native protease levels can also be accomplished by applying various growth manipulation strategies, including fungal morphology manipulation as well as optimisation of medium composition and fermentation protocols [39–42]. However, the selection of culture conditions to reduce protease activity is not straightforward as some factors involved are interrelated and might have unexpected antagonistic or synergistic effects [39].

Although proteolysis of heterologous proteins during storage can be overcome by adding a protease inhibitor cocktail to improve protein stability, however, this strategy is expensive and economically unprofitable for a large-scale production [43]. Thus, the use of Δ*prt*T mutant that exhibits a low extracellular protease activity as a host to produce heterologous proteins could be an alternative way as compared to the use of protease inhibitors in minimising degradation and lengthening the shelf life of recombinant proteins during storage.

Nevertheless, the basal proteolytic activity observed in An$\Delta prtT$ culture filtrates indicates that proteases are still produced by the mutant. This suggests that other transcription factors are involved in the expression of secreted proteases by *A. niger*. Wide domain regulatory genes such as *creA* (carbon catabolite repressor) and *areA* (nitrogen metabolite repressor) also affect the expression of proteases [44]. In *Saccharomyces cerevisiae*, the heat shock proteins Hsp12 and Hsp26 are regulated by both Hsf1p and the Msn2/4p transcription factors [45]. Proteomics-based secretome analyses of An $\Delta prtT$ and other regulatory mutants would facilitate assigning proteins to regulons.

5 Conclusion

In summary, this work shows that the deletion of Δ*prt*T transcription factor resulted in the reduction of major extracellular proteases produced by *A. niger* during growth. The lack of these proteases in the medium resulted in the improved activity and stability of recombinant cutinase produced by this fungus.

Acknowledgements This research project is funded by the Ministry of Science, Technology and Innovation, Malaysia, under the Grant 09-05-MGI-GMB003 and FP 0813B029[K2]. Nurhaida Kamaruddin is supported financially by Universiti Pendidikan Sultan Idris, Perak, Malaysia. We thank Mohd Khairul Ikhsan dan Mohd Rohaizad for kindly providing the *G. cingulata* anti-cutinase polyclonal antibody.

References

1. Meyer, V.; Fiedler, M.; Nitsche, B.; King, R.: The cell factory *Aspergillus* enters the big data era: opportunities and challenges for optimising product formation. Adv. Biochem. Eng. Biotechnol. **149**, 91–132 (2015)
2. Zhang, H.; Wang, S.; Zhang, X.X.; Ji, W.; Song, F.; Zhao, Y.; Li, J.: The *amyR*-deletion strain of *Aspergillus niger* CICC2462 is a suitable host strain to express secreted protein with low background. Microb. Cell Factories **15**, 68 (2016)
3. Sharma, R.; Katoch, M.; Srivastava, P.S.; Qazi, G.N.: Approaches for refining heterologous protein production in filamentous fungi. World J. Microbiol. Biotechnol. **25**(12), 2083–2094 (2009)
4. Wang, Y.; Xue, W.; Sims, A.H.; Zhao, C.; Wang, A.; Tang, G.; Qin, J.; Wang, H.: Isolation of four pepsin-like protease genes from *Aspergillus niger* and analysis of the effect of disruptions on heterologous laccase expression. Fungal Genet. Biol. **45**, 17–27 (2008)
5. Zhu, L.; Nemoto, T.; Yoon, J.; Maruyama, J.; Kitamoto, K.: Improved heterologous protein production by a tripeptidyl peptidase gene (*AosedD*) disruptant of the filamentous fungus *Aspergillus oryzae*. J. Genet. Appl. Microbiol. **58**(3), 199–209 (2012)
6. Bartling, S.; van den Hombergh, J.P.T.W.; Olsen, O.; von Wettstein, D.; Visser, J.: Expression of an *Erwinia* pectate lyase in three species of *Aspergillus*. Curr. Genet. **29**, 474–481 (1996)
7. Berka, R.M.; Ward, M.; Wilson, L.J.; Hayenga, K.J.; Kodama, K.H.; Carlomagno, L.P.; Thompson, S.A.: Molecular cloning and deletion of the gene encoding aspergillopepsin a from *Aspergillus awamori*. Gene **86**, 153–162 (1990)
8. Inoue, H.; Kimura, T.; Makabe, O.; Takahashi, K.: The gene and deduced protein sequences of the zymogen of *Aspergillus niger* acid proteinase A. J. Biol. Chem. **266**, 19484–19489 (1991)
9. Krishnan, S.; Vijayalakshimi, M.A.: Purification of an acid protease and a serine carboxypeptidase from *Aspergillus niger* using metal-chelate affinity chromatography. J. Chromatogr. **329**, 165–170 (1985)
10. Mattern, I.E.; van Noort, J.M.; van den Berg, P.; Archer, D.B.; Roberts, I.N.; van den Hondel, C.A.: Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. Mol. Gen. Genet. **234**, 332–336 (1992)
11. van den Hombergh, J.P.T.W.; Jarai, G.; Buxton, F.P.; Visser, J.: Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. Gene **151**, 73–79 (1994)
12. Jarai, G.; Kirchherr, D.; Buxton, F.P.: Cloning and characterization of the *pepD* gene of *Aspergillus niger* which codes for a subtilisin-like protease. Gene **139**, 51–57 (1994)
13. Frederick, G.D.; Rombouts, P.; Buxton, F.P.: Cloning and characterisation of *pepC*, a gene encoding a serine protease from *Aspergillus niger*. Gene **125**, 57–64 (1993)
14. Jarai, G.; van den Hombergh, J.P.T.W.; Buxton, F.P.: Cloning and characterization of the *pepE* gene of *Aspergillus niger* encoding a new aspartic protease and regulation of *pepE* and *pepC*. Gene **145**, 171–178 (1994)
15. Punt, P.J.; Schuren, F.H.; Lehmbeck, J.; Christensen, T.; Hjort, C.; van den Hondel, C.A.: Characterization of the *Aspergillus niger* *prtT*, a unique regulator of extracellular protease encoding genes. Fungal Genet. Biol. **45**, 1591–1599 (2008)
16. Bergmann, A.; Hartmann, T.; Cairns, T.; Bignell, E.M.; Krappmann, S.: A regulator of *Aspergillus fumigatus* extracellular proteolytic activity is dispensable for virulence. Infect. Immun. **77**(9), 4041–4050 (2009)
17. Sharon, H.; Hagag, S.; Osherov, N.: Transcription factor *prtT* controls expression of multiple secreted proteases in the human pathogenic mold *Aspergillus fumigatus*. Infect. Immun. **77**(9), 4051–4060 (2009)
18. Pel, H.J.; de Winde, J.H.; Archer, D.B.; Dyer, P.S.; Hofmann, G.; Schaap, P.J.; Turner, G.; de Vries, R.P.; Albang, R.; Albermann, K.; Andersen, M.R.; Bendtsen, J.D.; Benen, J.A.E.; van den Berg, M.; Breestraat, S.; Caddick, M.X.; Contreras, R.; Cornell, M.; Coutinho, P.M.; Danchin, E.G.J.; Debets, A.J.M.; Dekker, P.; van Dijck, P.W.M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A.J.M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G.S.P.; de Groot, P.W.J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J.P.T.W.; van den Hondel, C.A.M.J.J.; van der Heijden, R.T.J.M.; van der Kaaij, R.M.; Klis, F.M.; Kools, H.J.; Kubicek, C.P.; van Kuyk, P.A.; Lauber, J.; Lu, X.; van der Maarel, M.J.E.C.; Meulenberg, R.; Menke, H.; Mortimer, M.A.; Nielsen, J.; Oliver, S.G.; Olsthoorn, M.; Pal, K.; van Peij, N.N.M.E.; Ram, A.F.J.; Rinas, U.; Roubos, J.A.; Sagt, C.M.J.; Schmoll, M.; Sun, J.; Ussery, D.; Varga, J.; Vervecken, W.; van den Vondervoort, P.J.J.; Wedler, H.; Wosten, H.A.B.; Zeng, A.P.; van Ooyen, A.J.J.; Visser, J.; Stam, H.: Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. **25**(2), 221–231 (2007)
19. Bos, C.J.; Debets, A.J.; Swart, K.; Huybers, A.; Kobus, G.; Slakhorst, S.M.: Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr. Genet. **14**, 437–443 (1988)
20. Storms, R.; Zheng, Y.; Li, H.; Sillaots, S.; Martinez-Perez, A.; Tsang, A.: Plasmid vectors for protein production, gene expression and molecular manipulations in *Aspergillus niger*. Plasmid **53**, 191–204 (2005)
21. Kafer, E.: Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. **19**, 33–131 (1977)
22. Sambrook, J.; Russel, D.W.: Molecular Cloning: A Loboratory Manual. Cold Spring Harbor Laboratory Press, New York (2001)
23. Master, E.R.; Zheng, Y.; Storms, R.; Tsang, A.; Powlowski, J.: A xyloglucan-specific family 12 glycosyl hydrolase from *Aspergillus niger*: recombinant expression, purification and characterization. Biochem. J. **411**, 161–170 (2008)
24. Oh, S.S.L.; Bakar, F.D.A.; Adnan, A.M.; Mahadi, N.M.; Hassan, O.; Murad, A.M.A.: Isolation and characterization of glyceraldehydes-3-phosphate dehydrogenase gene of *Trichoderma virens* UKM1. Biotechnology **8**, 194–203 (2009)
25. van den Hombergh, J.P.T.W.; van de Vondervoort, P.J.J.; van der Heijden, N.C.B.A.; Visser, J.: New protease mutants in *Aspergillus niger* result in strongly reduced in vitro degradation of target proteins: genetical and biochemical characterization of seven complementation groups. Curr. Genet. **28**, 299–308 (1995)
26. Bradford, M.M.: A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 1105–1112 (1976)
27. Kumar, S.; Kikon, K.; Upadhyay, A.; Kanwar, S.S.; Gupta, R.: Production, purification and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. Protein Expr. Purif. **41**, 38–44 (2005)
28. van den Hombergh, J.P.T.W.; Gelpke, M.D.S.; van de Vondervoort, P.J.I.; Buxton, F.P.; Visser, J.: Disruption of three acid proteases in *Aspergillus niger*—effects on protease spectrum, intracellular proteolysis, and degradation of target proteins. Eur. J. Biochem. **247**(2), 605–613 (1997)

29. Moralejo, F.J.; Cardoza, R.E.; Gutierrez, S.; Sisniega, H.; Faus, I.; Martin, J.F.: Overexpression and lack of degradation of thaumatin in an aspergillopepsin A-defective mutant of *Aspergillus awamori* containing an insertion in the *pepA* gene. Appl. Microbiol. Biotechnol. **54**, 772–777 (2000)
30. Chiba, Y.; Midorikawa, T.; Ichishima, E.: Cloning and expression of the carboxypeptidase gene from *Aspergillus saitoi* and determination of the catalytic residues by site-directed mutagenesis. Biochem. J. **308**, 405–409 (1995)
31. van den Hombergh, J.P.T.W.; van de Vondervoort, P.J.I.; Fraissinet-Tachet, L.; Visser, J.: *Aspergillus* as a host for heterologous protein production: the problem of proteases. Trends Biotechnol. **15**(7), 256–263 (1997)
32. Jaton-Ogay, K.; Paris, S.; Huerre, M.; Quadroni, M.; Falchetto, R.; Togni, G.; Latge, J.P.; Monod, M.: Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. Mol. Microbiol. **14**, 917–928 (1994)
33. Lehmbeck, J.: Alkaline protease deficient filamentous fungi. US patent US6291209 (2001)
34. vanKuyk, P.A.; Cheetham, B.F.; Katz, M.E.: Analysis of two *Aspergillus nidulans* genes encoding extracellular proteases. Fungal Genet. Biol. **29**, 201–210 (2000)
35. Yoon, J.; Maruyama, J.; Kitamoto, K.: Disruption of ten protease genes in the filamentous fungus *Aspergillus oryzae* highly improves production of heterologous proteins. Appl. Microbiol. Biotechnol. **89**(3), 747–759 (2011)
36. Krappmann, S.: Gene targeting in filamentous fungi: the benefits of impaired repair. Fungal Biol. Rev. **21**(1), 25–29 (2007)
37. Li, Z.-H.; Du, C.-M.; Zhong, Y.-H.; Wang, T.-H.: Development of a highly efficient gene targeting system allowing rapid genetic manipulations in *Penicillium decumbens*. Appl. Micobial. Biotechnol. **87**(3), 1065–1076 (2010)
38. Maruyama, J.-I.; Kitamoto, K.: Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (ΔligD) in *Aspergillus oryzae*. Biotechnol. Lett. **30**, 1811–1817 (2008)
39. Braaksma, M.; Smilde, A.K.; van der Werf, M.J.; Punt, P.J.: The effect of environmental conditions on extracellular protease activity in controlled fermentations of *Aspergillus niger*. Microbiology **155**(10), 3430–3439 (2009)
40. O'Donnell, D.; Wang, L.; Xu, J.; Ridgway, D.; Gu, T.; Moo-Young, M.: Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. Biochem. Eng. J. **8**(3), 187–193 (2001)
41. Papagianni, M.; Joshi, N.; Moo-Young, M.: Comparative studies on extracellular protease secretion and glucoamylase production by free and immobilized *Aspergillus niger* cultures. J. Ind. Microbiol. Biotechnol. **29**(5), 259–263 (2002)
42. Papagianni, M.; Moo-Young, M.: Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. Process Biochem. **37**(11), 1271–1278 (2002)
43. Pillay, P.; Schluter, U.; van Wyk, S.; Kunert, K.J.; Vorster, B.J.: Proteolysis of recombinant proteins in bioengineered plant cells. Bioengineered **5**(1), 15–20 (2014)
44. Braaksma, M.; Punt, P.J.: *Aspergillus* as a cell factory for protein production: controlling protease activity in fungal production. In: Goldman, G.H., Osmani, S.A. (eds.) The *Aspergilli*: Genomics, Medical Aspects, Biotechnology and Research Methods, pp. 441–455. CRC Press, Boca Raton (2008)
45. Wu, W.S.; Li, W.H.: Identifying gene regulatory modules of heat shock response in yeast. BMC Genom. **9**, 439 (2008)