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Short Communication

Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy

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ABSTRACT

The filamentous fungus *Trichoderma reesei* has received attention as a host for heterologous protein production because of its high secretion capacity and eukaryotic post-translational modifications. However, the heterologous production of proteins in *T. reesei* is limited by its high expression of proteases. The pH control strategies have been proposed for eliminating acidic, but not alkaline, protease activity. In this study, we verified the expression of a relatively major extracellular alkaline protease (GenBank accession number: EGR49466.1, named *spw* in this study) from 20 candidates through real-time polymerase chain reaction. The transcriptional level of *spw* increased about 136 times in response to bovine serum albumin as the sole nitrogen source. Additionally, extracellular protease activity was reduced by deleting the *spw* gene. Therefore, using this gene expression system, we observed enhanced production and stability of the heterologous alkaline endoglucanase EGV from *Humicola insolens* using the Δ*spw* strain as compared to the parental strain RUT-C30.

1. Introduction

The filamentous fungus *Trichoderma reesei* is widely investigated for its effective production of cellulolytic enzymes (Zhong et al., 2012). This fungus produces a variety of enzymes, including cellobiohydrolases (CBH, EC 3.2.1.91), endo-β-1,4-glucanases (EG, EC 3.2.1.4), and β-glucosidases (BGL, EC 3.2.1.21), which are required for complete biomass hydrolysis (Singhania et al., 2013). Moreover, *T. reesei* has an excellent extracellular secretion capacity and can secrete large amounts of protein (20–100 g/l) compared to other microbial production/secretion systems, such as *Escherichia coli* and *Saccharomyces cerevisiae* (Martinez et al., 2008). Therefore, *T. reesei* is thought to be one of the most effective hosts for protein production.

However, the production of heterologous proteins in *T. reesei* has only proven to be moderately successful (Peterson and Nevalainen, 2012), and compared with high yields of native proteins, yields of heterologous proteins are very low (Cherry and Fidantsef, 2003; Nevalainen et al., 2005). This may be because of the low secretion rate of incorrectly folded proteins and degradation by proteases (Collén et al., 2005; Arvas et al., 2006). Indeed, in one study, truncation of recombinant proteins produced in *T. reesei* QM9414 was found to be due to protease activity (Dienes et al., 2007).

Several strategies have been developed to increase heterologous proteins yields in filamentous fungi, including modification of codon usage in heterologous genes (Te'o et al., 2000), introduction of a large number of gene copies

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(Withers et al., 1998), the use of strong promoters and efficient fungal secretion signals (Lv et al., 2012), and the development of vacuolar protein sorting receptor-deficient host strains (Yoon et al., 2010). One of the most successful strategies involves the construction of gene fusions of the target gene to genomic sequences encompassing the complete or partial coding region of a highly expressed fungal gene, such as *T. reesei* cellobiohydrolase 1 (CBH1) (Zou et al., 2012). However, even with successful secretion of higher levels of heterologous proteins, the problem of extracellular proteases remains.

At pH 5 and lower, the major protease of *T. reesei* has been shown to be acid aspartic protease (Haab et al., 1990). Eneyskaya et al. (1999) purified an acid aspartic protease that was responsible for limited proteolysis of fungal carbohydrases. Between pH 2.7 and 3.5, the proteolytic reaction was limited, while proteins were completely digested at lower pH (Eneyskaya et al., 1999). The reduction of aspartic protease secretion through bioprocessing strategies, such as nitrogen source and pH control, has been investigated systematically (Haab et al., 1990; Eneyskaya et al., 1999). When pH was controlled at 6.0, Dienes et al. (2007) purified a trypsin-like alkaline serine protease instead of an acid aspartic protease, as reported by Eneyskaya et al. (1999). All of these studies show that transcription of the extracellular acid protease is inhibited in medium having a neutral pH. The pH control strategy seems to be a simple approach for eliminating acidic protease activity (Haab et al., 1990; Eneyskaya et al., 1999). However, the protein products may be exposed to neutral and alkaline proteases using this strategy (Dienes et al., 2007). Therefore, we hypothesized that knockout of the major alkaline protease gene coupled with a pH control strategy may improve the production of heterologous proteins.

In this study, we verified the expression of the gene encoding a relatively major alkaline protease, *spw* (accession: EGR49466.1), from 20 candidates. SPW is a major extracellular protease of *T. reesei* RUT-C30. Additionally, we constructed an *spw* mutant strain by insertional inactivation. As a result, the extracellular protease activity of Δ*spw* strains was reduced, and the production and stability of the alkaline endoglucanase EGV from *Humicola insolens* were improved. Therefore, *T. reesei* Δ*spw* strains will be useful for future scientific research or industrial heterologous protein production.

2. Materials and methods

2.1. Strains and media

E. coli DH5α was used as the host strain for the recombinant DNA manipulations. *T. reesei* RUT-C30 (ATCC 56765), a hypersecreting and catabolite derepressed mutant (Peterson and Nevalainen, 2012), was used as a host for heterologous protein expression. *H. insolens* (Schülein, 1997) was used as the source of the alkaline endoglucanase EGV. Luria–Bertani (LB) medium was used to culture *E. coli* and *Agrobacterium tumefaciens*. Basal fermentation medium (BFM) (20∼50 g/l a carbon source, 0.6 g/l urea, 10 g/l $(NH_4)_2SO_4$, 5 g/l KH_2PO_4, 0.5 g/l $CaCl_2.2H_2O$, 0.6 g/l $MgSO_4.7H_2O$, 3 g/l peptone, 1 g/l yeast extract, 6 g/l corn paddle, 1 g/l Tween 80, 10 mg/l $FeSO_4.7H_2O$, 3.2 mg/l $MnSO_4.4H_2O$, 2.8 mg/l $ZnSO_4.7H_2O$ and 20 mg/l $CoCl_2.6H_2O$) was used for fungal cultures and alkaline endoglucanases fermentations.

Cultures were grown at 28 °C in shaking flasks with protease-inducible medium (PIM) (20 g/l a nitrogen source, 20 g/l glucose, 4.0 g/l KH_2PO_4, 0.5 g/l $CaCl_2.2H_2O$, 0.6 g/l $MgSO_4.4H_2O$, 10 mg/l $FeSO_4.7H_2O$, 3.2 mg/l $MnSO_4.4H_2O$, 2.8 mg/l $ZnSO_4.7H_2O$ and 20 mg/l $CoCl_2.6H_2O$) for RNA extraction and protease production.

2.2. Identification and sequence analysis of protease genes from T. reesei

Sixty predicted protease genes from *T. reesei* were identified by searching the NCBI protein database (see Supplementary material S1). After SignalP v4.1 prediction analysis (http://www.cbs.dtu.dk/services/SignalP/), 20 genes were selected for testing as the secreted protease (see Supplementary material S2).

2.3. RNA preparation

T. reesei strains were ground in PIM containing 20 g/l $(NH_4)_2SO_4$ at 28 °C and pH 6.0. After 2 days of cultivation, mycelia were centrifuged and supplied with fresh PIM containing 20 g/l bovine serum albumin (BSA) protein for 4 h. Then, about 400 mg of mycelia was used for extraction of RNA. Total RNA was extracted using the TRI Reagent Solution (Applied Biosystems) and purified with an additional on-column DNase digestion using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed with 500 ng of total RNA using PrimeScript 1st strand cDNA Synthesis Kit (TianGen) according to the manufacturer's instructions. Integrity of RNA preparations was checked with an Agilent 2100 bioanalyzer and quantification was done on a ND-1000 spectrophotometer (Thermo).

2.4. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

For qRT-PCR, the UltraSYBR Mixture (CWBIO) was used with 320 nM of forward and reverse primers (see Supplementary material S2) and 5 μl of 50-fold diluted cDNA in a final volume of 25 μl. Thermocycling was carried out in a ABI StepOne thermocycler. Every qRT-PCR was done in triplicates on 96 well microplates including negative (water) and positive controls (genomic DNA) and analyzed with ABI software. In addition, for all samples negative controls using an RT mix without reverse transcriptase were performed in order to exclude contamination of samples with genomic DNA. Primers were validated by creating standard curves with tenfold serial dilutions of genomic DNA, a primer pair being considered as valid if amplification efficiency ranged between 85% and 115%. Melting curves were realised after each qRT-PCR run, to confirm the specificity of amplification and the absence of primer dimers. The qRT-PCR program consisted of an