論文の内容の要旨

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論文題目

The study on heterologous protein production by disrupting a tripeptidyl peptidase gene *AosedD* in *Aspergillus oryzae*

（麹菌 *Aspergillus oryzae* におけるトリペプチジルペプチダーゼ遺伝子の破壊による異種タンパク質生産の解析）

Introduction

The filamentous fungus *Aspergillus oryzae* has been used for Japanese food fermentation industry for more than a thousand years. The fungus possesses the exceptional capacity of secreting large amounts of proteins into the culture medium, and it is a “generally regarded as safe” (GRAS) microorganism. For these reasons, *A. oryzae* is considered to be one of the most prominent hosts for recombinant production of heterologous proteins. So far, various industrially useful proteins have been successfully produced by *A. oryzae*.

However, in general, the production levels of animal and plant proteins by *A. oryzae* are typically much lower than those of fungal proteins probably due to the proteolytic degradation. Hence, the construction of protease-deficient strains to avoid the proteolytic degradation is one of the important strategies to improve the productivity. However, the genome sequencing project revealed that *A. oryzae* has 134 protease genes. In our laboratory, a number of targets for gene disruption were identified by DNA microarray analysis of *A. oryzae* protease genes, and a generated disruptant of ten protease genes (Δ*tppA* Δ*pepE* Δ*nptB* Δ*dppIV* Δ*dppV* Δ*alpA* Δ*pepA* Δ*AopepAa* Δ*AopepAd* Δ*cpI*) showed 3.8- and 3.2-fold higher levels of bovine chymosin (CHY) and human lysosome (HLY), respectively, than the wild-type strain. Among them, disruption of the *tppA* gene encoding a tripeptidyl peptidase is the most effective for improving the production level, suggesting that tripeptidyl peptidases play a major role in degradation of the heterologous protein. Three tripeptidyl peptidase genes were found in *A. oryzae* genome database, however, it remains to examine the effect of other tripeptidyl peptidases on the heterologous protein production.

On the other hand, our laboratory obtained a hyper-producing mutant strain of *A. oryzae*,

AUT1, which produced 3.2- and 2.6-fold higher levels of CHY and HLY, respectively. Meanwhile, disruption of the *Aovps10* gene encoding a vacuolar protein sorting receptor increases the production levels of CHY and HLY by 3.0- and 2.2-fold, respectively.

In this thesis, I examined the effect of disrupting a tripeptidyl peptidase gene *AosedD* (AO090166000084) on the heterologous protein production in *A. oryzae*. Furthermore, I attempted to further enhance the heterologous protein production by performing double genes disruption of *AosedD* and *Aovps10* in the hyper-producing *A. oryzae* strains.

Chapter 1. Effect of disrupting a tripeptidyl peptidase gene *AosedD* on heterologous protein production

DNA microarray analysis previously performed in our laboratory indicates that the *AosedD* was expressed at the highest level among the three tripeptidyl peptidase genes in *A. oryzae*. Hence, *AosedD* gene was selected as a candidate potentially degrading the heterologous protein.

To identify the feature of AoSedD, a fusion protein AoSedD-EGFP was expressed and characterized in *A. oryzae*. In Western blotting analysis, the fusion protein together with an EGFP fragment were detected in the culture medium; while in fluorescence microscopic analysis, the AoSedD-EGFP was observed at the septa where the secreted proteins of *A. oryzae* localize. In addition, an *AosedD* gene disruptant was constructed, and the tripeptidyl peptidase activities in the culture medium of the disruptant were decreased as compared with those of the wild-type strain. Collectively, these results suggested that AoSedD is a secreted protein.

To test the effect of *AosedD* gene disruption on the heterologous protein production, CHY or HLY expressing strains were constructed in the *AosedD* gene disruptant. The maximum yields of CHY and HLY produced by the *AosedD* disruptants in the culture supernatant were 77.0 and 20.7 mg/L, which were 2.9- and 1.7-fold increases, respectively, as compared with the wild-type strains. These increases are the highest among those of the single protease gene disruptions reported in *A. oryzae*, suggesting that AoSedD is one of the important proteases involved in the proteolytic degradation of heterologous proteins.

Chapter 2. Effect of disrupting the *AosedD* gene in multiple protease genes disruptants on heterologous protein production

In this chapter, to further improve the heterologous protein production, the *AosedD* gene was disrupted in the ten protease genes disruptant NSlD-ΔP10 (NSlD-tApEnBdIVdVaApApAapAdcI) background, and CHY or HLY expressing strains were constructed in this disruptants. As a result, the production levels were increased by 1.5-fold (from 88.5 mg/L to 129.1 mg/L) in CHY and by 2.0-fold (from 35.3 mg/L to 70.0 mg/L) in HLY

as compared with those of the ten protease genes disruptants. The results indicated that *AosedD* gene disruption in the ten protease genes disruptants was effective on enhancing the production levels of heterologous proteins.

Chapter 3. Effect of double genes disruption (Δ*AosedD* Δ*Aovps10*) in a hyper-producing mutant AUT1 on heterologous protein production

Recently, our laboratory developed a hyper-producing strain AUT1 by performing double protease genes disruption (*tppA* and *pepE*) by curable niaD marker and random mutagenesis, leading to ~3-fold increases in production of human lysozyme (HLY) and bovine chymosin (CHY) compared with the wild-type strain.

In this chapter, I performed *AosedD* gene disruption in this hyper-producing *A. oryzae* mutants AUT1, for further enhancing the heterologous protein production. Here, I disrupted the *AosedD* gene in a strain AUT1-lD applicable to multiple genes disruption by *pyrG* marker recycling with highly efficient gene-targeting background (Δ*ligD*). *AosedD* gene disruption in the hyper-producing mutant background of AUT1 increased the production yields of CHY and HLY to 139.5 mg/L and 74.0 mg/L, which were 1.4- and 1.9-fold higher than the parent strain, respectively.

To further enhance protein production, double disruption of the *AosedD* and *Aovps10* genes was constructed, as it was anticipated that both protease deletion and protein sorting pathway modification would have a combinatorial effect on increasing protein levels. The double genes disruption (Δ*AosedD* Δ*Aovps10*) showed significant increases in the production levels of CHY and HLY to 152.7 mg/L and 81.7 mg/L by 1.6- and 2.1-fold, respectively, as compared with the parent strain. Thus, we successfully generated an *A. oryzae* host for further enhancing the production ability of heterologous proteins by combining mutational and molecular breeding techniques.

Although in Chapter 1 it was suggested that AoSedD is a secreted protein, tripeptidyl peptidase activity in the culture medium was unexpectedly increased in the disruptant of vacuolar sorting receptor gene *Aovps10*. Hence, I hypothesized that there is some relevance of vacuolar sorting receptor AoVps10 for the secretion of tripeptidyl peptidases in *A. oryzae*. For localization analysis, two tripeptidyl peptidases AoSedD and TppA were expressed as EGFP fusion proteins in the wild-type strain and *Aovps10* gene disruptant. In the wild-type strain, AoSedD-EGFP and TppA-EGFP were detected at the septa similarly to other secreted proteins of *A. oryzae* such as α-amylase (AmyB) and ribonuclease T1 (RntA). However, the fluorescence was not observed at the hyphal tip where other secreted proteins accumulate. In contrast, fluorescence of AoSedD-EGFP and TppA-EGFP was present at the hyphal tip in the *Aovps10* gene disruptant. These results suggest that AoVps10 might inhibit accumulation of AoSedD and

TppA at the hyphal tip.

In addition, when the cultivation was prolonged until vacuoles were developed, the fluorescence of AoSedD-EGFP and TppA-EGFP expressed in the wild-type strain were also detected in the vacuoles. In the *Aovps10* gene disruptant, no fluorescence was detected in the vacuoles, suggesting that AoSedD and TppA are new cargoes of AoVps10. The localization analysis together with AoCpyA, a known cargo of AoVps10, revealed that the delivery of AoSedD and TppA to the vacuoles was delayed as compared with that of AoCpyA. This suggests additional machinery differentially sorting AoSedD/TppA and AoCpyA to the vacuoles besides AoVps10.

These results indicate that the tripeptidyl peptidases AoSedD and TppA possess both of features of secreted and vacuolar proteins, and that their secretion and vacuolar sorting might be regulated by the vacuolar protein sorting receptor AoVps10.

Conclusion

In this study, I obtained the results supporting the hypothesis that tripeptidyl peptidases play an important role in the degradation of heterologous proteins produced by *A. oryzae*. Moreover, I succeeded in further enhancing the production ability of *A. oryzae* by performing the double genes disruption (Δ*AosedD* Δ*Aovps10*) in the hyper-producing strains. These attempts finally led to approximately 6-fold increase in the production level as compared with the wild-type strain. Thus, the further enhanced host strain generated in this study is expected to contribute to providing large amounts of industrially useful heterologous proteins.

On the other hand, I found the tripeptidyl peptidases AoSedD and TppA as new cargoes of the vacuolar sorting receptor AoVps10 in filamentous fungi. These two peptidases uniquely showed combined features of the classical secreted and vacuolar proteins, implying the diverse protein sorting mechanism in the highly polarized morphology of filamentous fungi.

Publications

1) Zhu L, Nemoto T, Yoon J, Maruyama J, Kitamoto K (2012) Improved heterologous protein production by a tripeptidyl peptidase gene (*AosedD*) disruptant of the filamentous fungus *Aspergillus oryzae*. *Journal of General Applied Microbiology* Vol. 58:199-209.

2) Zhu L, Maruyama J, Kitamoto K. (2013) Further enhanced production of heterologous proteins by double-gene disruption (*ΔAosedD ΔAovps10*) in a hyper-producing mutant of *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* in press.