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Cellular Response to the Overexpression of Mutated or Exogenous Secretory Proteins in *Aspergillus oryzae*

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Research aims

Filamentous fungi have been used as hosts that produce useful proteins because of their ability to secrete proteins at a very high level. The protein expression systems have been developed in several filamentous fungi and had a great success in secreting the recombinant proteins derived from eukaryotic microbes. However, the efficiency of secretion of higher eukaryotic proteins was quite poor in many cases. One of the reasons for a decreased production could be the instability of exogenously expressed proteins in the secretory pathway. In eukaryotic cells, it is known that aberrant secretory proteins are subjected to a protein quality control and degraded intracellularly to maintain their cellular homeostasis. Therefore, the protein production, a situation where exogenous proteins are overexpressed, might put stresses on host cells. In this research, I used *Aspergillus oryzae* as a host strain to analyze the cellular response to the overexpression of a mutated secretory protein.

Methods

The plasmids used in this study were listed in Table 1. Each plasmid was integrated at the *niaD* locus of *A. oryzae* NS4 to generate the strain expressing the wild-type or mutant 1,2-α-mannosidase. *A. oryzae* cells were grown in YPM medium (1% yeast extract, 1% peptone, 2% maltose) at 30°C for 24 hours. Northern blot analysis and cDNA microarray were performed as described by Maeda *et al*.[1)]

Results

Tatara *et al*.[2)] showed that the cystein residue-443 of 1,2-α-mannosidase from *Aspergillus saitoi* (msdS) was important to maintain its conformation and the substitution of Cys-443 to Phe (C443F) resulted in a marked decrease in its secretion from *A. oryzae* cells, suggesting that the mutant enzyme was subjected to a protein quality control in the host cells. Therefore, I decided to express the mutant version of msdS in *A. oryzae* to analyze the cellular response to aberrant proteins loaded into the secretary pathway. Since the expression level is an important consideration to analyze the response to the expression of aberrant proteins, two different promoters were used in this study, i.e. one was the strong No. 8142 promoter, and the other was a more moderate *enoA* promoter. Northern blot analysis verified that the transcription level of *msdS* from No. 8142 promoter was much higher than that from *enoA* promoter.

Table 1. The plasmids used in this study.

Name	Promoter	Target cDNA for expression
pNE	*enoA*	none
pNE-AM1	*enoA*	wild-type f-*msdS*
pNE-AM1-C443F	*enoA*	C443F f-*msdS*
pNAN8142	No. 8142	none
pNAN-AM1	No. 8142	wild-type f-*msdS*
pNAN-AM1-C443F	No. 8142	C443F f-*msdS*

Using these strains, I examined the production of the wild-type and C443F msdS proteins with SDS-PAGE and immunoblotting. The wild-type msdS proteins were efficiently secreted to the medium with both promoters, although amount of msdS protein was more than tenfold higher from the No. 8142 promoter than the *enoA* promoter. On the other hand, the mutant msdS protein was not detected in the medium by Coomassie Brilliant Blue staining. Therefore, I tried to detect the mutant protein by immunoblotting. The mutant enzyme was still undetectable even by immunoblot analysis when expressed from the *enoA* promoter. In contrast, when overexpressed, the mutant enzymes were found in the medium and extensively subjected to the hyperglycosylation. To test the possibility that the mutant enzyme was accumulated inside the cells, the total cell extract was subjected to immunoblot analysis. At the moderate expression level, only a faint band of the C443F enzyme was detected, although a significant amount of the wild-type enzyme was observed, suggesting that the mutant enzyme was degraded rather than accumulated inside the cells. No obvious difference in amounts of enzymes was seen when overexpressed. Interestingly, the mutant enzyme observed in the cell extract was not hyperglycosylated, suggesting that the mutant enzymes with the hyperglycosylation were efficiently secreted to the medium.

Next, I examined the cellular response to the overexpression of the mutant msdS proteins at the transcription level. Using cDNA microarrays comprising about 5,000 non-redundant EST clones from *A. oryzae*, the transcriptions were compared between the strains expressing wild-type and mutant enzymes. The transcriptions of *bipA* and *pdiA*, which might be involved in protein folding in ER, were increased in the cells expressing the mutant enzyme, suggesting that the overexpression of the mutant msdS induced the unfolded protein response (UPR). Northern analysis confirmed that the overexpression of the mutant enzyme induced the transcriptions of ER chaperons, while the moder-

ate expression did not.

Conclusion

Only one amino acid substitution (Cys-443 to Phe) caused instability of 1,2-α-mannosidase in *A. oryzae*. At the moderate expression level, the mutant protein might be degraded through the secretory pathway, although the mechanism of how it is degraded is unclear. The hyperglycosylation of secretory proteins occurs at the Golgi apparatus in yeast. Therefore, I reasoned that the degradation might occur at ER because no hyperglycosylated mutant protein was detected in the cell lysate. When overproduced, the mutant proteins could bypass the ER-associated degradation and transport to Golgi apparatus where the hyperglycosylation occurs. The hyperglycosylated mutant proteins were efficiently secreted to the medium. The mutant protein detected in the cell lysate might be localized at ER because they were not hyperglycosylated, and the accumulated aberrant proteins could induce the UPR.

References

1) Maeda H., Sano M., Maruyama Y., Tanno T., Akao T., Totsuka Y., Endo M., Sakarada R., Yamagata Y., Machida M., Akita O., Hasegawa F., Abe K., Gomi K., Nakajima T., and Iguchi Y. (2004) Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags. *Appl. Microbiol. Biotechnol*. **65**, 74–83.

2) Tatara Y., Yoshida T., and Ichishima E.. (2005) A single free cysteine residue and disulfide bond contribute to the thermostability of *Aspergillus saitoi* 1,2-α-mannosidase. *Biosci. Biotechnol. Biochem*. **69**, 2101–2108.