Functional analysis of SM proteins for heterologous protein production in *Aspergillus oryzae*

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Research aims

In eukaryotic cells, secretory proteins are transported to the extracellular space via vesicles detached from the endoplasmic reticulum (ER), Golgi apparatus, through the plasma membrane. In this vesicular trafficking pathway, soluble *N*-ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) and Sec1/Munc18 (SM) proteins are required for fusion of the vesicle with the target membrane. SNARE proteins localize on target or vesicle membranes, where they form complexes that aid in membrane fusion; SM proteins act on SNARE protein complex to assist in this function. SM proteins have been well analyzed in the model eukaryote *Saccharomyces cerevisiae*, a type of yeast. In this model, the protein Sec1p functions from Golgi to the plasma membrane, and the Sly1p protein functions from ER to Golgi. It has been previously reported that *S. cerevisiae* cells that overexpressed SM proteins produced greater amounts of α-amylase derived from *Aspergillus oryzae*[1]. In contrast, in the model filamentous fungus *A. oryzae*, no information about the effect of SM proteins has been reported. *A. oryzae* has been approved as the Japanese national fungus because it is important in the Japanese fermentation and brewing industry and because it can produce abundant secretory proteins, including α-amylase. In this study, I aimed to elucidate the role of SM proteins in *A. oryzae* that are thought to function in the secretory pathway, using functional analysis as a basic study and trying to produce valuable proteins as an applied study.

Methods

In the genome database of *A. oryzae* (http://www.aspgd.org/), we found four predicted SM proteins, in which two proteins were orthologs of *S. cerevisiae* secretory SM proteins. One was an ortholog of Sec1p, named AoSec1 (AO090010000460), and the other was an ortholog of Sly1p, named AoSly1 (AO090701000606). We determined that the amino acid sequences of both *A. oryzae* proteins contain a Sec1 domain, suggesting that these *A. oryzae* SM proteins have a conserved function. *Aosec1* and *Aosly1* were cloned into an *egfp*-containing vector to generate strains expressing each EGFP-fused protein for

localization analysis.

In the *S. cerevisiae* genome database (https://www.yeastgenome.org/), it was reported that *SEC1* and *SLY1* are essential genes. Therefore, *Aosec1* or *Aosly1* conditionally-expressing strains were produced where each gene was regulated by the *nmtA* promoter (P*nmtA*), which has been successfully used for the analysis of essential genes[2].

SM protein-overexpressing strains, where the *amyB* promoter (P*amyB*) is used to express each SM protein-encoding gene, were generated to analyze the effect of secretory protein amount. α-Amylase (AmyB) activity in the culture supernatant of these SM protein-overexpressing strains was analyzed as a homologous secretory protein using an α-amylase measuring kit (Kikkoman), and total secretory protein amount was determined using Bradford dye reagent (Takara).

Results

1. Localization analysis of *A. oryzae* SM proteins

The AoSec1 or AoSly1 encoding gene was cloned, and strains expressing each C-terminally EGFP-tagged protein were cultured for observation under a fluorescent microscope. AoSec1-EGFP was observed to locate in the cytoplasm, while AoSly1-EGFP was observed to exist as punctate structures, suggesting Golgi localization (Fig. 1).

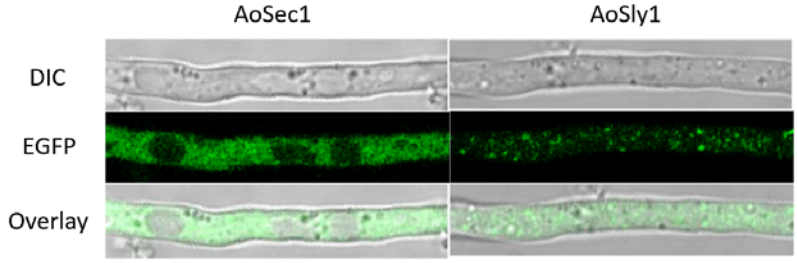

Fig. 1 Localization of SM proteins

AoSec1-EGFP was observed to localize in cytoplasm. AoSly1-EGFP was observed as punctate structures, suggesting Golgi localization.

2. Analysis of conditional mutants of *A. oryzae* SM proteins

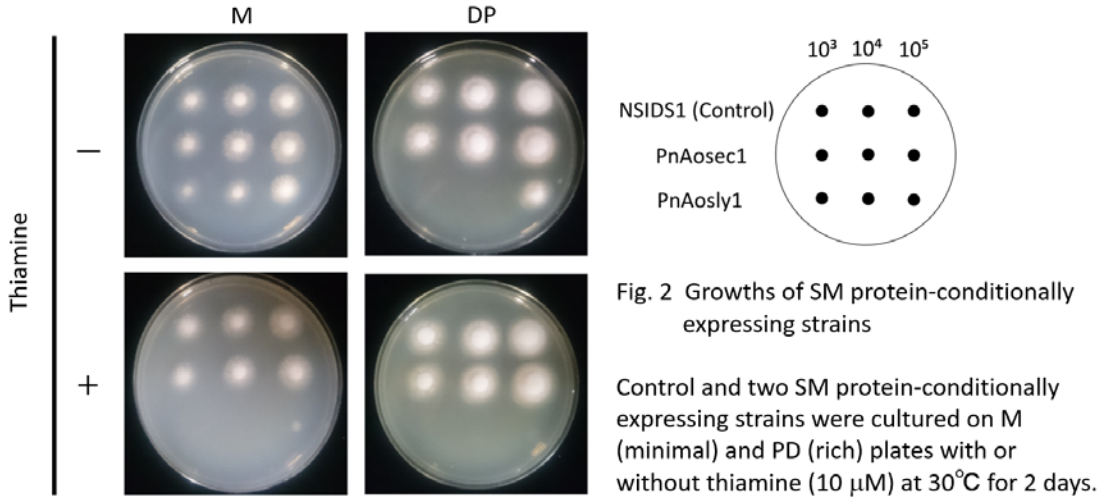

Fig. 2 Growths of SM protein-conditionally expressing strains

Control and two SM protein-conditionally expressing strains were cultured on M (minimal) and PD (rich) plates with or without thiamine (10 μM) at 30℃ for 2 days.

Conditional mutants, in which expression of *Aosec1* or *Aosly1* is regulatable by P*nmtA*, were generated, and their growths were analyzed on plates of M (minimal) or PD (rich) medium, supplemented with or without 10 μM thiamine. In *Aosec1*-repressed conditions, no obvious growth defect was observed. In contrast, in *Aosly1*-repressed conditions, severe growth defect was observed (Fig. 2). In *S. cerevisiae*, *SEC1* and *SLY1* are essential genes; however, in *A. oryzae*, *Aosec1* was shown to be non-essential, at least under the examined conditions.

3. Analysis of protein secretion in strains overexpressing *A. oryzae* SM proteins

Aosec1- or *Aosly1*-overexpressing strains were cultured in DPY medium for 2 days, and total secretory protein amount was measured in the culture supernatant (Fig. 3). SM protein-overexpressing strains could produce approximately 1.3-fold more secretory protein than a control strain. However, no increase in α-amylase activity was observed. Heterologous protein (bovine chymosin) production will be examined in the future study.

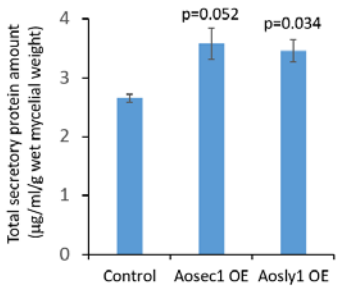

Fig. 3 Total secretory protein amount in SM protein-overexpressing strains

Strains were cultured at 30°C for 2 days, and each supernatant was used for measuring total secretory protein amount (n=3).

Conclusion

AoSec1 localizes in the cytoplasm and AoSly1 likely localizes to Golgi apparatus. In *Aosec1*-repressed conditions, no obvious growth phenotype was observed; in contrast, in *Aosly1*-repressed conditions, a severe growth defect was observed. Both *Aosec1*- and *Aosly1*-overexpressing strains produced more secretory proteins, suggesting that SM proteins in *A. oryzae* function in the secretory pathway. In a future study, the effect of SM protein-repressed or -overexpressed conditions on heterologous protein production will be examined.

References

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