Contribution of membrane lipid unsaturation to temperature-dependent changes of secreted protein in the filamentous fungus *Aspergillus oryzae*

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Research objective

Filamentous fungi are eukaryotic microorganisms characterized by a long filamentous cell morphology. These fungi are widely used in industrial fermentation applications because of their ability to secrete various degradative enzymes into the extracellular space. The high extracellular secretory capacity of filamentous fungi for various degradative enzymes is due to active vesicular transport, through which biomembrane components and cell wall synthases are continuously transported to the mycelial tip. Phospholipids are the main component of the biomembrane that forms the transport vesicles. There are various types of phospholipids, depending on the hydrophilic head and hydrophobic tail. During industrial use, filamentous fungi are exposed to various stresses, including heat stress generated by fungi. Environmental temperature significantly affects biomembrane properties. However, the effects of changes in phospholipid acyl chains on protein secretion in filamentous fungi are unclear. This study aimed to clarify the relationship between incubation temperature, biomembrane phospholipid composition, and protein secretion in *Aspergillus oryzae*, an industrially important filamentous fungus.

Methods

Generation of gene disruptant strains: The regulation of acyl chains of phospholipids in filamentous fungi is unclear. In this study, I focused on *Aspergillus nidulans*, which has long been used as a model organism for filamentous fungi, and *A. oryzae*. Candidate gene-disruption strains will be generated from these strains.

Lipidome analysis: Phospholipids were extracted from cultured mycelia using the BUME method[1)] and subjected to quantitative phospholipid analysis using liquid chromatography-tandem mass spectrometry (LC/MS/MS).

Examination of extracellular protein secretion capacity To analyze the extracellular protein secretory capacity, the size of the halo after the iodine-starch reaction using a plate medium containing starch was quantified using ImageJ software.

Results

Regulation of phospholipid unsaturation in *A. nidulans*

Lipidome analysis was performed using phospholipids extracted from *A. nidulans* cultured in minimal liquid medium. Phospholipids having 34 and 36 carbons on the two constituent acyl chains were detected. Focusing on phospholipids with the 36-carbon acyl chain, the number of double bonds in the acyl chain ranged from zero to six. There were up to three double bonds in the fatty acids comprising the phospholipid acyl chain, indicating that phospholipids with two multiple double-bond fatty acids are universally present in filamentous fungi. Previous studies have demonstrated that filamentous fungi show growth patterns in which conidia germinate after non-polar swelling of the conidia. I examined the phospholipid composition during the life cycle and discovered a transient increase in highly unsaturated phospholipids, with a maximum value for the time at which the conidia germinated. Furthermore, lipidome analysis of mycelia generated on a solid medium revealed the abundance of highly unsaturated phospholipids in areas where many cells displayed filamentous morphology.

In *A. nidulans*, *sdeA* and *sdeB* encode Δ9 desaturases that introduce the first double bond to fatty acids, *odeA* encodes Δ12 desaturase that introduces the second, and *odeB* encodes Δ15 desaturase that introduces the third. However, the effects of these genes on biomembrane phospholipids have not been analyzed in *A. nidulans*. In this study, I generated single disruptant strains of these genes and analyzed the composition of their phospholipid acyl chains. This *in vivo* analysis revealed that the *odeA*- or *odeB*-disrupted strains did not produce phospholipids with five or more double bonds or three or more double bonds, respectively.

Effects of genes regulating phospholipid unsaturation on protein secretion in *A. oryzae*

In *A. oryzae*, *AO090005000456*, *AO090102000339*, *AO090026000799*, and *AO090103000283* are putative Δ9 desaturase genes, *AO090001000224* is a putative Δ12 desaturase gene, and *AO09000010000714* is a putative Δ15 desaturase gene. These were designated *sdeA*, *sdeB*, *sdeC*, *sdeD*, *odeA*, and *odeB* in *A. oryzae*, respectively. The *sdeA*- and *odeA*-disrupted strains showed severe growth retardation at 20°C, and significant growth retardation at 25°C and 30°C (Figure panel A). In *A. nidulans*, the effects of gene disruption differed between *A. oryzae* and *A. nidulans*; the *sdeA*-disrupted strain grew more poorly at higher temperatures and the *odeA*-disrupted strain grew to a certain extent at lower temperatures. When *A. oryzae* and *A. nidulans* were cultured at 20°C, 30°C, and 37°C and analyzed for biomembrane phospholipid composition, the number of double bonds in phospholipids increased at low temperatures in these two strains. The two strains exhibited similar changes in acyl chain composition in response to temperature changes.

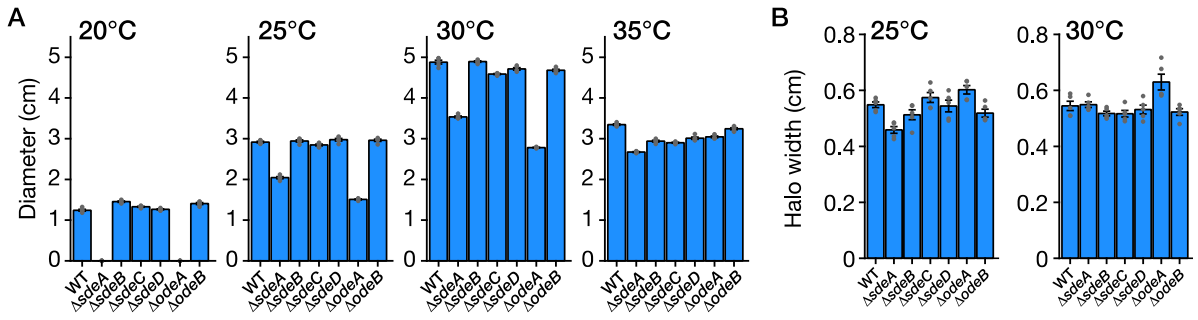

Figure. Phenotypes of fatty acid desaturase deficient strains in *A. oryzae*. (A) Conidia (10^3) of the wild-type (WT), Δ*sdeA*, Δ*sdeB*, Δ*sdeC*, Δ*sdeD*, Δ*odeA*, and Δ*odeB* were inoculated onto CD medium and incubated for 3.5 days at 20°C, 25°C, 30°C, or 35°C. The sizes of the colonies were measured. (B) Conidia (10^3) of these strains were inoculated onto starch-containing CD medium and incubated for 3.5 days at 25°C or 30°C. The sizes of the halos produced in the iodine-starch test were measured. Bars indicate the mean of five independent experiments and dots indicate raw data. Error bars represent standard error.

These disruption strains were cultured in a starch-containing plate medium, and the ability to secrete proteins was evaluated based on the size of the halo in the iodine-starch test. The Δ*sdeA* strain incubated at 20°C showed a significantly smaller halo; on the other hand, Δ*odeA* strain incubated at 30°C showed a significantly larger halo (Figure panel B). Because the Δ*odeA* strain exhibited growth retardation even at 30°C in liquid CD medium, the phospholipid composition of the Δ*odeA* strain has not yet been analyzed. In *A. nidulans*, *odeA* significantly increased phospholipids with two oleic acids as acyl chains. Therefore, to evaluate the effect of oleic acid on protein excretion, the halo size of the wild-type strain was measured in starch-containing CD medium with oleic acid. A slight increase in the halo was observed. However, the same effect was observed when other fatty acids were added. Therefore, the cause of the larger halo in the Δ*odeA* strain was unclear.

Conclusion

In *A. oryzae*, the halo sizes at 30°C were significantly larger in the Δ*odeA* strain. There were no significant differences in halo size between the wild-type and Δ*sdeA* strains at 30°C. The Δ*sdeA* strain showed growth retardation at both temperatures, as did the Δ*odeA* strain. Therefore, poor growth is not expected to affect halo size. In the future, it will be necessary to clarify how the secretion of other proteins is altered, and the mechanism responsible for this change in the Δ*odeA* strain.