Elucidation of the Molecular Weight Control Mechanism of Fungal Cell Wall Polysaccharide Nigeran and Establishment of a Nigeran Fermentation Production System

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Research objective

Filamentous fungi of the genus *Aspergillus* belonging to the *Nigri* section, including *Aspergillus luchuensis*, produce the fungal cell wall component, nigeran, an unbranched D-glucan with alternating α-1,3- and α-1,4-glucoside linkages, under nitrogen starvation conditions. We previously identified the *A. luchuensis* nigeran synthase gene, *nisA*[1]. Furthermore, we reported that the disruption of two genes adjacent to *nisA*, α-glucanotransferase gene (*agtC*) and a gene of unknown function (*gnsA*), affected nigeran production and its molecular weight. In this study, we expressed both *agtC* and *gnsA* as recombinant enzymes and examined their effects on the molecular weight of nigeran.

In recent years, biomass-based plastics made from polysaccharides have attracted considerable attention as substitutes for plastics. We investigated whether nigeran produced by the *A. oryzae nisA*-overexpressing strain could be used as a biomass-based plastic material[2]. We attempted to obtain strains with high nigeran production that constitutively expressed *nisA*.

Methods

1. Characterization of recombinant AgtC and GnsA

The α-glucanotransferase gene (*agtC*) was amplified using polymerase chain reaction (PCR) and expressed as a recombinant enzyme using the *A. oryzae* expression system. The gene of unknown function (*gnsA*) was also amplified using PCR and expressed as a recombinant enzyme using the *Escherichia coli* expression system. rAgtC and rGnsA were reacted with α-glucans, such as nigeran, starch, and α-1,3-glucan, and the molecular weights of α-glucans were analyzed using size exclusion chromatography. Furthermore, we analyzed the structures of the reaction products of rAgtC and nigeran oligosaccharides prepared via acid hydrolysis.

2. Construction of a nigeran fermentation production system

We replaced the *A. luchuensis nisA* promoter with an improved enolase promoter (P-*enoA*142), which is highly expressed in the glycolytic system of *A. oryzae*, and obtained a

transformant that constitutively expressed *nisA*. *A*. *oryzae* was transformed with a plasmid in which the high-expression vector pNEN142 and *nisA* were ligated. The hosts used for transformation included a strain (AGΔ), in which all three α-1,3-glucan synthase (AG) genes were disrupted, and another strain, in which the galactosaminogalactan (GAG) synthase and AG genes were disrupted (AG-GAGΔ). The yield and molecular weight of nigeran obtained from *nisA*-overexpressing strains were analyzed.

Results

1. Characterization of recombinant AgtC and GnsA

Purified rAgtC and rGnsA were reacted with nigeran, and the change in their molecular weights was analyzed using size exclusion chromatography. We found that the molecular weight of nigeran was reduced by rAgtC treatment. In contrast, rGnsA did not affect the molecular weight of nigeran. When rAgtC and rGnsA were combined and reacted with nigeran, the molecular weight of nigeran decreased, as in the case of rAgtC alone. Reacting both rAgtC and rGnsA with nigeran resulted in a similar reduction in the molecular weight of nigeran compared to rAgtC alone. In a previous study, both an increase and decrease in nigeran molecular weight were observed in the *A*. *luchuensis* Δ*gnsA* strain (AgtC functions). In contrast, the molecular weight of nigeran did not change in the Δ*agtC* strain, in which GnsA functioned. This is consistent with the results obtained in this study that rAgtC reduces the molecular weight of nigeran, whereas rGnsA does not affect its molecular weight. In addition, starch or α-1,3-glucan reacted with rAgtC and rGnsA, but the molecular weights of these glucans did not change, suggesting that rAgtC acts specifically in nigeran.

Fungal α-glucanotransferases (Agt) act mainly on α-1,4-glucosidic bonds in α-1,4-glucan and maltooligosaccharides[3)]. To clarify whether rAgtC acts on α-1,4- or α-1,3-glucosidic bonds in nigeran, a nigeran oligosaccharide mixture prepared via the acid hydrolysis of nigeran was reacted with rAgtC. We found that rAgtC did not act on nigeran oligosaccharides with an estimated degree of polymerization (DP) of less than 6, and only hydrolyzed the nigeran oligosaccharides with an estimated DP of more than 6. The obtained product was fractionated, and its structural analysis is underway.

2. Construction of a nigeran fermentation production system

A comparison of nigeran production by the *A*. *luchuensis* parental strain and *nisA*-overexpressing strain with the promoter replaced revealed that the *nisA*-overexpressing strain produced approximately twice as much nigeran as the parental strain. Compared to the *nisA*-overexpressing strain generated from *A*. *oryzae* niaD300, the *nisA*-overexpressing strains generated from AGΔ and AG-GAGΔ strains produced 2–3 times more nigeran. Based on the high homology between *nisA* and fungal α-1,3-glucan synthase genes, we suggest that the

synthetic substrates of nigeran and α-1,3-glucan are the same. Because the *A*. *oryzae* AGΔ strain does not synthesize α-1,3-glucan, it is presumed that its nigeran production was higher than that of the *A*. *oryzae* niaD300 strain, which synthesizes α-1,3-glucan.

The molecular weight of nigeran was determined using size-exclusion chromatography. The nigeran molecular weight of the *nisA*-overexpressing strain generated from the *A*. *oryzae* AG-GAGΔ strain was the highest, followed by the *A*. *oryzae* niaD300, *A*. *oryzae* AGΔ, *A*. *luchuensis nisA*-overexpressing, and *A*. *luchuensis* parent strains.

Conclusion

The mechanism of nigeran synthesis is unclear compared to that of other fungal cell wall polysaccharides. The characterization of nigeran synthesis-related enzymes in this study has provided fundamental knowledge leading to an understanding of nigeran synthesis mechanisms. To clarify the significance of nigeran production in fungi, we will continue to conduct extensive research on the enzymes and genes involved in nigeran synthesis.

Here, we found that the *A*. *oryzae nisA*-overexpressing strain produced more nigeran in a shorter culture period than the wild-type *A*. *luchuensis* strain. Furthermore, we obtained a *nisA*-overexpressing strain that produced higher-molecular-weight nigeran. Therefore, the results of this study can contribute to the future research on biomass-based plastics using nigeran.

References

1) Uechi, K., Yaguchi, H., Tokashiki, J., Taira, T., and Mizutani, O. (2021) Identification of genes involved in the synthesis of the fungal cell wall component nigeran and regulation of its polymerization in *Aspergillus luchuensis*. *Appl*. *Environ*. *Microbiol*. **87(21)**: e0114421.
2) Togo, A., Uechi, K., Mizutani, O., Kimura, S., and Iwata T. (2021) Synthesis and characterization of α-1,3-*alt*-α-1,4-glucan (nigeran) ester derivatives. *Polymer* **214**: 123343.
3) Kaaij, R. M. van der et al., (2007) Two novel, putatively cell wall-associated, and glycosylphosphatidylinositol-anchored α-glucanotransferase enzymes of *Aspergillus niger*. *Eukaryot. Cell* **6(7)**: 1178-1188.