Creation and Exploratory Research of the Longest Bacterial Polyynes with a Pentayne Structure

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Research objective

Bacterial polyynes, which have a conjugated carbon-carbon triple bond (C≡C) starting from the terminal alkyne, are natural products with strong biological activities, such as antifungal, antioomycete, and nematocidal activities. Natural products with polyyne structures are used as medicines, traditional medicines, and health-promoting nutritional agents. Therefore, bacterial polyynes are an attractive group of compounds in chemistry and biology but also for industrial applications. However, only five classes of bacterial polyynes have been found thus far, with protegenins and caryoynencins, which have a tetrayne structure, being the longest polyynes (four C≡C repeats).[1,2] Our efforts to discover novel bacterial polyynes and analyze their biosynthesis have laid the groundwork for the creation of genetically engineered bacterial polyynes. In this study, we tested whether enzymes (fatty acyl-ACP ligase and acetylenase) in the protegenin biosynthetic gene cluster could be artificially modified to construct pentyne structures or even longer polyyne structures.

Methods and results

1. Biosynthetic mechanism of the teterayne structure in protegnins

Protegnins are bacterial polyynes biosynthesized by *Pseudomonas protegens* strain Cab57.[2] We found that protegnins are biosynthesized by a *pro* gene cluster. However, the function of each of these genes remains unknown. Therefore, we generated a strain deficient in each gene. Deletion of *proA* (ligase), *proB* (desaturase), and *proD* (ACP) resulted in a loss of the ability to produce protegenin A. Intermediate levels of accumulation were not observed. The deletion of *proH* slightly reduced the production of protegenin A. Deletion of *proF*, which encodes rubredoxin, greatly reduced the production of protegenin A.

The *pro* gene clusters were then expressed in *E. coli* to test whether the enzymes/proteins they encoded could also function in *E. coli*. The entire cluster was cloned by PCR and expressed in *E. coli* BL21(DE3) using the pET-21a(+) vector, after which the bacteria acquired the ability to produce protegenin A, confirming that the enzymes in the cluster can function within *E. coli*. To examine in detail how polyyne formation occurs, we optimized the induction of *proD* expression alone and then co-expressed *proD* and *proA* and examined the progression of the acylation of ProD, an ACP, by SDS-PAGE, western blotting, and LCMS. Expression

and acylation of ProD were confirmed. We are currently expressing the remaining modified enzymes and investigating the sequence of unsaturated fatty acids bound to ProD.

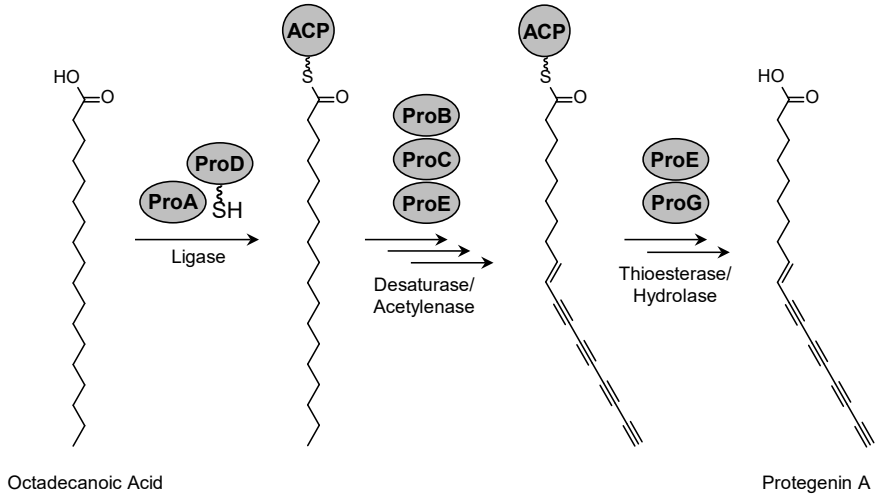

Fig 1. Proposed biosynthetic pathway for protegein A

2. Introduction of fatty acy-ACP ligase using C20 fatty acids as substrates

Previous studies have shown that bacterial polyynes are biosynthesized from C16 fatty acids with a triyne structure, and from C18 fatty acids with a tetrayne structure. Therefore, if the substrate specificity of fatty acyl-ACP ligase (also known as fatty acyl-AMP ligase) in the biosynthetic gene cluster could be changed from C18 to C20, it may be possible to create bacterial polyynes with a pentagonal structure.

Although several reactions that use C20 fatty acids as substrates in secondary metabolism in bacteria are known, we focused on a specific fatty acyl AMP ligase from a gram-negative bacterium and examined its expression in the Cab57 strain. However, the expected conversion was not observed, indicating that additional enzymes should be investigated.

3. Investigation of bacterial polyynes detection by induced Raman analysis

Because of their unique structure, polyyne compounds exhibit Raman absorption at positions that are different from those of other biological components in Raman spectroscopy. Utilizing this structure, we expected that bacterial polyynes in cells could be detected selectively and with high sensitivity.

To verify this, we performed induced Raman microscopy analysis and found that the *P. protegens* Cab57 strain produced relatively high levels of protegenin A. We constructed an experimental system using these cells as model, but we were unable to achieve the expected sensitivity and were only able to detect protegenin A using a combination of integration counts and image processing. The reason for this is that, although protegenin A was produced at a higher level than other bacterial polyynes, the amount was not sufficient to be detected by

induction Raman microscopy.

Conclusion

Knowledge of the protegenin A biosynthetic pathway has progressed; however, the mechanism by which desaturases/acetylenases are involved in the formation of the polyyne moiety remains unclear. The use of induced Raman spectroscopy to efficiently search for bacterial species producing bacterial polyynes was considered, but the sensitivity was not as high as expected; therefore, other methods need to be considered.

References

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