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Genome-wide Analysis and Control of Microbial Hosts for a High-level Production of Therapeutic Proteins

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Abstract

The formation of insoluble aggregation of the recombinant kringle fragment of human apolipoprotein(a), *rh*LK8, in endoplasmic reticulum was identified as the rate-limiting step in the *rh*LK8 secretion in *Saccharomyces cerevisiae*. To analyze the protein secretion pathway, some of yeast genes closely related to protein secretion was rationally selected and their oligomer DNA were arrayed on the chip. The expression profiling of these genes during the induction of *rh*LK8 in fermentor fed-batch cultures revealed that several foldases including *pdi1* gene were up-regulated in the early induction phase, whereas protein transport-related genes were up-regulated in the late induction phase. The coexpression of *pdi1* gene increased *rh*LK8-folding capacity. Hence, the secretion efficiency of *rh*LK8 in the strain overexpressing *pdi1* gene increased by 2-fold comparing in its parental strain. The oligomer DNA chip arrayed with minimum number of the genes selected in this study could be generally applicable to the monitoring system for the heterologous protein secretion and expression in *Saccharomyces cerevisiae*. With the optimization of fed-batch culture conditions and the alteration of genetic background of host, we obtained extracellular *rh*LK8 at higher yields than with *Pichia pastoris* systems, which was a 25-fold increased secretion level of *rh*LK8 compared to the secretion level at the initiation of this study.

Introduction

S. cerevisiae was one of the most popular microorganism for expression of heterologous genes because of its safety for human uses and a long history of industrial applications (1, 2). However, the productivity of therapeutic proteins in *S. cerevisiae* was often insufficient to fulfill the expectations in the industrial mass production systems. This phenomenon seems to be derived from its middle capability of protein expression and secretion in comparison with other expression hosts such as *E. coli* and *P. pastoris*, respectively. In some aspects, *P. pastoris* seemed to be more applicable to industrial purposes in that it can be easily achieved high cell density culture and high-level secretion, although stories are different from each target protein (3, 4). In this respect, *S. cerevisiae* has many rooms to be improved in their expression/secretion

efficiencies (5).

S. cerevisiae genome was firstly sequenced and was revealed its functions all over the other microorganisms in the aid of genomics and functional analyses of each gene. Efficient use of these databases may pave the way for enhancing production, or secretion level of heterologous proteins by a genome-wide analysis and control of *S. cerevisiae.*

The therapeutic dosage of anti-angiogenic proteins, including *rh*LK8, is so high that development of an economical production system is a prerequisite for a successful commercialization. In this study, the feasibility of *S. cerevisiae* as a host strain for the production of *rh*LK8, a novel recombinant anti-angiogenic protein (6, 7, 8, 9), was assessed by a profiling gene expression related to protein secretion, co-expression of foldase, and optimizations of fed-batch fermentation conditions.

Materials and Methods

The yeast genes affecting the over-production and secretion of *rh*LK8 was intensively analyzed and consequently the host cell was remodeled for the maximizing of productivity of target protein. The genes related to proteolysis and truncation of *rh*LK8 also was analyzed and disrupted to improve the productivity of *rh*LK8. Using this *S. cerevisiae* expression system, fermentation processes was optimized with an aid of a rationale design of induction strategy. The protein production processes was scaled up enough to execute the pre-clinical and clinical trial for the commercialization of *rh*LK8, Greenstatin ^{TM}.

Results and Discussion

The goals of this study are 1) development of yeast expression system for commercialization of Greenstatin^{TM}, *rh*LK8, 2) development of a pilot-scale fermentation and purification process, and 3) achievement of *rh*LK8 expression/secretion level of over 300 mg/L by an aid of the re-engineering of host strain, yeast gene expression profiling, and optimization of fed-batch culture processes.

Production systems for *rh*LK8 had been established previously for studying its efficacy for anti-angiogenic activities using *P. pastoris* and *E. coli* expression system. However, *E. coli* system had intrinsic problems such as laborious refolding process during scale-up study. Although the successful application of *P. pastoris* system to scaled-up processes, the use of *P. pastoris* system burdened us with a heavy license fee and the royalty rate that pulls down the international competitiveness of commercialization of *rh*LK8 as an anti-angiogenic agent.

Consequently, to overcome these problems and propel the commercialization of *rh*LK8, the expression system using *S. cerevisiae* was established. *S. cerevisiae* was chosen as a candidate for production of *rh*LK8 because it is an eukaryotic, pyrogen-free, and GRAS (Generally Regarded as Safe) microorganism and has an extensive genomic databases, which may afford us to remodel host genome. The yeast genes affecting the over-production and secretion of *rh*LK8 was analyzed and the host cell was remodeled for the maximizing of productivity of target protein. Using this *S. cerevisiae* expression vector and host system, fermentation processes was optimized with an aid of genome-wide analyses during fermentation.

As results of studies concerning the optimization of fed-batch culture systems and the alteration of genetic background of host, we obtained a 25-fold increased secretion level of *rh*LK8 compared to the secretion level at the initiation of this study.

This expression system could be used as the platform technologies for the scaled-up process enough to afford the materials for the pre-clinical and clinical trials for the commercialization of *rh*LK8 and other therapeutic proteins in the near future.

Conclusions

1. *rh*LK8, a novel anti-angiogenic protein, was successfully expressed in both *P. pastoris* and *S. cerevisiae.* The two expression systems are comparable to each other in the levels of *rh*LK8 secretion.

2. Each expression system had its own different critical factors influencing overall protein production yield. For example, a C-terminal truncation by protease is the case for *P. pastoris* and the intracellular accumulation as insoluble forms is for *S. cerevisiae* expression system.

3. Over-expression of PDI1 (chaperones/foldase) resulted in the increase of the intracellular protein solubility and secretion level.

4. As results of studies concerning the optimization of fed-batch culture systems and the alteration of host genome, the secretion level of *rh*LK8 increased by 25-fold compared to the one at the initiation of this study.

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