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Production of a Platelet Aggregation Inhibitor, Salmosin, by High Cell Density Fermentation of Recombinant *Escherichia coli*

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Optimal conditions for a high cell density fermentation were investigated in a recombinant *Escherichia coli* producing salmosin, a platelet aggregation inhibitor. The optimized carbon and nitrogen sources were glycerol 10 g/l, yeast extract 30 g/l, and bacto-tryptone 10 g/l, yielding the dry cell weight (DCW) of 10.61 g/l in a 500 ml flask culture. The late-stage induction with 1% L-arabinose in a 5 l jar fermentor showed the highest DCW of 65.70 g/l after 27 h of the fed-batch fermentation. Around 2,200 mg/l of the protein was expressed as an inclusion body that was then refolded to obtain the active salmosin of 96 mg/l. We also confirmed the inhibitory activity against platelet aggregation of the active salmosin from the high cell density fermentation.

Keywords: High cell density fermentation, platelet aggregation inhibitor, recombinant *Escherichia coli*, salmosin

Disintegrin is a cysteine-rich low-molecular-weight polypeptide containing the Arg-Gly-Asp (RGD) sequence, which is recognized by various integrins. Disintegrin has a function of inhibiting the aggregation of fibrinogen-dependent platelets by selectively binding to integrins GP IIb-IIIa, which are expressed in platelets [4, 6, 21]. To date, several disintegrins have been isolated from snake venoms and their inhibitory activities against platelet aggregation proved; applaggin from *Agkistrodon piscivirous* [21], halysin from *Agkistrodon halys* [9], and saxatilin from *Gloydius saxatilis* [8]. As another disintegrin, salmosin composed of 73 amino acids was isolated from *Agkistrodon halys brevicaudus* [11] and its functions demonstrated to inhibit platelet aggregation and tumor angiogenesis, and suppress metastatic tumor growth as well as induce apoptosis by disorganizing focal adhesions [7].

High cell density fermentation is a useful process for improving the production yield of valuable recombinant proteins. In the high cell density fermentation, however, there are several problems including nutrient depletion, exhaustion of dissolved oxygen, and by-products formation causing the inhibition of cell growth and decrease of the desired proteins. To address these problems, the fed-batch fermentation has been applied together with the developments of nutrient feeding strategies including constant feeding, exponential feeding, and indirect feeding such as pH-stat and DO-stat [18].

Escherichia coli has proven to be an appropriate host for recombinant protein expression and production because of the relatively easy constructions of foreign proteins, simple cultivation for their productions using inexpensive medium, and the easy scale-up by a short fermentation cycle [10]. However, the heterologous expressions of foreign eukaryotic proteins in *E. coli* often lead to the formation of inclusion bodies, by which the number of downstream processes such as solubilization, refolding, and several purification steps could be needed, causing the loss of production yields [3]. Despite these bottlenecks, the recombinant *E. coli* system has been widely used for the mass productions of foreign proteins with a high cell density.

The high cell density fermentation of recombinant *E. coli* with the inducible promoter system is usually achieved with two separate phases [19]. The cells are grown to a high cell density under the optimized growth conditions such as culture medium, pH, and temperature in the growth phase, where the expression of target protein is kept at a

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minimum. To express the target protein upon an induction, the cells enter the production phase where it is necessary to determine the optimal induction time as well as concentration of inducer.

In this paper, we optimized the culture medium and determined the optimal inducer concentration and induction time for the fed-batch high cell density fermentation of recombinant *E. coli* producing salmosin, the platelet aggregation inhibitor.

The recombinant *E. coli* MC1061 harboring pMASIN used in this study was kindly provided by the Cardiovascular Research Institute, Yonsei University, Korea. This recombinant *E. coli* had been reported to express the salmosin as a form of inclusion body [11], the size of which with fusion protein was approximately estimated to be 28 kDa, confirmed before further experiments in this study (data not shown). We focused on the maximization of cell density of recombinant *E. coli*, based on the hypothesis that the production yield of expressed inclusion body is dependent on the cell density, which is therefore one of the major requirements for the high productivity of recombinant protein [2, 24]. To determine the optimal nitrogen sources for the high cell density, the cultures were prepared in a 500 ml flask with various ratios of yeast extract to bacto-tryptone, which is typically used in the *E. coli* culture, since one or more nitrogen sources usually increases the cell density as well as the cell growth rate [1]. As the results, the maximum cell density was observed with the ratio of 3% yeast extract to 1% bacto-tryptone (Supplementary Table S1). We also investigated the effect of carbon source on the cell density of recombinant *E. coli*. Generally, the *E. coli* cells generate acetic acid as a by-product and consequently reduce the pH when the cells grow above a threshold growth rate using glucose as the limiting nutrient [5]. Therefore, we investigated the effect of glycerol in place of glucose as the carbon source on the cell density of recombinant *E. coli* MC1061, resulting in that the cell density was highest at 1% glycerol (Supplementary Fig. S1).

The optimal inducer concentration was investigated with various concentrations of L-arabinose used to induce the heterologous expression of recombinant salmosin (Fig. 1). The recombinant *E. coli* MC1061 harboring ΔpMASIN was grown in a 500 ml flask with 100 ml of the medium containing 3% yeast extract, 1% bacto-tryptone, and 0.5% NaCl, with 100 μg/ml of ampicillin, at 37°C to the optical density at 600 nm (OD_{600}) of 1.0–1.5. After addition of L-arabinose, the cells were further cultivated at 37°C up to 18 h. The cell density was not affected by the variation of L-arabinose concentration, ranging from 8.06 g/l to 8.95 g/l. On the other hand, total protein concentration was increased, depending on the L-arabinose concentration. However, the expressed protein yield per L-arabinose concentration (8.6 mg/g-L-arabinose) was highest at 1% L-arabinose, which was therefore used in further experiments with a 5 l jar fermentor (KMJ-5C; Mitsuwa Co., Japan).

We also examined the effect of induction time on the cell density in the jar fermentor (Fig. 2). The feeding medium containing concentrated glycerol was fed at the constant rate of 30 ml/l/h to obtain the high cell density when the initial glycerol was almost consumed in the growth phase. The 1% L-arabinose was then added into the culture broth at three different stages of the cell density; early stage (around DCW of 21 g/l after 6 h culture), mid stage (45 g/l after 11 h culture), and late stage (54 g/l after 16 h culture). The cell growth rate was reduced after the

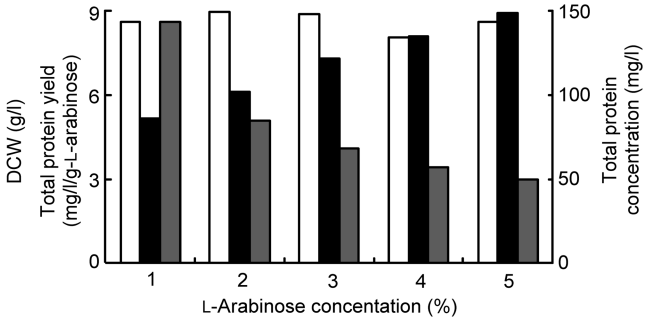

Fig. 1. Effect of L-arabinose concentration on the cell growth and total protein production of recombinant *E. coli*.
The DCW (white bars) was determined according to the predetermined calibration curve; DCW=0.6887×OD_{600}. Total protein yield (gray bars) was calculated as the total protein concentration (black bars) per L-arabinose concentration.

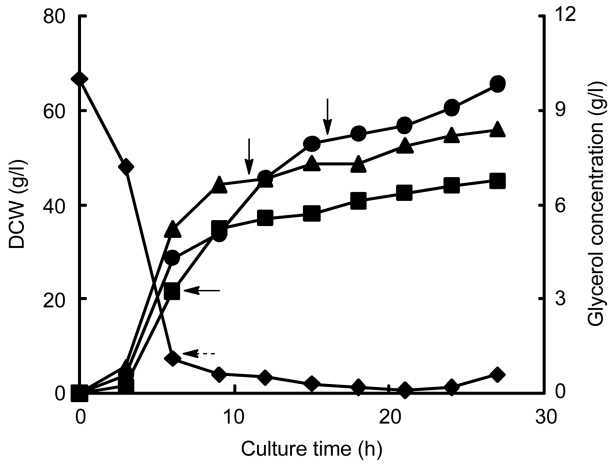

Fig. 2. Effect of induction time on the cell growth of recombinant *E. coli*.
The fermentation conditions are described in the Materials and Methods in the supplementary information. The dashed and solid arrows indicate the points for adding the feeding medium and L-arabinose, respectively. The 1% L-arabinose was fed at the early stage (squares), mid stage (triangles), or late stage (circles) of the cell density. The glycerol concentration in the culture broth during the fed-batch fermentation with the late-stage induction was monitored (diamonds) by using a glycerol determination kit (Sigma).

addition of 1% L-arabinose for all cases, which could have resulted from the utilization of cellular machinery for the expression of target protein, salmosin, instead of the cell growth [1]. The induction at the early stage during the fed-batch fermentation significantly inhibited the cell growth of recombinant *E. coli*, with DCW of 45.18 g/l. However, when the recombinant *E. coli* was induced at the mid stage and late stage, the cell growth was increased by 23.93% and 45.42%, respectively, showing the highest DCW of 65.70 g/l in the case of induction at the late stage. These results are in agreement with the previous studies showing that the induction at a late stage during the recombinant *E. coli* culture improved the expression levels of TGF-α-PE40 and D-amino acid oxidase, depending on the cell density [13, 14]. Our results indicate that the cell growth of recombinant *E. coli* is strongly influenced by the induction time. In addition, the induction was proved to be preferably done in the late stage of the cell growth phase for the achievement of high cell density of recombinant *E. coli* [1].

To refold the salmosin expressed as an inclusion body, the harvested cells were disrupted by sonication to recover the inclusion body, which was then solubilized in the buffer containing 8 M urea. Subsequently, a Sephadex G-25 desalting column was employed to recover the solution containing the refolded salmosin (Supplementary Fig. S2A). This simple refolding process by one gel filtration step has already been proved to be effective for removing the urea and allowing the target protein to refold into its native conformation [20]. The process of refolded salmosin into an enzymatically active protein was achieved by treatment with an urokinase-type plasminogen activator (uPA), confirming the single band of approximately 8 kDa on SDS–PAGE (Supplementary Fig. S2B), as already reported in a previous study [12]. The yield of recombinant protein at each step is shown in Table 1. The overall recovery of the active salmosin was 4.4% with its final concentration of 96 mg/l. The low recovery could be due to the aggregation of inclusion body in the solubilization step where the recovery was 19.5%. Because of the high initial protein concentration (2,200 mg/l) in this study, the aggregation rate could be more than the folding rate in the solubilization buffer, finally causing the decrease of the folded protein yield [22].

Table 1. Yields of the total protein from recombinant *E. coli*.

Steps	Total protein (mg/l)[a]	Recovery (%)[b]
Inclusion body	2,200	100
Solubilized inclusion body	430	19.5
Refolded salmosin	380	17.3
Active salmosin	96	4.4

The preparation of recombinant active salmosin is described in the Materials and Methods in the supplementary information.

[a]Total protein concentration was determined by the Bradford method using the Bio-Rad protein assay kit (Hercules, CA, USA).

[b]The recovery (%) was calculated as the protein concentration at each step per initial protein concentration.

We also checked the existence of inhibitory activity of the active salmosin against the platelet aggregation, since the native salmosin was already known to have this biological activity [11]. The control experiments using the whole-blood pre-mixture with collagen showed the platelet aggregation of 95% after incubation for 8 min, whereas the addition of 50 μl of the active salmosin to the pre-mixture demonstrated the inhibition of platelet aggregation, exhibiting an aggregation of 15% (Fig. 3). These results suggest that the recombinant salmosin produced by the high cell density fermentation was well expressed, and refolded with the active form without the loss of platelet aggregation-inhibitory activity.

The developments and productions of platelet aggregation inhibitors from natural products [15, 23] have been considered to be substitutes for the already well-known compounds such as aspirin and indomethacin [17]. To accommodate these changes, several disintegrins from snake venoms have been developed as novel platelet aggregation inhibitors [8, 11]. However, little has been reported about the mass productions of these integrins. Recent study reported the fed-batch fermentation of recombinant *Pichia pastoris* for enhancing the production of saxatilin [16]. Our results obtained in this study could give useful information for the commercial mass production of the platelet aggregation

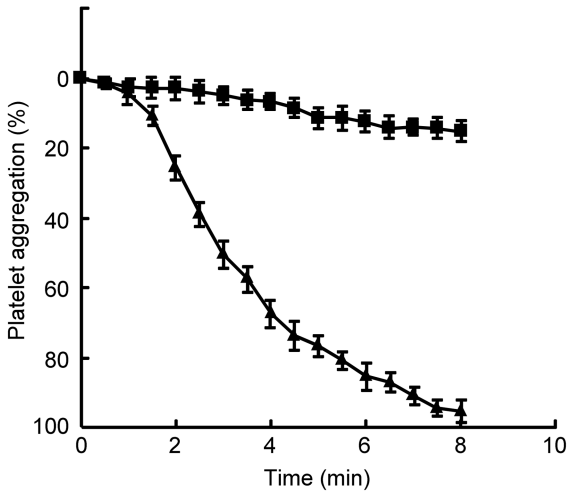

Fig. 3. Inhibitory activity against the platelet aggregation of the recombinant active salmosin in whole blood.

The method of platelet aggregation assay is described in the Materials and Methods in the supplementary information. The aggregation levels with (squares) or without (triangles) the recombinant active salmosin were monitored. The means with standard deviations of three independent experiments are shown.

inhibitor using the high cell density fermentation of recombinant *E. coli* system.

References

1. Bae, C. S., M. S. Hong, S. G. Chang, D. Y. Kim, and H. C. Shin. 1997. Optimization of fusion proinsulin production by high cell-density fermentation of recombinant *E. coli*. *Biotechnol. Bioprocess Eng.* **2:** 27−32.
2. Curtis-Fisk, J., R. M. Spencer, and D. P. Weliky. 2008. Isotopically labeled expression in *E. coli*, purification, and refolding of the full ectodomain of the influenza virus membrane fusion protein. *Protein Expr. Purif.* **61:** 212−219.
3. Dasari, V. K. R., D. Are, V. R. Joginapally, L. N. Mangamoori, and K. S. B. R. Adibhatla. 2008. Optimization of the downstream process for high recovery of rhG-CSF from inclusion bodies expressed in *Escherichia coli*. *Process Biochem.* **43:** 566−575.
4. Dennis, M. S., W. J. Henzel, R. M. Pitti, M. T. Lipari, M. A. Napier, T. A. Deisher, S. Bunting, and R. A. Lazarus. 1990. Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: Evidence for a family of platelet-aggregation inhibitors. *Proc. Natl. Acad. Sci. USA* **87:** 2471−2475.
5. Eiteman, M. A. and E. Altman. 2006. Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends Biotechnol.* **24:** 530−536.
6. Gould, R. J., M. A. Polokoff, P. A. Friedman, T. F. Huang, J. C. Holt, J. J. Cook, and S. Niewiarowski. 1990. Disintegrins: A family of integrin inhibitory proteins from viper venoms. *Proc. Soc. Exp. Biol. Med.* **195:** 168−171.
7. Hong, S. Y., H. Lee, W. K. You, K. H. Chung, D. S. Kim, and K. Song. 2003. The snake venom disintegrin salmosin induces apoptosis by disassembly of focal adhesions in bovine capillary endothelial cells. *Biochem. Biophys. Res. Commun.* **302:** 502−508.
8. Hong, S. Y., Y. S. Koh, K. H. Chung, and D. S. Kim. 2002. Snake venom disintegrin, saxatilin, inhibits platelet aggregation, human umbilical vein endothelial cell proliferation, and smooth muscle cell migration. *Thromb. Res.* **105:** 79−86.
9. Huang, T. F., C. Z. Liu, C. H. Ouyang, and C. M. Teng. 1991. Halysin, an antiplatalet Arg-Gly-Asp-containing snake venom peptide, as fibrinogen receptor antagonist. *Biochem. Pharmacol.* **42:** 1209−1219.
10. Jeong, K. J. and M. Rani. 2011. High-level production of a single chain antibody against anthrax toxin in *Escherichia coli* by high cell density cultivation. *Bioprocess Biosyst. Eng.* **34:** 811-817.
11. Kang, I. C., K. H. Chung, S. J. Lee, Y. D. Yoon, H. M. Moon, and D. S. Kim. 1998. Purification and molecular cloning of a platelet aggregation inhibitor from the snake (*Agkistrodon halys brevicaudus*) venom. *Thromb. Res.* **91:** 65−73.
12. Kang, I. C., Y. D. Lee, and D. S. Kim. 1999. A novel disintegrin salmosin inhibits tumor angiogenesis. *Cancer Res.* **59:** 3754−3760.
13. Kim, S. J., N. J. Kim, C. Shin, and C. W. Kim. 2008. Optimization of culture condition for the production of D-amino acid oxidase in a recombinant *Escherichia coli*. *Biotechnol. Bioprocess Eng.* **13:** 144−149.
14. Lee, C., W. J. Sun, B. W. Burgess, B. H. Junker, J. Reddy, B. C. Buckland, and R. L. Greasham. 1997. Process optimization for large-scale production of TGF-α-PE40 in recombinant *Escherichia coli*: Effect of medium composition and induction timing on protein expression. *J. Ind. Microbiol. Biotechnol.* **18:** 260−266.
15. Ma, L. Y. and P. G. Xiao. 1998. Effect of *Panax notoginseng* saponins on platelet aggregation in rats with middle cerebral artery occlusion or *in vitro* and on lipid fluidity platelet membrane. *Phytother. Res.* **12:** 138−140.
16. Min, C. K., J. W. Lee, K. H. Chung, and H. W. Park. 2010. Control of specific rate to enhance the production of a novel disintegrin, saxatilin, in recombinant *Pichia pastoris*. *J. Biosci. Bioeng.* **110:** 314−319.
17. Packham, M. A. and J. F. Mustard. 1977. Clinical pharmacology of platelets. *Blood* **50:** 555−573.
18. Park, Y. C., C. S. Kim, C. I. Kim, K. H. Choi, and J. H. Seo. 1997. Fed-batch fermentations of recombinant *Escherichia coli* to produce *Bacillus macerans* CGTase. *J. Microbiol. Biotechnol.* **7:** 323−328.
19. Passarinha, L. A., M. J. Bonifácio, and J. A. Queiroz. 2009. Application of a fed-batch bioprocess for the heterologous production of hSCOMT in *Escherichia coli*. *J. Microbiol. Biotechnol.* **19:** 972−981.
20. Quintas-Granados, L. I., E. Orozco, L. G. Brieba, R. Arroyo, and J. Ortega-López. 2009. Purification, refolding and autoactiviation of the recombinant cysteine proteinase EhCP112 from *Entamoeba histolytica*. *Protein Expr. Purif.* **63:** 26−32.
21. Savage, B., U. M. Marzec, B. H. Chao, L. A. Harker, J. M. Maraganore, and Z. M. Ruggeri. 1990. Binding of the snake venom-derived proteins applaggin and echistatin to the arginine-glycine-aspartic acid recognition site(s) on platelet glycoprotein IIb.IIIa complex inhibits receptor function. *J. Biol. Chem.* **265:** 11766−11772.
22. Singh, S. M. and A. K. Panda. 2005. Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.* **99:** 303−310.
23. Wu, T. S., Y. Y. Chan, M. J. Liou, F. W. Lin, L. S. Shi, and K. T. Chen. 1998. Platelet aggregation inhibitor from *Murraya euchrestifolia*. *Phytother. Res.* **12:** S80−S82.
24. Yee, L. and H. W. Blanch. 1992. Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Nat. Biotechnol.* **10:** 1550−1556.