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 [21]. Generally, the recombinant E. coli MC1061 harboring APMASIN 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-SC; 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