재조합 대장균의 유가배양을 통한 spider silk protein의 효율적인 고농도 생산 및 정제

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Efficient High-Level Production of Spider Silk Protein by Fed-Batch Cultivation of Recombinant *Escherichia coli* and Its Purification

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Introductions

Dragline spider silk has a number of notable mechanical properties such as high tensile strength and elasticity. Strong natural fibers with good tensile and compressive properties would be useful for many applications in medicine, such as sutures, membranes and temporary scaffolds for tissue engineering. This silk protein is composed of two proteins, MaSP 1 and 2, whose partial cDNAs have been cloned and sequenced (Lewis *et al.* 1992). Recently, recombinant spider silks have been generated from synthetic spider silk genes using *Escherichia coli* as the heterologous host. While recombinant proteins have been successfully generated from these genetically engineered systems (Winkler *et al.* 1999 Winkler *et al.* 2000, Szela *et al.* 2000), a significant limitation in these studies has been the difficulties in high-level producing genetically engineered silk-like proteins (Lewis *et al.* 1992, S. Winkler *et al.* 2000). Presently the *in vivo* production of artificial polymeric proteins is just starting and yields are still low. Thus, a need to overcome the low expression is indispensable. In this study, we describe the efficient process development during high-cell density cultivation of recombinant *E. coli* for the production of recombinant silk protein. We also describe simple procedure for purification of silk protein. The methodology described will allow production of sufficient quantities of the protein for many potential uses including production of artificial synthetic fibers.

Materials and Methods

Bacterial strain and plasmid

The *E. coli* strain BL21(DE3) (F- *ompT hsdSB* (rB-m B-) *gal dcm (DE3)*) (Novagen, Madison, WI) harboring the recombinant plasmid, pSH16A (Stefan *et al*. 1999), was used as a host strain for the high cell density cultivation. The plasmid, pSH16A, which encode silk proteins of 54 kDa (16 repeats of the monomer sequence) was kindly provided by Dr. Kaplan.

Culture condition

Flask cultures were carried out in a 500 mL flask containing 100 mL of semidefined or defined medium in a shaking incubator at 37 É and 200 rpm. The semidefined medium used was R/2 medium (pH 6.8) supplemented with 2 g/L yeast extract, 10 g/L glucose, and 30 mg/L kanamycin (Km) (Park *et al*. 1999). The R/2 medium (pH 6.8) contains per liter: 2 g of (NH4)2HPO4, 6.75 g of KH2PO4, 0.85 g of citric acid, 0.7 g of MgSO4·7H2O, and 5 ml of a trace metal solution that contains (per liter of 5 M HCl) 10 g of Fe SO4·7H2O, 2.25 g of ZnSO4·7H2O, 1 g of CuSO4·5H2O, 0.5 g of MnSO4·5H2O, 0.23 g of Na2B4O7·10H2O, 2 g of CaCl2·2H2O, and 0.1 g of (NH4)6MO7O24. The feeding solution used for fed-batch culture

contains per liter: glucose, 500 g; MgSO4·7H2O, 20 g; and yeast extract, 100g; Km, 30 mg/L was added to the growth medium. At an optical density at 600 nm (OD600) of 0.6, isopropyl-¥â-D-thiogalactside (IPTG) (Sigma Chemical Co., St. Louis, Mo) was added to a final concentration of 0.1, 1.0, 2.0 and 5.0 mM respectively. Cells were cultivated for another 4 h and then were harvested by centrifugation at 6,000 ×g for 10 min at 4 É. The cells were disrupted by sonication (Sonics & Materials Inc. Newtown, CT) for 10 min at 15% output. After centrifugation at 10,000 ×g for 10 min at 4 É, the supernatant fluid (soluble protein fraction) was saved for SDS-PAGE analysis.

Fed-batch cultivation

Fed-batch cultures were grown at 37 É in a 6.6 L jar fermenter (Bioflo 3000; New Brunswick Scientific Co., Edison, N.J.) containing 1.8 liters of R/2 medium. A seed culture (200 mL) was prepared in the same medium. The culture pH was controlled at 6.8 by the addition of 28% (v/v) ammonia water. Except for periods when the pH increase due to glucose depletion. The dissolved oxygen concentration was kept at 40% of air saturation by automatically increasing the agitation speed to 1,000 rpm and by changing the percentage of pure oxygen. A nutrient feeding solution was added by using the pH-stat (with high limit) feeding strategy. When the pH rose to a value greater than its set point (pH 6.8) by 0.08 due to the depletion of glucose, the appropriate volume of the feeding solution was automatically added to increase the glucose concentration in the culture broth to 5 g/L. Expression of the synthetic silk gene was induced by adding IPTG to a final concentration of 2 mM.

Purification of silk protein

Cells were harvested from 50 mL of culture by centrifugation at 6,000 ×g for 10 min at 4 É. The cells were resuspended in 50 mL of resuspension buffer (50 mM K2HPO4, 300 mM NaCl, pH 8.0) containing 200 §¶/mL lysozyme and Mini Complete protease inhibitor (Boehringer Mannheim, Mannheim, German) and were disrupted by sonication for 30 min at 45% output. The lysate was centrifuged at 14,000 ×g for 30 min at 4 É to remove cell debris. Finally, silk protein was purified by immobilized metal affinity chromatography. The protein solution was loaded onto a Ni-chealating resin (Qiagen, Valencia, CA) that had been preequilibrated with 50 mM K2HPO4, 300 mM NaCl, 5 mM imidazole, pH 8.0, washed extensively with equilibrium buffer and then protein was eluted with a linear 5 to 250 mM imidazole gradient in the same buffer at a rate of 120 ml/hr. The protein concentration in each fraction was monitored with a UV detector (Bio-Rad).

Analytical methods

During fed-batch cultivation, cell growth was monitored by measuring the OD600. Cell dry weight was determined as described previously (Lee and Chang 1994). Protein samples were analyzed by electrophoresis on SDS-PAGE gels containing 10% (w/v) polyacrlyamide as described by Laemmli (Laemmli 1970). The gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). The protein bands on the SDS-PAGE gels were quantified by densitometry (ImagerMaster; Pharmacia Biotech, Uppsala, Sweden). The amount of soluble protein was determined with a protein assay kit (Bio-Rad) by using bovine serum albumin as the standard. The purity of silk protein was analyzed by SDS-PAGE.

Results and Discussion

Fed-batch cultures

pH-stat fed-batch cultures of *E. coli* BL21(DE3) harboring pSH16A were grown as described in materials and methods. To examine the effect of cell density at the time of induction on production of silk protein, cells were induced with IPTG at OD600 value of 26, 60. Figure 1(A), 1(B) shows the time profiles for cell density (OD600), cell dry weight, and silk protein content expressed as a percentage of the total protein content. When cells were induced at the low cell density (OD600, 26), the fraction of silk protein in the total protein increased for 8 h

after induction and then decreased slightly (Fig. 1A). The cell dry weight and the maximal silk protein content were 33.8 ± 0.7 g/liter and 13% ± 0.3% of the total protein, respectively. When cells were induced at the intermediate cell density(OD600, 60), the fraction of silk protein content again increased for 8 h after induction and then decreased. The cell dry weight and the maximal silk protein content were 59.0 ± 2.9 g/liter and 12% ± 0.4% of the total protein, respectively (Fig.1B). Therefore, recombinant silk protein was most efficiently produced when cells were induced at the intermediate cell density.

Purification of recombinant silk protein

Recombinant silk protein was purified from 50 ml of culture broth obtained from a fed-batch culture induced at the intermediate cell density as described in materials and methods. The final amount and the recovery yield of purified silk protein were 9.2 mg and 26.3%, respectively. The purity of the silk protein as determined by SDS-PAGE was greater than 80%

Genes for fibrous proteins, whether of natural origin or synthetic, are likely to be long and repetitive. Their protein products comprise restricted amino acid composition. The propagation and expression of such genes in *E. coli* present difficulties not usually encountered with the more commonly produced pharmaceutical protein.

In this study, silk protein was high-level expressed by fed-batch cultivation. Fed-batch cultivation has most often been used for production of various recombinant proteins at high concentrations with high productivities (Lee 1996). There are several nutrient-feeding strategies that are available, and the pH-stat strategy was employed in this study for the following reason. In order to prevent excessive feeding of glucose, which is known to cause acetate production, a predetermined amount of the nutrient feeding solution was added only when the glucose concentration dropped to zero (indicated by an increase in the pH). Formation of recombinant proteins is also affected by the time of induction and inducer (IPTG) amount. Generally, recombinant protein was most efficiently produced when cells were induced at the intermediate cell density. The amount of inducer required to titrate the repressor molecules is proportional to the total cell mass and the optimal specific concentration of the inducer therefore needs to be determined for maximizing the silk protein synthesis at any cell concentration. When genetically engineered *E. colis* were induced with a range of specific amount of inducer (IPTG) (0.1 to 1 mM), recombinant proteins have been successfully expressed. In this study, in an attempt to produce repetitive proteins, induction with higher IPTG concentrations was more efficient (data not shown). Although the reason for this is not clear, it may be due to the increase demands of IPTG dosage per cell because of genetic deletion, duplication and processivity errors in RNA and protein synthesis. However, for the large-scale production of recombinant proteins, induction with higher IPTG concentrations is not ideal owing to toxicity and cost. Induction with effective amount of IPTG (2 mM) at the intermediate cell density (OD600, 60) resulted in the best production of recombinant silk protein, whose concentration was as high as 6.49 g/L.

In this report, we describe the production of large quantities of recombinant silk protein by fed-batch fermentation and efficient purification of this silk protein. The production of a high concentration of silk protein when cells are induced at an intermediate cell density during fed-batch cultivation should be useful for development of strategies for efficient production of other artificial fiber proteins.

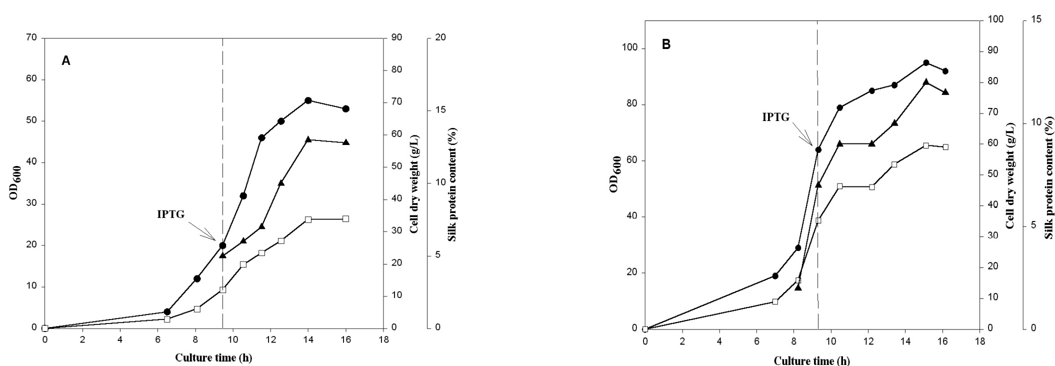

Fig. 1(A). Time profiles for cell density (OD600) (●), cell dry weight (▲), and silk protein content (□) during fed-batch cultivation with induction at the low cell density

Fig. 1(B) Time profiles for cell density (OD600) (●), cell dry weight (▲), and silk protein content (□) during fed-batch cultivation with induction at the intermediate cell density.

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