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Phase analysis in single-chain variable fragment production by recombinant *Pichia pastoris* based on proteomics combined with multivariate statistics

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The proteomics technique, which consists of two-dimensional gel electrophoresis (2-DE), peptide mass fingerprinting (PMF), gel image analysis, and multivariate statistics, was applied to the phase analysis of a fed-batch culture for the production of a single-chain variable fragment (scFv) of an anti-C-reactive protein (CRP) antibody by *Pichia pastoris*. The time courses of the fed-batch culture were separated into three distinct phases: the growth phase of the batch process, the growth phase of the fed-batch process, and the production phase of the fed-batch process. Multivariate statistical analysis using 2-DE gel image analysis data clearly showed the change in the culture phase and provided information concerning the protein expression, which suggested a metabolic change related to cell growth and production during the fed-batch culture. Furthermore, specific proteins, such as alcohol oxidase, which is strongly related to scFv expression, and proteinase A, which could biodegrade scFv in the latter phases of production, were identified via the PMF method. The proteomics technique provided valuable information about the effect of the methanol concentration on scFv production.

[**Key words:** Phase analysis; Single-chain variable fragment; *Pichia pastoris*; Proteomics; Cancerd2; Two-dimensional gel electrophoresis; Peptide mass fingerprinting]

Two-dimensional electrophoresis (2-DE) is a valuable tool for precise protein separation and can be effectively applied to proteome analysis based on peptide mass fingerprinting (PMF) using MALDI-TOF MS (1). In previous work, proteome analysis with the aid of artificial neural networks was successfully applied to recognize the sufficiency of chemical elements based on the protein spots resolved in 2-DE (2). The recognition of culture phases in a culture process would be important if the determination of different culture operations in each phase had a critical advantage for the improvement of bioproduction (3,4). We have developed a novel image analysis method combined with recently developed powerful computing hardware, in which the time series of the image data of silver staining gel taken during the developmental process was used for analysis (5). This image analysis method can check the spots of the same protein in each image at different times of development, and was modified in the present study in order to compare corresponding protein spots from two different gel images. The modified image analysis also contains multivariate analysis, which examines the relationship between the spots of two gel images.

In the present study, a proteomic technique with a modified image analysis was applied to phase recognition during the fed-batch culture production of a single-chain variable fragment antibody (scFv). *Pichia pastoris* is widely used as a host strain for the production of foreign proteins, and many eukaryotic proteins have been produced in the supernatant outside cells (6–11). We also used a strain of *P. pastoris* (GS115) expressing the scFv of anti-CRP antibody as a model strain in the present study, and the fed-batch cultures were carried out under the control of methanol concentration during the production phase. In the fed-batch culture, the control of methanol feeding is important because methanol is used not only for the induction of protein expression but also for the growth of the strain. The starvation of the carbon source mortally inhibits both the growth of the strain as well as the production of foreign protein. Furthermore, an excessively high methanol concentration in the culture broth severely affects the metabolism in the strain. Therefore, we constructed a methanol feeding control system with the aid of a semi-conductor gas sensor (7,8). As a result of the fed-batch culture using a 2 *l* jar-fermentor with a control system, we obtained 73 g/*l* of the dry cell weight (DCW) and 2.1 g/*l* of the scFv concentration in the supernatant of the culture broth, which was 5 times higher than the maximum product concentration obtained in the flask cultivation. The obtained scFv concentration in the present study is one of the highest concentrations reported in scFv production (6,8,11–15). In the fed-batch culture with a methanol control system, the scFv production started soon after the startup of the methanol feeding operation. The production continued for approximately 100 h, during which the production rate was kept almost constant. We analyzed the proteins contained in the cell in the growth and production phases using two-dimensional electrophoresis (2-DE) in order to investigate the effect of the methanol feeding operation on production.

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We also examined the protein expression amounts in each phase and compared them statistically based on multivariate analysis.

The images obtained from the cells in the different phases were remarkably different from one another, which suggested that the expression protein of the cells had seriously changed with the phase transition. Furthermore, many proteins in the cell were identified using a MALDI TOF MASS with a MASCOT database system, and some of them were strongly related to the expression of scFv with an AOX1 promoter and the scFv biodegradation process. These results could provide crucial information for the determination of an optimum operation for scFv production.

MATERIALS AND METHODS

Culture conditions *P. pastoris* GS115 was obtained from Invitrogen (Carrlsbad, CA, USA). *P. pastoris* is particularly well-suited for fermentative growth, and has the ability to reach very high cell densities during fermentation, which may improve overall protein yields. The transformation of the strain for the scFv production was carried out based on the information from references (7,10), as follows.

The anti-CRP scFv gene with a His tag was inserted into the Snab I site of a vector plasmid pPIC9 (Invitrogen), which contained the AOX1 promoter and α-factor signal genes for expression in *P. pastoris*. The inserted plasmid was digested with Sal I in a HIS 4 gene and introduced into *P. pastoris* GS115 by electroporation after competent cell preparation, as shown in Supplementary Fig. S1.

The transformed strain was stocked in a 1 ml vial in a freezer at −80°C, and was used as a fermentation inoculum for the seed culture, which was carried out for 24 h in 500 ml flasks containing 50 ml YPD medium at 30°C with circular shaking at 170 rpm. A seed culture broth was used for the inoculum of the fed-batch culture using a 2-*l* Jar fermentor (BMJ-O2PI; Biott, Tokyo) with a 1-*l* medium solution (glycerol, 40 g/*l*; K_2SO_4, 18.2 g/*l*; $CaSO_4 \cdot 2H_2O$, 0.93 g/*l*; $MgSO_4 \cdot 7H_2O$, 14.9 g/*l*; KOH, 4.13 g/*l*; H_3PO_4, 26.7 ml/*l*) and 4.4 ml of a trace metal solution ($CuSO_4 \cdot 5H_2O$, 6 g/*l*; KI, 0.08 g/*l*; $MnSO_4 \cdot 5H_2O$, 3 g/*l*; H_3BO_3, 0.02 g/*l*; $MoNa_2O_4 \cdot 2H_2O$, 0.20 g/*l*; $CoCl_2 \cdot 6H_2O$, 0.916 g/*l*; $ZnCl_2$, 20 g/*l*; $FeSO_4 \cdot 7H_2O$, 65 g/*l*; D-biotin, 0.2 g/*l*; H_2SO_4, 5.0 ml/*l*). The pH of the medium was automatically controlled at 5.0 with the addition of an ammonium aqueous solution, and the dissolved oxygen (DO) level was maintained above 20% air saturation by control of the agitation speed (400–1000 rpm).

On-line control of the methanol concentration Semi-conductor gas sensor models TGS813 and TGS822 (Figaro Engineering Inc., Osaka) were used for the on-line monitoring of the methanol concentration in the exhausted gas from the fermentor (7,8). The output signal from the sensor was amplified and converted to digital signals and transferred to a personal computer, which could graphically display the methanol concentration in the culture broth, and could control the feed rate of the methanol tubing pump with a proportional-integral-derivative control system. TGS813 was used for the fed-batch culture at 5, 10 and 25 g/*l* of methanol concentration, and TGS822 was used for the fed-batch culture at 3 g/*l* of methanol concentration.

Operation of the fed-batch culture The fed-batch culture process was divided into the following operational phases. In the first phase, the batch operation was carried out for initial growth using glycerol as a carbon source. When the glycerol in the initial medium was perfectly consumed, and the DO value jumped up for the exhaustion of the carbon source, the second operation phase was started as follows. The glycerol fed-batch culture was carried out for high cell density, and an 80% glycerol aqueous solution with a 2.5% volume of trace element solution was used for the feeding medium. The feed pump was on/off controlled in such a manner that its switch would be on when the DO value was higher than 60%, and its switch would be off when the DO value was less than 60%. The temperature, pH, agitation speed, and aeration rate were maintained at 30°C, 5.0, 900 rpm, and 1.2 vvm, respectively, during cultivation.

The transition process was carried out as follows. Glycerol feeding was terminated when the optical density at 600 nm (OD_{600}) exceeded 130. Initiation of the induction of the protein production was carried out by adding 10 g of a 100% methanol solution containing 25 ml PTM trace salts per liter of methanol 30 min after the DO sudden increase, which indicated glycerol starvation. After 1.5 h, 10 g of methanol was added again, and after another 1.5 h the methanol feeding was started using the control system of methanol concentration with the on-line detection system of methanol concentration, as shown in Fig. 1. We carried out the culture experiment with various methanol concentrations in order to examine the effect on the growth and the scFv production.

Measurement of scFv concentration in the culture broth A sample solution from the culture broth was centrifuged at 4500 ×*g* for 10 min. The supernatant solution was harvested and centrifuged again at 4500 ×*g* for 5 min and the 2nd supernatant was harvested. The His-tagged scFv was purified from the 2nd supernatant solution using the affinity chromatography system (ÄKTA system) with a His Trap HP affinity column (GE Healthcare). The supernatant was applied to the affinity column, which was pre-equilibrated with buffer A (pH 7.2) containing 20 mM imidazole and 2 × PBS buffer containing 16 g/*l* NaCl, 0.4 g/*l* KCl, 5.8 g/*l* $Na_2HPO_4 \cdot 12H_2O$ and 0.4 g/*l* KH_2PO_4. After washing the column with buffer A, the bound proteins were eluted with buffer B (pH 7.2) containing 400 mM imidazole and 2 × PBS buffer, and the eluates were harvested using a fraction collector. The harvested solution was treated by dialysis using a cellulose membrane tube with 1 × PBS buffer as the dialysate. The protein amount in the dialysis-treated solution was measured based on the Lowry's method using a Dc protein assay kit (Bio-Rad).

The cell concentration in the culture broth was calculated from the optical density at 600 nm using a V-630 spectrophotometer (JASCO, Tokyo). The methanol concentration was intermittently measured using a gas chromatograph (model GC-14B, Shimadzu Kyoto), which was equipped with a flame ionization detector and a Chromatopac C-R8A (Shimadzu). The initial column temperature was 80°C, the temperature was increased at 4°C/min up to 120°C, and the temperature was held at 120°C for 2 min. The temperature of the injector and detector were maintained at 140°C and 120°C, respectively. N_2 gas (0.2 MPa) was used as a carrier gas. A propanol solution was used as the internal standard liquid.

SDS-PAGE SDS-PAGE was carried out in order to investigate the protein mixture content in the supernatant of the culture broth using 13 cm × 13 cm acryl amide gels, which contained a stacking gel zone and a separating gel zone. The electric currency during electrophoresis was set at 30 mA/gel.

Coomassie Brilliant Blue G-250 (0.1%) was used to stain the separated protein.

2-DE analysis The overall procedure for 2-DE was based on the protocol listed in a review by Görg et al. (16) and on the technical information for a 2-D PAGE in SWISS-2DPAGE (http://world-2dpage.expasy.org/swiss2dpage/docs/protocols/). The culture broth in the fed-batch culture was diluted to 30 g/*l* DCW, and 1.6 ml of this diluted solution was centrifuged for 5 min at 800 ×*g*. Precipitated cells were washed 9 times in 1/15 M phosphate buffer (pH 7.0) with centrifugation for 5 min at 4500 ×*g* after each washing. Cell washing using a phosphate buffer should be carried out carefully in order to thoroughly remove the ionized components. The cell pellet was resuspended in 0.8 ml of lysis buffer containing 8 M urea, 2 M thiourea, 2% w/v 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 0.3 M dithiothreitol (DTT), 32 µl of IPG buffer (GE Healthcare Japan, Tokyo) and trace amounts of Bromophenol Blue. The sample solution was transferred into a screw-capped tube with 0.92 g of glass beads (diameter, 1.0 mm). Cell disruption should be carried out

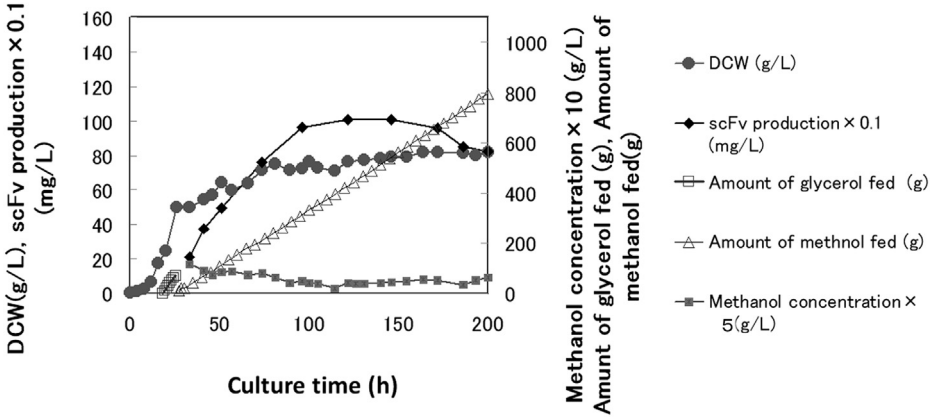

FIG. 1. Time courses of a fermentation system with methanol concentration controlled at 5 g/*l*.