Production and purification of human menin from *Drosophila melanogaster* S2 cells using stirred tank reactor

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Abstract

A process was developed for producing human menin from transformed *Drosophila* Schneider 2 cells. Protein expression was achieved after inducing the metallothionein promoter by adding copper sulfate to cells growing in suspension in a stirred-tank reactor. Experiments in shake flasks showed that the production of menin was improved when the induction was conducted late in the exponential phase of cell growth at a concentration of $1-2 \times 10^7$ cells ml⁻¹, with a copper concentration of 0.2 mM for no more than 24 h. This observation was confirmed by experiments in bench-scale fermentors. Subsequently, a pilot-scale fermentation yielded 1 mg l⁻¹ culture of purified menin.

Introduction

The *Drosophila* expression system has been available for several years (Schneider, 1972), but until recently not much attention has been given to its potential as a method for expressing heterologous proteins (Deml et al., 1999). Like other eucaryotic expression systems, the *Drosophila* cells are able to synthesize proteins with post-translational modifications (Hansen et al., 1998), but compared with other methods, this process allows the rapid selection of a stable line with up to 500 copies of a gene in a single transfection event (Johansen et al., 1989), and the suspended cells can grow to much higher densities (between 2 and 6×10^7 cells per ml) in a relatively simple medium (Sondrgaard 1966). In addition, the heterologous protein expression is under the well-studied and tightly-regulated metallothionein (Mtn) promoter (Johansen et al., 1989), which is easier to activate by the addition of the copper sulfate to the medium.

Several proteins have been successfully produced using the *Drosophila* expression system. These include human plasminogen (Nilsen and Castellino 1999), an analog of human tissue plasminogen-

activator (Olson et al., 1992), a human MHC class II molecule (Hansen et al., 1998), a human dopamine β -hydroxylase (Li et al., 1966) and mouse endostatin (Park et al., 1999). Very little information is available on the actual propagation process, on optimizing the production strategy (Bunch et al., 1988), on the effect of various environmental factors such as dissolved oxygen and pH, and on ways to handle large-scale production of recombinant proteins.

Human menin was cloned and expressed in the *Drosophila melanogaster* S2 cells. Menin is a 610 amino acid protein with a molecular weight of 67 KDa expressed by the *MEN 1* gene. The protein may act as a potential tumor suppressor in multiple endocrine neoplasia type 1, (Candrasekharappa et al., 1977), an autosomal dominant familial cancer syndrome. The characterization of the production process of this protein, which includes a pre-induction phase and a post-induction phase, is described in this report. An attempt was made to optimize both the proper conditions for achieving high cell densities and for expression and production of the protein.

Materials and methods

Cells, culture conditions, and analytical methods

The *Drosophila* Expression System (Invitrogen, U.S.A.) was used to establish stable, inducible *Drosophila* Schneider 2 cells (S2) for over-expression of human menin. The menin cDNA was amplified by PCR using pCMV-Sport-Menin (Sci) with forward

(5'-CCAGAATTCGCCGCCATGGGGCTGAAG-GCCGCCCAG-3') and reverse (3'-CCAGTACCGGT-GAGGCCTTTGCGCTGCCGCTTGAG-5') primers. The former and latter primers contained *Eco* RI and *Age* I sites, respectively, to facilitate ligation into the pMT/V5-HisA expression vector. Subsequently, the menin insert was entirely sequenced to verify that the construct junctions were correct and that no changes inadvertently occurred during amplification with amplitag gold. S2 cells were transfected with pMT/HisA-menin and pCoHYGRO at a ratio of 19:1 using calcium phosphate. After two days of growth in complete *Drosophila* Expression Medium (Invitrogen, USA), the transfected cells were selected with hygromycin B at 23 °C. Stably transfected *Drosophila* Schneider 2 cells were cultured in 250 ml shake flasks shaken at 100 rpm on an orbital shaker (New Brunswick Scientific, U.S.A.) in a 22 °C incubator (Forma, U.S.A.) with HyQ SFX Serum-free Insect Cell Medium (Hyclone SH30278.02, U.S.A.) supplemented with 100 U ml⁻¹ Penicillin, 100 µg ml⁻¹ amphotericin B, 10 µg ml⁻¹ gentamicin and 200 µg ml⁻¹ Hygromycin B. The viable cell densities of these stock cultures were kept above 10⁶ cells ml⁻¹ by continuous passage. Viable-cell density was determined by dye-exclusion cell counts using 0.4% Trypan Blue (Gibco 15250-012) and an improved Neubauer hemacytometer (Hauser Scientific, U.S.A.). Glucose and lactate concentrations were determined using a biochemistry analyzer (YSI 2700, OH, U.S.A.). Full length menin was detected by western blotting using an antibody (SQV) raised against synthetic peptide corresponding to the C terminal 28 amino acids of menin (Guru et al., 1998).

Optimization experiments

Full factorial design was used to study the effect of induction timing, inducer concentration and induction duration on the amount of the menin recovered. This design allows for the use of two arbitrary levels of each variable and accounts for individual effects as well as interactive effects (Box et al., 1987). In the

experiments, an inoculum of S2 cells was grown in two 250-ml shake flasks shaken at 90 rpm. When the cells reached the mid-to-late exponential growth phase, they were transferred to a 1 l spinner flask (with a working volume of 600 ml) at a concentration of ~10⁶ cells ml⁻¹. This flask was stirred continuously at 90 rpm in a 22 °C incubator. When the induction times were reached, 50 ml of the cell suspension were transferred to another shake flask where they were induced with a CuSO₄ solution (100 mM) at the desired concentration. At the prescribed time, each flask was harvested, the resulting cell pellet was washed once with PBS containing EDTA-free protease inhibitors (Boehringer Mannheim, Germany), resuspended in 1 ml of the same buffer solution and stored at -20 °C.

Menin detection and quantitation

The intracellular menin concentration was determined by homogenizing the cell pellets with a Polytron (Brinkman, U.S.A.) for 30 s, followed by 15 min centrifugation at 14 000 rpm in a microcentrifuge (Eppendorf, Germany). The resultant cell extracts were purified using immobilized metal affinity chromatography (IMAC) according to the following procedure: 0.5 ml of the extract was mixed for one hour at 4 °C with 1 ml Ni-NTA resin (Qiagen, U.S.A.) and 10 ml PBS containing 10 mM imidazole. The slurry was packed into a 1 cm diameter column, washed with 10 ml wash buffer (50 mM NaH₂PO₄; 300 mM NaCl; 20 mM imidazole, pH 8) and eluted with 4 ml in 1 ml aliquots of elution buffer (50 mM NaH₂PO₄; 300 mM NaCl; 200 mM imidazole, pH 8). The eluted fractions were pooled and menin was detected by Western blotting with the SQV antibody. Quantitative analysis was done using a dot blot according to the following procedure: pre-cut nitrocellulose membranes (Novex, U.S.A.) were wetted with PBS and placed in a Minifold filtration manifold (Schleicher and Schuell, U.S.A.); 100 µL well each of standards and samples were loaded; and the membrane was then blocked with 10% milk; the blots were probed with the polyclonal SQV antibody (1:1000 dilution) followed by a secondary alkaline phosphatase-conjugated, goat anti-rabbit antibody, 1:2000 dilution (Kirkegaard and Perry Laboratories, U.S.A.), and developed with a BCIP/NBT solution (Kirkegaard and Perry Laboratories). The blots were scanned and analyzed with the Scion Image Analysis software.

Fermentation

Fermentations were conducted in 2 and 10 l bench-top fermentors equipped with a pitched blade impeller (B. Braun). Initial cell concentration was $\sim 10^6$ ml $^{-1}$, agitation was constant at 90 rpm, dissolved oxygen concentration was controlled at 30% by sparging air or pure oxygen, and the pH was maintained at 6.4 with CO $_2$.

Results

High-density growth of *Drosophila* cells

High-density growth of the *Drosophila* cells was achieved by propagation in shake flasks, spinner flasks or stirred-tank reactor. Figure 1 demonstrates a typical growth process. The cells grew exponentially at a rate of 0.3–0.4 h $^{-1}$ for four days reaching a maximum cell density of 3×10^7 cells ml $^{-1}$. During this period the cells consumed glucose, glutamate and lactate at a specific rates of 8×10^{-12} g h $^{-1}$ cell $^{-1}$, $1\text{--}5 \times 10^{-11}$ mmoles h $^{-1}$ cell $^{-1}$, and $9\text{--}10 \times 10^{-13}$ g h $^{-1}$ cell $^{-1}$, respectively. Viability remained at 95% even at 100 h after the stationary phase was reached. Ammonia was slowly produced during the exponential growth phase and was rapidly consumed once the stationary phase was reached.

Improving menin expression

Table 1 summarizes the level of expressed menin using a full factorial design experiment. Three variables were examined: the time of induction along the cell growth curve, the concentration of the inducer (CuSO $_4$) and the length of the induction phase. A preliminary set of experiments (not shown), indicated that amongst the induction condition tested the highest expression of menin was obtained when the induction occurred during a late exponential growth phase, when the cell concentration was 3×10^7 ml $^{-1}$, at a copper sulfate concentration of 0.5 mM; and for a period of 24 h. Based upon these results, a second set of experiments was performed (Table 1), confirming the previous results and showing that amongst the condition tested the best expression was observed when the induction occurred during the late exponential growth phase, when the cell number was 2.5×10^7 ml $^{-1}$; at a lower copper sulfate concentration of 0.2 mM; and for a period of 24 h.

Menin expression in stirred tank reactors

Process development

The process for menin expression in a 2-l bench-top fermentor is shown in Figures 2a and 2b. The expression was done at two conditions: a) induction at an early logarithmic growth phase when the cell concentration was 6×10^6 ml $^{-1}$; and b) induction at a late logarithmic phase when their concentration was 2×10^7 ml $^{-1}$. This process was conducted after the first optimization experiment and therefore the protein expression was induced with 0.5 mM copper sulfate which resulted in rapid production of menin in the first 12 h post induction. Induction at the higher cell concentration yielded initially twice as much menin as induction at the lower cell concentration, but this was followed by 30% decline 12 h later. These results confirmed the findings obtained in optimization experiments performed in shake flasks, in which higher menin amounts were obtained when induction was done late in the growth phase. Initial specific menin production rates were measured in this experiment and were found to be similar at the two conditions: 8×10^{11} μ g cell $^{-1}$ h $^{-1}$ when the induction was done early in the growth phase and 6×10^{11} μ g cell $^{-1}$ h $^{-1}$ when the induction was done later in the growth phase, menin concentration was higher 12 h after induction. It is likely that harvesting at 12 h post-induction is better than harvesting at 24 h after induction, though harvesting this early was not attempted in the shake flask experiments.

Pilot scale production of menin

A fermentation process for production of menin using a 10-l fermentor is shown in Figure 3. The fermentor was inoculated with 2 l of cultured S2 cells that had been grown in a 2-l fermentor; medium was added gradually, reaching a final volume of 10 l 150 h after inoculation. After a cell density of 1.2×10^7 ml $^{-1}$ was reached, menin expression was induced by the addition of 0.2 mM copper sulfate for 12 h, 0.2 mM copper sulfate replaced the 0.5 mM concentration since the second optimization experiment indicated that this concentration is better. The overall production process is described in Figure 4. Following induction, the cells were collected and processed as described in the material and method section. Between 1 and 2 mg of pure protein were recovered from each liter of culture. Figure 5 shows the SDS-PAGE of the protein elution from an IMAC column.

Figure 1. Propagation process of S2-menin cells in a 250-ml shake flask; cell density (●), viability (—), glucose concentration (■), lactate concentration (▲), glutamate concentration (▼) and ammonia concentration (◆).

Table 1. Factorial design for menin production as a function of: cell concentration, inducer concentration and induction duration

Culture	Cell concentration at induction (10^7 ml^{-1})	CuSO_4 concentration at induction (mM)	Harvest time days post induction)	Menin (%)*
1	0.5	0.2	1	3.93
2	0.5	0.7	1	2.35
3	0.5	0.2	6	1.29
4	0.5	0.7	6	52.08
5	1.5	0.45	3.5	69.52
6	1.5	0.45	3.5	18.35
7	2.5	0.2	1	100
8	2.5	0.7	6	3.56
9	2.5	0.2	6	1.73
10	2.5	0.7	1	84.80

* 100% Menin was the highest obtained in this experiment.

Figure 2. Production of menin from S2 cells in 2 l fermentor. (a) Induction with 0.5 mM CuSO_4 at early exponential growth phase (b) Induction with 0.5 mM CuSO_4 at late exponential growth phase; cell density (●), menin concentration (■).

Figure 3. Production of menin from S2 cells in a 10 l fermentor. The cells were grown in a fed batch mode and induced with 0.2 mM CuSO₄; cell density (●) culture volume (—), glucose concentration (▲), lactate concentration (■).

Figure 4. Production process of menin from 10 l fermentation.

Discussion and conclusions

The *Drosophila* Expression System was investigated as an additional eukaryotic expression system for the production of heterologous proteins. A production process for human menin was established. Menin is a 67 KDa protein that may function as a potential tumor suppressor in multiple endocrine neoplasia type 1. The protein was expressed intracellularly through induction of stably transfected cells growing in suspension culture, extracted from the cells, and purified by IMAC. Approximately 1 mg of purified protein per liter of culture was obtained, which is a relatively low amount when compared with the level of expression of other human proteins in *E. coli*. But it is similar to expression levels of several human proteins in mammalian cell systems (Castillo et al., 1998; Watanabe et

al., 1998). Unlike mammalian cells, *Drosophila* cells easily grow to high densities. Concentrations of $2 \times 10^7 \text{ ml}^{-1}$ are easily obtained in serum-free medium in a stirred-tank reactor using simple batch growth strategies, sparging air and oxygen directly into the medium. It was found that the amount of protein produced is dependent on the time of induction, the concentration of the copper sulphate and the induction duration. Two optimization experiments were conducted which indicated that higher amount of protein is produced if the induction is done later in the growth, and for a period of 12 h. The experiment was done in two stages the first indicated that the best conditions were 0.5 mM copper sulphate and 24 h induction, when another set of conditions was tested, lower concentration of copper sulphate 0.2 mM and 12 h induction time were better. Similar to other eucaryotic

Figure 5. SDS-PAGE of menin eluted from IMAC column. Lane 1-flow through, lane 2-molecular weight markers, Lane 8–3 elution fractions 1–6.

expression systems, the expressed menin is soluble, but because it accumulates intracellularly the recovery process is quite cumbersome. Higher amounts of menin were produced when the induction was done late in the growth phase when the cell concentration was higher and the specific production was similar to the specific production obtained when the induction was done early in the growth phase. Improving the specific expression level and driving the protein outside the cells should improve the process significantly, resulting in wider application of this system.

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