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Optimization of HEK293 Cell Culture in a New Brunswick™ CelliGen® 115 Bioreactor for the Production of Recombinant GPCR

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Abstract

Heterologous expression of membrane proteins remains a bottleneck for structural characterization by x-ray crystallography. Such proteins represent approximately 30 % of the proteome and are not sufficiently represented in the Protein Data Bank (PDB)[1]. G-protein-coupled receptors (GPCRs) are an area of particular interest as it is estimated that one third of current FDA-approved drugs act through this class of receptors.

Introduction

We have been studying rhodopsin with an interest in determining the conformational change that leads to signal transduction in this class of receptors. Although there has been some success in expressing select members of the large GPCR family in bacterial systems, the best characterized expression systems have generally been in mammalian tissue culture. In our case, we isolated stable cell lines in which the desired receptor is expressed upon exposure to tetracycline. The cell line was derived from HEK293 cells, which can be grown in suspension. Attempts to scale up production of recombinantly-expressed protein by the use of spinner flasks were unsuccessful.

Based on our initial experiments using tissue culture plates, we had expected approximately 1 mg of recombinant protein for 1 L of cells grown in suspension, but found that expression levels in spinner flasks were closer to 0.1 mg per L. Use of a stirred-tank bioreactor allowed for optimization of cell growth, as described below, and resulted in higher cell densities with concomitant higher levels of expression of our recombinant protein.

The BioFlo/CelliGen 115 bioreactor equipped with a thermal mass flow controller and four-gas mix module.

Materials and methods

Cell line

The cell line, HEK293 GnT (N-acetylglucosaminyl-transferase I), was a generous gift from Phillip Reeves and H. Gobind Khorana[2]. It is a derivative of the standard HEK293 cell line that was selected by mutagenesis and ricin treatment to be deficient in N-acetylglucosaminyl-transferase I activity. GPCRs expressed in this cell line have a more uniform pattern of glycosylation which should result in a higher likelihood of crystallization. We also utilized the vector that Reeves, Callewaert et al. have described[2] which places receptor expression under the control of tetracycline exposure to the cells.

Bioreactor

We used the New Brunswick™ BioFlo®/CelliGen® 115 bioreactor equipped with a thermal mass flow controller and four-gas mix module. A pitched-blade impeller was driven by a magnetic motor, and the cultures were grown in a 5 L (2.0 - 5.6 L working volume) water-jacketed vessel.

Culture media

DMEM/F12 supplied as a powder from Atlanta Biologicals (Lawrenceville, Georgia, USA) was used as the base media. This was supplemented with sodium bicarbonate (3.7 g/L), Primatone® RL-UF (0.3 g/L), 10 % heat-treated FBS, penicillin G (100 units/mL), streptomycin (100 µg/mL), glutamine (292 µg/mL), dextran sulfate (300 µg/mL), and pluronic F-68 (0.1 % w/v). The media was sterilized by filtration through a 0.2 µm membrane and pumped into the vessel.

Control software

All equipment was monitored using New Brunswick BioCommand® software with data logging set at one-minute intervals.

Method

On the day before inoculation, the 5 L bioreactor vessel was filled with 4 L of phosphate buffered saline. The various ports were connected to appropriate tubing for removal of the saline, introduction of media, introduction of cells, and the pumping of the four-gas mix through the sparger. The pH electrode was calibrated and then disconnected and the protective cover was installed. The oxygen probe was examined and also covered by a protective cover. The jacket of the vessel was filled with water, and the assembly was set in autoclave for a 30-minute sterilization cycle. Afterward the vessel was returned to the tissue culture room and allowed to cool overnight. The following day the calibration of the pH electrode and oxygen sensor was checked after allowing the oxygen sensor to charge by the control unit.

A stable cell line which contains the expression cassette for the GPCR, under the control of a cytomegalovirus promoter/tetracycline-responsive promoter was selected using the neo gene. These cells were maintained in tissue culture plates with DMEM/F12 medium supplemented with 10 % fetal bovine serum, G418, and blasticin. For inoculation of a 5 L bioreactor vessel, thirty 15 cm plates were grown to approximately 80 % confluence. On the day of inoculation, 4 L of media were prepared and transferred to the vessel with a peristaltic pump after removal of PBS from the vessel.

All setpoints were programmed from BioFlo/CelliGen 115 control station as follows: temperature at 37 °C and a pH of 7.2. Oxygenation was maintained at 50 % using the four-gas mixture of air, nitrogen, oxygen, and carbon dioxide, and the thermal mass flow controller was set to deliver 0.5 L per minute. The pH was maintained by a combination of carbon dioxide and a solution of 7.5 % sodium bicarbonate that was controlled by pump 2. Agitation with the pitched blade impeller was set to 30 rpm.

The cells were recovered from the tissue culture plates by brief trypsinization and resuspension in the culture medium. The cells were pumped into the vessel with an auxiliary peristaltic pump. A small sample was removed and the starting cell density was determined with a hemocytometer.

Over the next five to seven days, the cell density was checked on a daily basis. Once the density reached 0.8 – 1.0 x 10^6, the culture was supplemented with 40 mL of 20 % (w/v) glucose and 120 mL of 10 % (w/v) Primatone RL-UF. The following day, expression was induced by the addition of tetracycline (2 µg/mL) and sodium butyrate (5 mM) to the culture (Figure 1). One day later, the cells were recovered from the bioreactor and pelleted by centrifugation. A 1 mL aliquot was reserved for analysis by Western Blot to determine the level of expression (Figure 2).

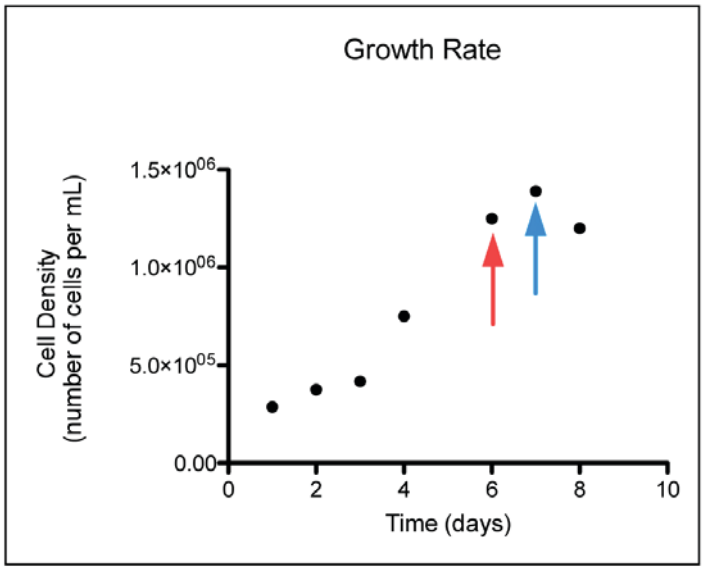

Figure 1. Growth curve for HEK293 cells in a CelliGen 115 bioreactor 5 L vessel. The red arrow indicates the addition of glucose and Primatone. The blue arrow indicates the addition of tetracycline and sodium butyrate for the induction of protein expression.

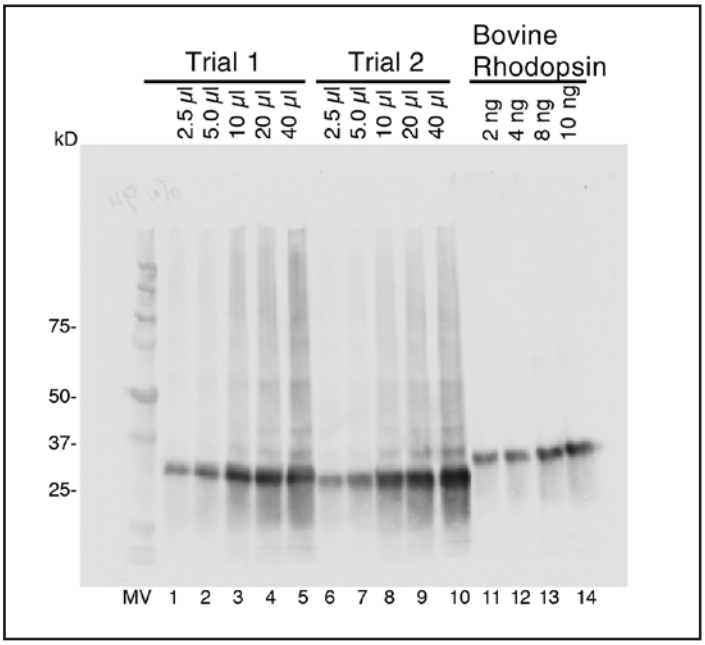

Figure 2. Western Blot with mAb for detection of GPCR protein expressed in HEK293 cells.

Results and discussion

The expressed GPCR was solubilized by lysing the cell pellet from the small aliquot with a buffer containing 1 % (w/v) dodecyl-maltoside. The expressed GPCR was detected using a Western Blot with a monoclonal antibody, and the signal detected was compared to rhodopsin purified from cow retinae. We detected approximately 1 mg of recombinant GPCR per L of cell culture. The migration of the recombinant protein was probably due to differences in glycosylation. This was a dramatically improved result when compared to cell growth in suspension with spinner flasks where a cell density above 0.5 x 10^6 was hard to achieve. In experiments using the same cell line performed in spinner flasks, the expression level of recombinant GPCR ranged from 0.1 – 0.2 mg/L of culture (Table 1).

Yields	7 L Spinner Flask	BioFlo/CelliGen 115 Bioreactor 4.5 L Run	BioFlo/CelliGen 115 Bioreactor Two 4.5 L Runs
Maximum Cell Density	0.5 x 10^6/mL	1.4 x 10^6/mL	1.4 x 10^6/mL
Protein Culture Volume	0.1–0.2 mg/L	1 mg/L	10 mg/L

Table 1. Recombinant protein (rGPCR) expression comparisons.

A large-scale prep (two 4.5 L runs) was subsequently performed, and 10 mg of purified rGPCR were obtained in a detergent solubilized form. A G-protein activation assay in which uptake of a radio-labeled non-hydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirmed the bioactivity of the recombinant protein. The reaction was started by the addition of GTPγS, and aliquots of the reaction were applied to nitrocellulose filters at various times. In the absence of a receptor, very little spontaneous uptake of the radio-labeled nucleotide was detected. The form of the receptor expressed in the experiments contained mutations in which residues were altered to cause constitutive activation. The receptor expressed in the bioreactor caused an increase in the rate of nucleotide uptake by transducin, as expected (Figure 3).

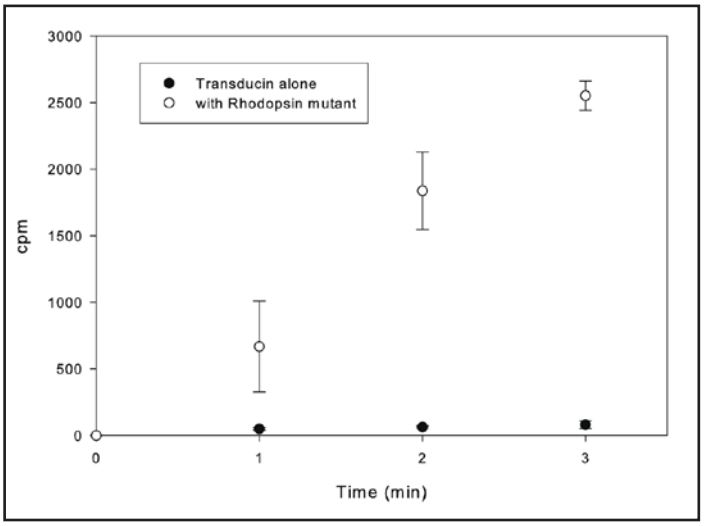

Figure 3. The bioactivity of the expressed rGPCR protein in the bioreactor was measured using G-protein activation assay using [^{35}S]-GTPS binding assay. Uptake of a radio-labeled nonhydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirming the bioactivity of the recombinant protein.

Conclusion

This study demonstrates that by being able to control the cell culture process parameters using a cell culture bioreactor, both the HEK293 cell density and expression levels of the rGPCR dramatically increased in comparison to using a spinner flask or tissue culture plates. The bioactivity of the rGPCR was good, however a change in the level of glycosylation of the recombinant protein was indicated by the positions of the rGPCR bands relative to the standard protein band in the Western Blots.

References

[1] **Worldwide Protein Data Bank information portal to biological structures:** http://www.rcsb.org/pdb/static.do?p=search/index.html.

[2] **Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line.** Reeves PJ, Callewaert N, et al. *Proc Natl Acad Sci USA* 2002; 99 (21): 13419 -24.

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