Optimizing Acetylcholinesterase Production by Altering Cell Line and Media Composition

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Background

The Clark Lab designs nanosensors for chemical imaging in living systems, including a DNA-based nanosensor for mapping and measuring the release of Acetylcholine in real-time in vivo. Acetylcholinesterase (AChE) is a key component of this sensor, detecting the neurotransmitter Acetylcholine by hydrolyzing it into Acetic Acid and Choline, which reduces the local pH. This change can then be detected by the nanosensor's fluorophores to signal the presence of Acetylcholine. In order to create and test this sensor experimentally, large quantities of AChE are required, and its price necessitates its production in-house. This is currently done through a culture of HEK-293T cells that were modified to produce and secrete recombinant AChE into the waste medium of the cell culture, which is then purified using affinity columns. This process is very labor-intensive and time consuming, and can be optimized for more efficient production of AChE. One way this process can be improved upon is by changing the composition of the cell medium used for this culture. Prior research shows medium with reduced amounts of Fetal Bovine Serum (FBS) can benefit recombinant protein production by removing interferer proteins, expediting purification and isolation of AChE from the collected waste medium. Additionally, a change in the cell line used could greatly improve the efficiency of AChE production. Previous research shows CHO-K1 cells to have much higher recombinant protein yield than HEK-293T cells, and are well-established as the most common non-human cell line used for therapeutic protein production. This project seeks to explore how a shift in the medium composition to reduced-serum media or a change in cell line to CHO-K1 cells could optimize the production of AChE for the Clark Lab.

Purification

In order to obtain recombinant AChE, cells were cultured in T-75 flasks and surface culture dishes in a variety of media. Waste media was collected every 2-3 days and replaced with fresh media and cells were passaged roughly twice a week. After collecting 300-500 mL of waste medium, the medium was purified using affinity columns and 100kD Millipore centrifugal filter tubes. After purification, the optical density for the purified waste medium at 412 nm in Ellman's assay was measured over time on a microplate reader. In Ellman's assay, AChE catalyzes a reaction between Acetylthiocholine and DTNB that results in a product with a yellow color. The rate of the reaction is therefore proportional to this color change, which can be measured using a microplate reader. Equation 1 was then used to calculate the enzyme activity based upon this reaction time.

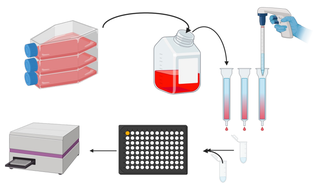
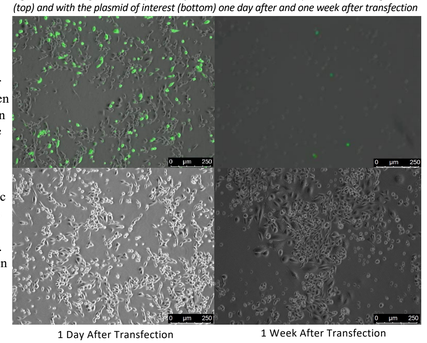


Figure 1: The above figure displays the general methodology of media collection, enzyme purification, and the use of Ellman's Assay to determine enzyme activity, that was used in this project.

Transfection

In order to induce AChE expression and secretion within a culture of CHO-K1 cells, cells were transfected from one well of a six well plate with a customized plasmid. Additionally, a separate transfection was performed on another well using a plasmid that codes for the expression of Green Fluorescent Protein (GFP). This served as a basis for verifying the success of the transfection. Images taken one day after transfection show successful expression of the plasmids of interest with GFP expressed in the cells shown in the top left image and sufficient cell attachment and proliferation in the bottom left. Following the transfection process, successfully transfected cells were selected for using the antibiotic Hygromycin B (600 µg/mL), which transfected cells carried resistance for, in the cell medium. The GFP plasmid did not contain this antibiotic-resistant gene. Images on the right display the results of the selection after one week, with most cells dying off due to the antibiotic in the well transfected with a GFP-producing plasmid, and transfected cells surviving and proliferating in the well of interest.

Figure 2: The images below depict transfected CHO-K1 cells with a plasmid containing GFP (top) and with the plasmid of interest (bottom) one day after and one week after transfection



1 Day After Transfection | 1 Week After Transfection

Adaptation

A sharp reduction in Fetal Bovine Serum deprived the culture of HEK-293T cells of many proteins involved in cell adhesion, resulting in widespread cell death and a lack of attachment to culture dishes. To combat this a gradual adaptation procedure was employed, wherein the percentage of FBS in the cell medium was reduced from 10% to 5% and eventually to 2%, with reductions occurring over the span of two weeks. The gradual adaptation allowed cells to adjust to the lack of attachment proteins in the environment and alter their own protein expression accordingly. Upon initial adaptation to 2% FBS, low cell attachment necessitated the use of Corning® CellBIND® culture dishes, specially designed to promote cell adhesion in serum-free or reduced-serum culture conditions. As serum was reduced in the flasks, cell morphology showed a corresponding reduction in cell attachment, while the implementation of these dishes showed clear improvements to cell attachment and proliferation.

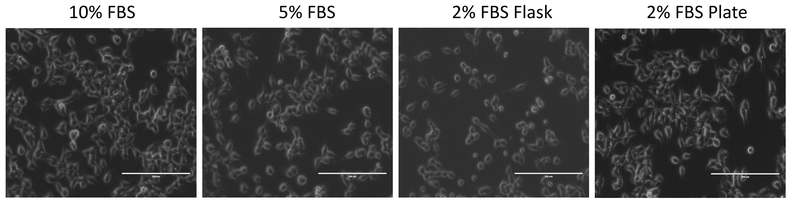


Figure 3: The above images depict HEK-293T cells cultured in medium containing 10% to 2% FBS throughout the adaptation process. The rightmost image shows a culture on a specially made plate for improved cell adherence while the left three display T-75 cultures. All picture legends are 200 µm.

Results

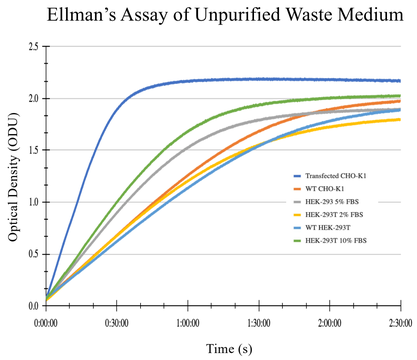


Figure 4: The above graph displays the optical density of several Ellman's assays with unpurified cell waste media over time based upon their absorbance at 412nm.

To provide a basis for comparison between different culture conditions and cell lines, Ellman's assay was run using a plate reader to measure the absorbance of waste medium from cells in each condition over the course of over two hours. Medium was normalized to a fixed enzyme concentration of 20 µM. Transfected CHO-K1 cells showed the greatest activity levels, reaching their maximum optical density in roughly half an hour, compared to the wild type CHO-K1 cells that took over two hours to reach its maximum value. This was followed by HEK-293T cells with 10%, 5%, and 2% FBS media, and lastly the wild type cells of both cell lines.

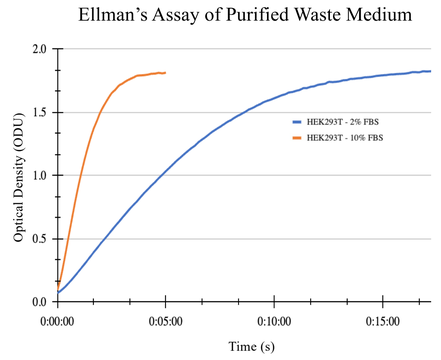


Figure 5: The graph below displays the optical density of Ellman's assays with purified cell waste media over time based upon their absorbance at 412nm.

In order to evaluate the enzyme activity of cell waste media from HEK-293T cells in 2% FBS and 10% FBS after purification another Ellman's assay was run. The concentrations of AChE in the purified waste of both can be found in the table below. The waste medium containing 10% FBS demonstrated a much higher enzyme activity, taking just over two minutes to reach its maximum optical density, while the 2% FBS medium took just under 20. The equations seen to the right were then used to calculate the enzyme activity and concentration of AChE in both solutions and the results were displayed in Table 1.

Results

Equation 1 $$Enzyme\ Activity\ \left(\frac{U}{mg}\right) = \frac{n}{mt}$$

$n = Amount\ of\ Acetylthiocholine\ Chlorine\ (nmol);\ m = Amount\ of\ AChE\ (mg);$
$t = Reaction\ time\ (min.)$

Equation 2 $$Beer - Lambert\ Law:\ A = \varepsilon bC$$

$A = Absorbance;\ \varepsilon = Molar\ absorption\ coefficient\ \left(\frac{1}{M * cm}\right);\ b = Path\ length\ (cm);$
$C = Concentration\ of\ AChE\ (\mu M)$

	Purified 10% FBS Waste Medium	Purified 2% FBS Waste Medium
Enzyme Activity	109.96	22.117
Concentration	4.690 µM	3.500 µM

Table 1: The table above reports the enzyme activity and concentration of purified waste media from HEK-293T cells cultured in 2% FBS and 10% FBS media. These values were calculated using the equations above.

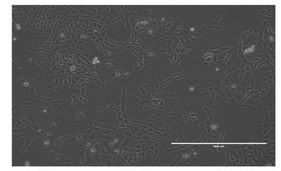


Figure 6: The above image depicts transfected CHO-K1 cells after two weeks of selection and passaging to a T-75 Flask. The cells demonstrate successful surface binding and proliferation.

Discussion & Conclusion

The first and most notable result was an increased enzyme activity seen in transfected CHO-K1 cells as opposed to HEK-293T cells under any conditions. This suggests that, as expected, this cell line produces AChE at a greater rate than HEK-293T cells when transfected with the same plasmid. Based upon this future culture work should utilize this cell line in order to more efficiently produce AChE of higher quality. Further verification is needed however to determine whether this trend is continued for the waste medium of CHO-K1 cells after purification, and to calculate an exact value of enzyme activity for AChE produced by the CHO-K1 culture.

The second major result from this experiment was a diminished production of AChE from HEK-293T cells cultured in 2% FBS medium, producing a much lower enzyme activity than that of HEK-293T cells in 10% FBS medium. This underperformance could have resulted from a variety of factors. One possibility is a change in gene expression from the cells due to stresses of adaptation or due to the different environmental conditions. Another possibility is the removal of proteins within FBS that contributed to the production of or influenced the activity of AChE. Despite its inferior output, 2% medium cultures did show benefit in reducing purification times, with waste medium taking roughly half as long to purify as 10% FBS waste medium, likely due to fewer contaminant proteins to be removed. Further research is needed to determine whether the trend of reduced enzymatically active AChE continues for reduced serum cultures as the amount of serum decreases further or the time after adaptation lengthens.

Future work could see more research done to verify the trends seen in this project and expand upon them by continuing adaptation further to completely serum-free medium and by collecting data on the enzyme activity of purified waste medium from transfected CHO-K1 cells. Additionally, the goal of optimization of methods and maximization of AChE production could be further explored through the design of different plasmids that additionally amplify the expression of AChE production and secretion genes or the use of suspension cultures in order to significantly increase the amount of cell medium collected from one culture and therefore the rate at which AChE can be collected.

References

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