Teaching biochemistry and molecular biology using dihydrofolate reductase as an expression system

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Introduction

Dihydrofolate reductase (DHFR), a key enzyme in the metabolism of folate, catalyzes reactions for the synthesis of purines, thymidyl acid and certain amino acids. The DHFR protein is expressed in a variety of organisms (both prokaryotic and eukaryotic), and serves as a model enzyme for scientists studying the correlation between protein structure and function. The structure of DHFR is highly conserved at the active sites throughout the domains of life. Due to the importance of DHFR in cellular proliferation, DHFR inhibitors are a target of many anti-cancer drugs, while the bacterial version has interest as antibacterial agents (Wright and Anderson, 2011). The overall objective of the project is to have students induce expression, purify and enzymatically assay for DHFR activity. Students also conduct novel experiments by designing PCR primers for site-directed mutagenesis and examine specific activity of their mutant DHFR.

Methods and Materials

Supplies needed: The Protein Expression and Purification Series™ kit, histidine (His) and glutathione S-transferase (GST), 1° antibodies and DHsu-T1 competent cells (Bio-Rad Laboratories). The GeneTailor® Site-Directed Mutagenesis kit (Invitrogen) and PCR primers (IDT) were used.

Week 1: Introduction to DHFR, guidelines for keeping a laboratory notebook and preparation of media stock solutions.

Week 2: Inoculate a single colony containing wild-type DHFR. Collect uninduced fraction from culture, measure OD600, subculture and induce DHFR expression. Collect pellet, lyse cells, separate insoluble from soluble protein fractions and analyze fractions by SDS-PAGE.

Week 3: Protein purification via IMAC (immobilized metal affinity chromatography) resin and analysis by SDS-PAGE.

Week 4: Measure enzyme activity of DHFR using spectrophotometry.

Week 5: Measure protein concentration by the Bradford method. SDS-PAGE and Western blotting transfer.

Week 6: Western blot analysis of His and GST tags.

Week 7: Each group designed PCR primers that were used for site-directed mutagenesis. No PCR product was obtained from the Glu→Lys mutation.

Week 8: Results obtained from class following enzyme digest of site-directed mutagenesis products. Lane 1-1 kb ladder; (a) Lanes 2 and 3-Pro-Xleu; Lanes 4 through 7- Trp→Cys. (b) Lanes 2 and 3-Arg→Thr.

Week 9: Summary of student results for specific activity of mutant DHFR enzyme compared to the wild-type DHFR.

Week 10: Protein sequence alignment of wild-type vs. mutant DHFR gene with mutations highlighted in yellow.

Results

Week 2:

Example of student’s SDS-PAGE electrophoresis of soluble and insoluble fractions. Lane 1: Precision Plus Protein Standard (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa); Lane 2- Uninduced fraction; Lane 3- Induced fraction; Lane 4- Soluble fraction; Lane 5- Insoluble fraction.

Week 3:

Example of student’s SDS-PAGE electrophoresis of purified GST-DHFR-His protein. Lane 1: Precision Plus Protein Standard (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa); Lane 2- Soluble fraction; Lane 3- Flow through fraction; Lane 4- Wash fraction; Lane 5- Eluted fraction; Lane 6- Desalted eluted fraction.

Week 4:

Enzymatic activity of wild-type DHFR protein. Two examples of results obtained from class.

Week 5:

Example of student’s BSA curve for measuring protein concentration. Protein concentrations from the Insoluble fraction ranged from 2.07-2.9 μg/μl. Soluble fraction from 0.38-4.97 μg/μl. Purified fraction from 0.29-0.76 μg/μl.

Week 6:

Western blot analysis of His and GST tags. Lane 1: Precision Plus Protein Standard; Lane 2- Insoluble fraction; Lane 3- Eluted fraction; Lane 4- Desalted eluted fraction.

(a) His tag at 1.5000 dilution (~50 kDa).
(b) GST tag at 1.3000 dilution (~25 kDa).

Week 7:

Each group designed PCR primers that were used for site-directed mutagenesis. No PCR product was obtained from the Glu→Lys mutation.

Week 8:

Wild-type Sequence

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<th>Nucleotide Position</th>
<th>Wild-type Sequence</th>
<th>Proposed Mutation</th>
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<tbody>
<tr>
<td>1</td>
<td>GCC (Pro)</td>
<td>TCC (Leu)</td>
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<tr>
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Week 9:

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Week 10:

Summary of student results for specific activity of mutant DHFR enzyme compared to the wild-type DHFR.

Week 11:

Each group designed PCR primers that were used for site-directed mutagenesis. No PCR product was obtained from the Glu→Lys mutation.

Week 12:

Summary of student results for specific activity of mutant DHFR enzyme compared to the wild-type DHFR.

Summary

In this project, students not only gain hands-on experience with a multitude of standard laboratory techniques (e.g. protein purification, measure of specific enzyme activity, Western blot analysis, PCR amplification, transformation into competent cells, restriction digest and bioinformatics), but they also have the opportunity to design and implement cutting edge research approaches to the study of a critical enzyme. All student groups successfully purified DHFR, and detected both His and GST tags by Western blot analysis. A PCR product was obtained by three out of four groups following site-directed mutagenesis (results from week 9; data not shown). Mutant DHFR showed a 32-122% decrease in specific activity compared to the wild-type protein. However, 18-155% increases have also been obtained (data not shown). DNA sequencing and protein alignments confirmed that mutants were successfully introduced into the DHFR gene.

Acknowledgements

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Literature Cited