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, thymidylic 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 objective of this 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 DH5α-T1 compentent 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: Inoculate a single colony containing wild-type DHFR. Plasmid purification and primer design for site-directed mutagenesis.

Week 8: Methylation of purified plasmid containing the wild-type DHFR gene and amplify gene using primers for site-directed mutagenesis.

Week 9: Analysis of mutagenic product via agarose gel electrophoresis and transform into competent cells for protein expression.

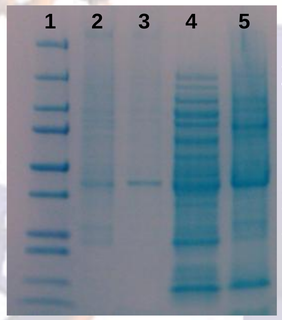
Week 10: Inoculate a single colony from mutagenesis reaction. Plasmid purification, measurement of plasmid concentration, restriction digest and confirmation of mutagenic product by agarose gel electrophoresis. Sequence DNA from purified plasmid.

Week 11: Inoculate wild-type and mutagenic colonies. Measure OD600 and subculture to induce protein expression. Collect pellet, lyse cells, separate insoluble from soluble fractions, purify protein.

Week 12: Measure enzyme activity of wild-type and mutant DHFR using spectrophotometry. Perform DNA sequence alignments, translate into protein sequence and align DHFR protein sequences.

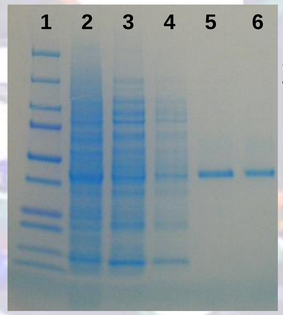
Week 13: Student presentations, notebooks and written reports due.

Results



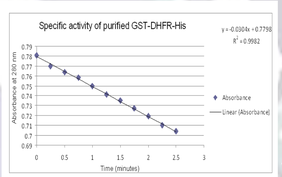
Week 2:

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 kD); Lane 2- Uninduced fraction; Lane 3- Induced fraction; Lane 4- Soluble fraction; Lane 5- Insoluble fraction.

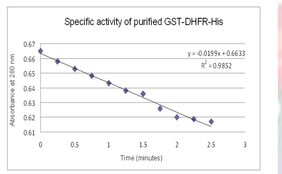


Week 3:

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 kD); Lane 2- Soluble fraction; Lane 3- Flow through fraction; Lane 4- Wash fraction; Lane 5- Eluted fraction; Lane 6- Desalted eluted fraction.



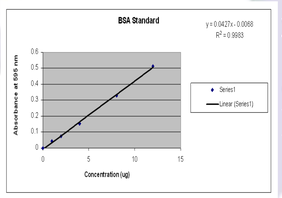
Specific activity = 2.28 µmol/min/mg protein



Specific activity = 0.58 µmol/min/mg protein

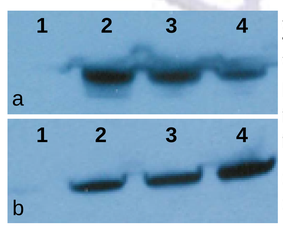
Week 4:

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 2.0-7.29 µg/µl; Soluble fraction from 0.38-4.97 µg/µl; Purified fraction from 0.29-0.76 µg/µl.



Week 6:

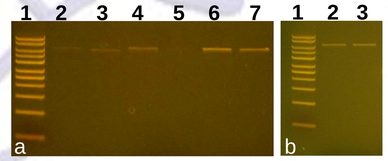
Western blot analysis of His and GST tags. Lane 1- Precision Plus Protein Standard; Lane 2- Insoluble fraction; Lane 3- Eluted fraction; Lane 4- Desalted eluted fraction.

(a) His tag at 1:5000 dilution (~50 kDa).
(b) GST tag at 1:3000 dilution (~25 kDa).

Group	Nucleotide Position	Wild-type Sequence	Proposed Mutation
1	477-479	CCC (Pro)	TTT (Leu)
2	190-192	AAT (Arg)	ACT (Thr)
3	169-171	TGG (Trp)	TCT (Cys)
4	93-95	CAA (Glu)	AAA (Lys)

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 10:

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

Wild-type Sequence	Proposed Mutation	Effect on Enzymatic Activity
CCC (Pro)	TTT (Leu)	Decreased specific activity by 32%
AAT (Arg)	ACT (Thr)	Decreased specific activity by 92%
TGG (Trp)	TCT (Cys)	Decreased specific activity by 122%
CAA (Glu)	AAA (Lys)	N/A (no clones)

Week 12:

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

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Group 1    VRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLV
Group 2    VRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLV
Group 3    VRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLV
Wild-type  VRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLV

Group 1    IMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLI
Group 2    IMGRKTWFSIPEKTRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLI
Group 3    IMGRKTQFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLI
Wild-type  IMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLI

Group 1    EQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPE
Group 2    EQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPE
Group 3    EQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPE
Wild-type  EQPELASKVDMVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPE

Group 1    IDLGKYKLLLEYPGVLSEVQEEKGIKYKFEVYEKK
Group 2    IDLGKYKLLPEYPGVLSEVQEEKGIKYKFEVYEKK
Group 3    IDLGKYKLLPEYPGVLSEVQEEKGIKYKFEVYEKK
Wild-type  IDLGKYKLLPEYPGVLSEVQEEKGIKYKFEVYEKK
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Week 12:

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

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

Special thanks to students in the 2010 Advanced Biochemistry course (BMB 401) who have been instrumental in helping with the development of this exercise. Much appreciation to James Kincaid for continuing with this project in 2011, and for hours spent designing and testing multiple primers for mutagenesis.

Literature Cited

Wright DL and Anderson AC. 2011. Antifolate agents: a patent review (2006-2010). Expert Opin Ther Pat 9:1293-308.