OVEREXPRESSION AND PARTIAL PURIFICATION OF RECOMBINANT HUMAN SERINE HYDROXYMETHYLTRANSFERASE: A POTENTIAL ANTICANCER TARGET

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I. INTRODUCTION

Serine hydroxymethyltransferase (SHMT) (E.C. 2.1.2.1) is a ubiquitous enzyme that plays a central role in the metabolism of growing tissues undergoing a high rate of DNA synthesis. This pyridoxal-5'-phosphate (PLP)-dependent enzyme provides precursors and cofactors for nucleotide biosynthesis by catalyzing the reversible interconversion of serine and tetrahydrofolate with glycine and N^5, N^{10}-methylenetetrahydrofolate. This aldolase reaction is unusual for a PLP-dependent enzyme and has been studied.[1] In addition, X-ray crystallographic studies on SHMT from various sources, including humans, have provided useful structural information.[2] The human cytosolic form of SHMT (483 AA) is a 53,083 Da homotetramer with a calculated pI of 7.61. Despite the structural knowledge present, there is still some question regarding the mechanism for this conversion.[1] One possibility is a retroaldol mechanism with free formaldehyde as an intermediate and that is

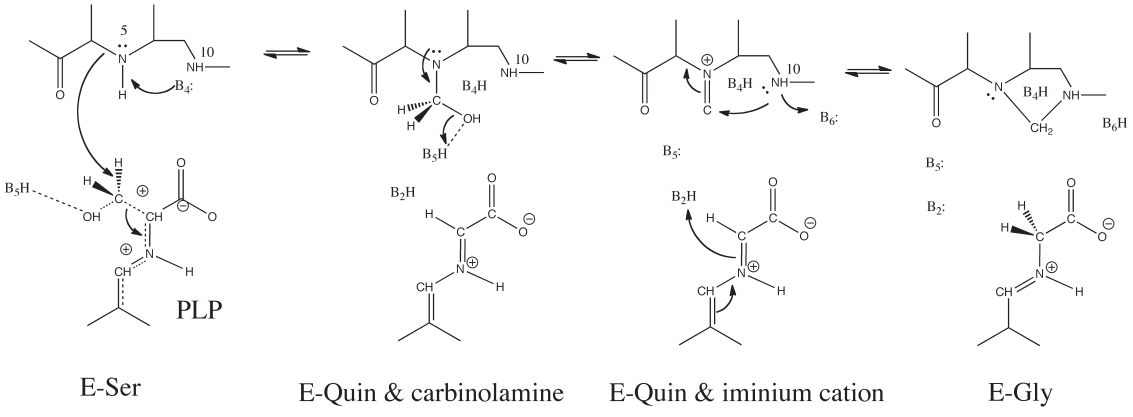

Figure 1. Proposed mechanism for the interconversion of serine and tetrahydrofolate with glycine and N^5,N^{10}-methylenetetrahydrofolate.

subsequently attacked by tetrahydrofolate.[1] Alternatively, the N^5 of tetrahydrofolate makes a nucleophillic attack on serine C^3 leading to the breakage of the C^3-C^2-bond of serine

rather that the C^3-hydroxyl bond (Figure 1).[1] Even though the first mechanism requires an active site base that is not apparent in the active site, it is not possible to definitively rule out this mechanism.

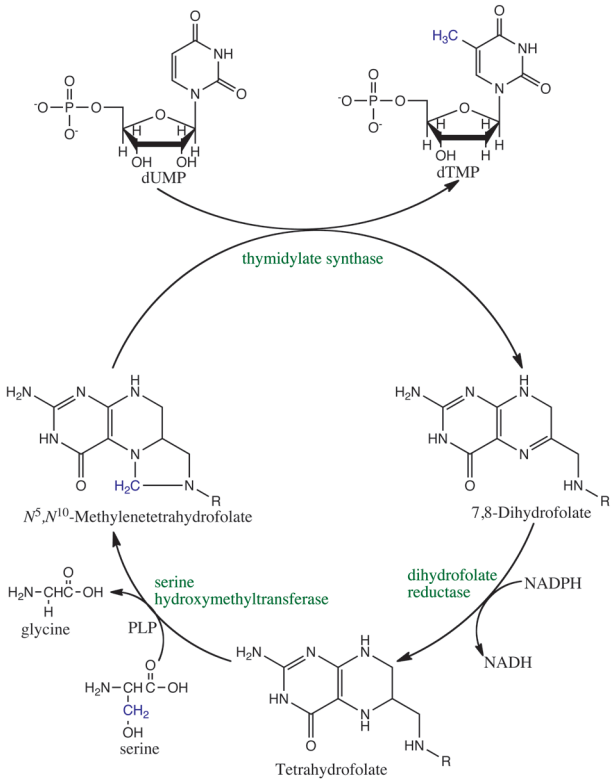

Figure 2. The thymidylate synthase cycle showing the transfer of a one carbon group from serine to N^5, N^{10}-methylenetetrahydrofolate by SHMT.

In the thymidylate synthase cycle the N^5, N^{10}-methylenetetrahydrofolate produced by SHMT is directly involved in pyrimidine biosynthesis by donating a methyl group used for methylating dUMP to dTMP (Figure 2). In addition, the glycine produced in the SHMT catalyzed reaction can serve as a precursor in purine biosynthesis by providing two carbons and a nitrogen to the purine ring. With both of the reaction products involved in nucleotide biosynthesis, it is not surprising that SHMT activity was shown to be up-regulated in various cancer cell types.[3,4] Furthermore, the inhibition of SHMT in myeloma cells in culture results in dose-dependent inhibition of cellular growth provides support that SHMT is a suitable target for anticancer pharmaceuticals.[5] The other two enzymes in the thymidylate synthase cycle, thymidylate synthase (TS) and dihydrofolate reductase

(DHFR), are anticancer targets. This is important because drugs that target TS and DHFR are susceptible to drug resistance, and they are toxic themselves.[6]

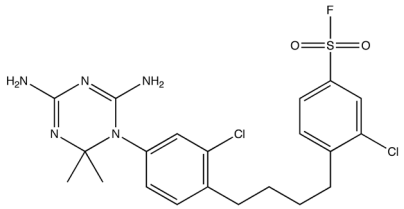

Figure 3. The chemical structure for triazine antifolate (NCS 127755).

Previous work on the classic inhibitors of DHFR viz. methatrexate and its structural analogs failed to inhibit SHMT activity significantly.[7] Triazine antifolate (NCS 127755) (Figure 3) was the only compound with reasonable inhibition of SHMT.[5] Formyltetrahydrofolate derivatives were found to be slow tight binding inhibitors of SHMT.[8] Serine analogs using putative mechanism based inactivators as possible chemotherapeutic agents have also previously been explored, but were not promising.[7] D-cycloserine (DCS) inhibited SHMT, and it was suggested as a possible chemotherapeutic agent.[6,9,10] However, DCS is a very general PLP dependent enzyme inhibitor and would also inhibit other PLP dependent enzymes. Structurally related compounds to DCS, such as O-amino-D-serine (OADS) inhibited SHMT with K_i values nearly 500 × less than K_m values of serine.[11] 4-chloro-L-threonine inhibited SHMT, but it was suggested that β-trifluoroallothreonine and β-trifluorothreonine might act suicide substrates and may be more specific and better inhibitors of SHMT.[12]

Mimosine is a plant amino acid (Figure 4) that was found to effectively inhibit SHMT, possibly both the mitochondrial and the cytosolic forms of SHMT, resulting in an inhibition of DNA replication in mammalian cells.[13] However, more work is required to test its effectiveness as a possible drug. Thiosemicarbazide (TSC) was shown to be a slow but tight binding inhibitor to SHMT.[14] Kinetic studies with substituted derivatives of TSC revealed that the presence of a $H_2NHN—C=S$ group in the inhibitor is important in

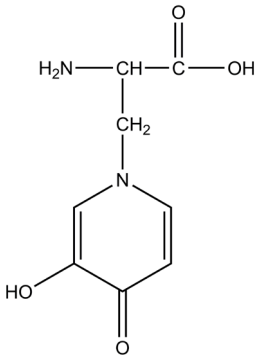

Figure 4. Chemical structure of mimosine.

the interaction. Based on this finding, it was suggested that serine and glycine derivatives containing $H_2NHN—C=S$ could be highly potent and specific inhibitors of SHMT with promise as chemotherapeutic agents.[15]

Although there are some potential candidates, the goal of this research is to perform enzymatic studies on hSHMT in order to identify and screen possible inhibitors that may be used as chemotherapeutics for the treatment of cancer. Pure hSHMT is required for performing the enzymatic studies for kinetically characterizing the physiological relevant aldolase reaction catalyzed by SHMT. The cDNA clone coding for human SHMT was heterologously overexpressed in *Escherichia coli* (BL21 strain) and the recombinant protein was partially purified. Attempts to affinity-purify hSHMT using a Ni^{+2} chelating column were unsuccessful and hSHMT was only partially purified by selective salt precipitations followed by both gel-filtration and anion-exchange chromatography. The cDNA clone was used as a template to amplify the gene coding for hSHMT, and was subcloned into a pET151/D-TOPO (Invitrogen) plasmid. This paper will focus on the purification of recombinant hSHMT, and the cloning process.

II. EXPERIMENTAL METHODS

Materials

L-(+)-arabinose was purchased from Sigma. All other commercial chemicals were purchased from Fisher. *PfuTurbo* DNA polymerase was purchased from Stratagene. The Champion pET151/D-TOPO vector was purchased from Invitrogen. *Taq* DNA polymerase, *Hin*dIII, and *Nde*I were purchased from New England BioLabs (NEB). *Kpn*I and *Bam*HI were purchased from Fisher Scientific. *Sac*I was purchased from Promega.

Overexpression of Recombinant hSHMT

Cells were scraped from a frozen stock of *Escherichia coli* BL21 and inoculated into 5 mL of Luria-Bertani (LB) medium with ampicillin (Ap) and chloramphenicol (Cm) to a final concentration of 50 μg mL^{-1} and 34 μg mL^{-1}, respectively, and grown with shaking at 37 °C overnight. The next morning, the entire overnight culture was aseptically transferred into 500 mL of sterile LB medium containing Ap and Cm to a final concentration of 50 μg mL^{-1} and 34 μg mL^{-1} respectively, and grown with shaking at 37 °C. At an optical density (OD) at 600 nm of 0.6, L-(+)-arabinose was added to a final concentration of 1% (w/v) to induce the overexpression of the plasmid-encoded gene product (SHMT). After five hours, the culture was removed and the cells were harvested at 4 °C by centrifugation for 15 minutes at 1,571 × g. The cell pellet was either stored at –70 °C, or resuspended in 20 mM Tris-HCl buffer (pH 7.5) and then stored at –70 °C.

Cell Lysis

After thawing the cells in ice, cold 20 mM Tris-HCl buffer (pH 7.5) was added to the cell pellet and vortexed until completely resuspended. Cells that were resuspended prior to storage at –70 °C were also vortexed to ensure that they were completely suspended. After the cells were completely suspended, lysozyme was added to a final concentration of

0.5 mg mL^{-1} and incubated with shaking for 20 to 30 minutes on ice. The cell suspension was disrupted in an ice-water bath with a Ultrasonic Processor XL (Heat Systems) at an intensity of 2.5, using 80 1.5 second treatments with a 10 second pause between each treatment. The cellular debris was removed at 4 °C by centrifugation for 20 minutes at 12,096 × g.

Purification of hSHMT by Anion-Exchange FPLC

Ammonium sulfate was slowly added to the lysate (supernatant after cell lysis) to 30% saturation at 0 °C and stirred for 10 minutes. The solution was equally distributed into 1.5 mL tubes and centrifuged at 4 °C for 15 minutes at 16,000 × g. The resulting supernatant was subjected to ammonium sulfate precipitation from 30 to 70 % saturation at 0 °C and stirred for 10 minutes. The precipitate was collected after centrifuging at 4 °C for 15 minutes at 16,000 × g and was resuspended in a minimal amount of 20 mM Tris-HCl buffer (pH 7.5). The resuspended protein sample was applied onto a Bio-Scale Mini Bio-Gel P-6 Desalting Cartridge (BioRad) and eluted with ice-cold 20 mM Tris-HCl buffer (pH 7.9). The fractions were analyzed with an OceanOptics USB 2000 spectrophotometer at 280 nm. The fractions with the highest absorbance at 280 nm were pooled and concentrated to about one milliliter using a 50,000 MWCO (Vivaspin) concentrator centrifuged at 2,000 × g (4 °C). The concentrated protein sample was further purified by anion-exchange on a UNO Sphere Q (Bio Rad) column connected to BioLogic DuoFlow FPLC system (Bio Rad) at 4 °C. The column was first equilibrated with 20 mM Tris-HCl buffer (pH 7.9), the 1 mL sample was injected onto the column, and the column was washed with 5 column volumes of the same buffer. The column eluted with a linear gradient of 0 M to 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.9) over 10 column volumes, followed by an isocratic flow of 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.9) for 5 column volumes. The absorbance at 280 nm was measured for all the fractions, and select fractions were analyzed by SDS-PAGE.

Enzyme Assay

The relative quantity of SHMT was estimated spectrophotometerically at room temperature by measuring the disappearance of NADH that accompanies the reduction of acetaldehyde formed from the SHMT catalyzed aldol cleavage of L-*allo*-threonine to glycine (Figure 5). The assay was prepared by incubating a protein sample with 1 mM

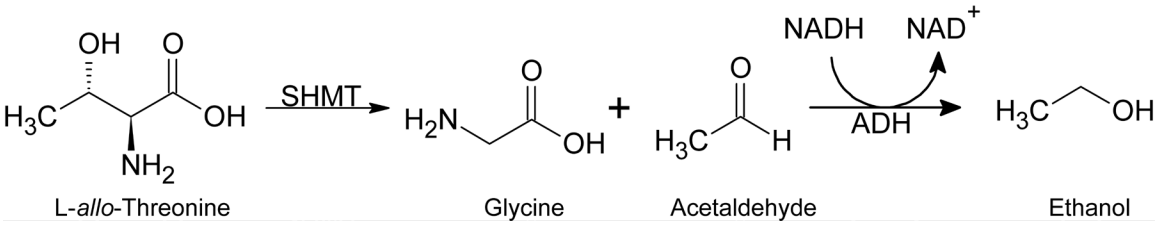

Figure 5. The reactions involved in the assay. L-*allo*-threonine is reacted overnight, and the activity is measured by the fast reaction of acetaldehyde to ethanol by ADH.

L-*allo*-threonine and 20 μM PLP in 50 mM phosphate buffer (pH 7.5) overnight. After the addition of 204 μM NADH, the initial absorbance at 340 nm was recorded, and the final the absorbance at 340 nm after incubating with 30,000 U of alcohol dehydrogenase (ADH) for 4 minutes. The relative quantity of SHMT was calculated by dividing the change in absorbance at 340 nm by the total amount of protein in that sample, which was estimated with a spectrophotometer at 280 nm using an extinction coefficient of 1.

Ni^{+2}-NTA Affinity-Chromatography

Affinity purification of SHMT from the lysate in a single step was originally attempted using Ni^{+2}-NTA His-Bind Superflow (Novagen) resin (0.5 mL bed volume) in a 5 mL polypropylene column. Lysate that was prepared in equilibration buffer (20 mM Tris-HCl, 10 mM Imidazole, 100 mM NaCl, pH 7.9) was loaded onto the column and the column was washed with 10 column volumes of ice-cold equilibration buffer. The column was eluted with 10 column volumes of ice-cold elution buffer (20 mM Tris-HCl, 500 mM Imidazole, pH 7.9) while collecting 1 mL fractions. The fractions were analyzed with an OceanOptics USB 2000 spectrophotometer at 280 nm and by SDS-PAGE.

Affinity purification of SHMT was then attempted using a Ni-Mac Cartridge (Novagen). Lysate that was prepared in MAC bind buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 8.0) was loaded onto the column, washed with 6 column volumes of ice-cold MAC wash buffer (300 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole, pH 8.0), and eluted with 6 column volumes of ice-cold MAC elute buffer (300 mM NaCl, 50 mM sodium phosphate, 250 mM imidazole, pH 8.0). The fractions having the highest absorbance at 280 nm were analyzed by SDS-PAGE and enzyme assay.

Subcloning of hSHMT into pET151/D-TOPO

SHMT cDNA was amplified by PCR using the following primers: (forward) 5′ CAC CAT GAC GAT GCC AGT CAA 3′ and 5′ GTG GTG AAG CTT TTA GAA GTC AGG CAG GCC AG 3′ (reverse). The reaction was carried out using the following concentrations: 2.5 U of *PfuTurbo* DNA Polymerase (Stratagene), 1.0 × *Pfu* reaction buffer, 1.0 mM dNTPs, 0.5 μM of forward primer, 0.5 μM of reverse primer, and 90 ng of plasmid DNA. The following cycling parameters were used: initial denature at 95 °C for 5 minutes; 95 °C for 60 seconds, 58–62 °C for 60 seconds, 72 °C for 60–90 seconds for 30 cycles; final extension at 72 °C for 10 minutes; hold at 4 °C. The PCR product was gel purified from a 1% agarose gel in 1 × TAE buffer by using a QIAprep kit.

Subcloning of hSHMT cDNA into a pET151D-TOPO (Invitrogen) vector was performed by adding the following into a sterile PCR tube: 5 ng of gel-purified PCR product, 1 μL of Champion pET151/D-TOPO (Invitrogen) vector, 2.75 μL of salt solution, and 1 μL of sterile water. This was gently mixed and incubated at room temperature for 10 minutes before transferring 3 μL to a vial of One Shot TOP10 Chemically Competent *E. coli* cells and incubating on ice for 10 minutes. The cells were heat-shocked for 30 seconds at 42 °C and immediately placed in ice before 250 μL of sterile room temperature LB medium was added. The cells were incubated while shaking at 37 °C for 1 hour before

transferring 200 μL and 100 μL onto individual pre-warmed LB/Ap plates. The cell culture was spread evenly, and incubated agar-side-up at 37 °C for 24 hours.

Colony Screening

Plasmid DNA was extracted from colonies, and was analyzed by using a combination of different restriction enzymes. All restrictions were performed overnight, using the recommended buffers for double digestions. The enzyme combinations used to cut once in the vector and once in the insert were *Hin*dIII and *Nde*I, *Kpn*I and *Sac*I, *Nde*I and *Bam*HI, *Kpn*I and *Sac*I. The plasmid was also restricted at the sites flanking the insert with *Nde*I and *Sac*I.

Colonies were also screened by PCR amplification of the insert using the following primers: (forward) 5′ CAC CAT GAC GAT GCC AGT CAA 3′ and 5′ GTG GTG AAG CTT TTA GAA GTC AGG CAG GCC AG 3′ (reverse). The reaction was carried out using the following concentrations: 1.25–5 U of *Taq* DNA Polymerase, 1.0 × of standard *Taq* reaction buffer (NEB), 200 μM–1000 μM dNTPs, 1–1.5 μM of forward primer, 1–1.5 μM of reverse primer, and 90 ng of plasmid DNA. The following cycling parameters were used: initial denature at 94 °C for 3 minutes; 94 °C for 45 seconds, 55–62 °C for 30 seconds, 72 °C for 90 seconds for 30 cycles; final extension at 72 °C for 10 minutes; hold at 4 °C. The following parameters suggested by the manufacturer were also used: initial denature at 95 °C for 30 seconds; 95 °C for 30 seconds, 60 °C for 30 seconds, 68 °C for 90 seconds for 30 cycles; final extension at 68 °C for 10 minutes; hold at 4 °C. The PCR products were analyzed by electrophoresis using a 1% agarose gel in 1 × TAE buffer.

III. RESULTS

Purification of hSHMT by Anion-exchange FPLC

Crude lysate from *E. coli* was purified by selective ammonium sulfate fractionation at 0–30% and 30–70% saturation, the protein sample was desalted and concentrated to 1 mL for purification by anion-exchange FPLC. The absorbance at 280 nm was measured for each fraction and an elution profile was constructed (Figure 6). The first peak from fraction 1 to fraction 6 is the flow-through peak that contains unbound proteins; the second and third peaks are not resolved. The coupled assay was used to identify active fractions containing active hSHMT from these elution peaks. The bulk of the hSHMT was eluted from the column from 20% to 76% of elution buffer, and was found in F24–F35. hSHMT was detected in fractions 4, 5, 9 during the isocratic flow of 20 mM Tris-HCl buffer (pH 7.9),

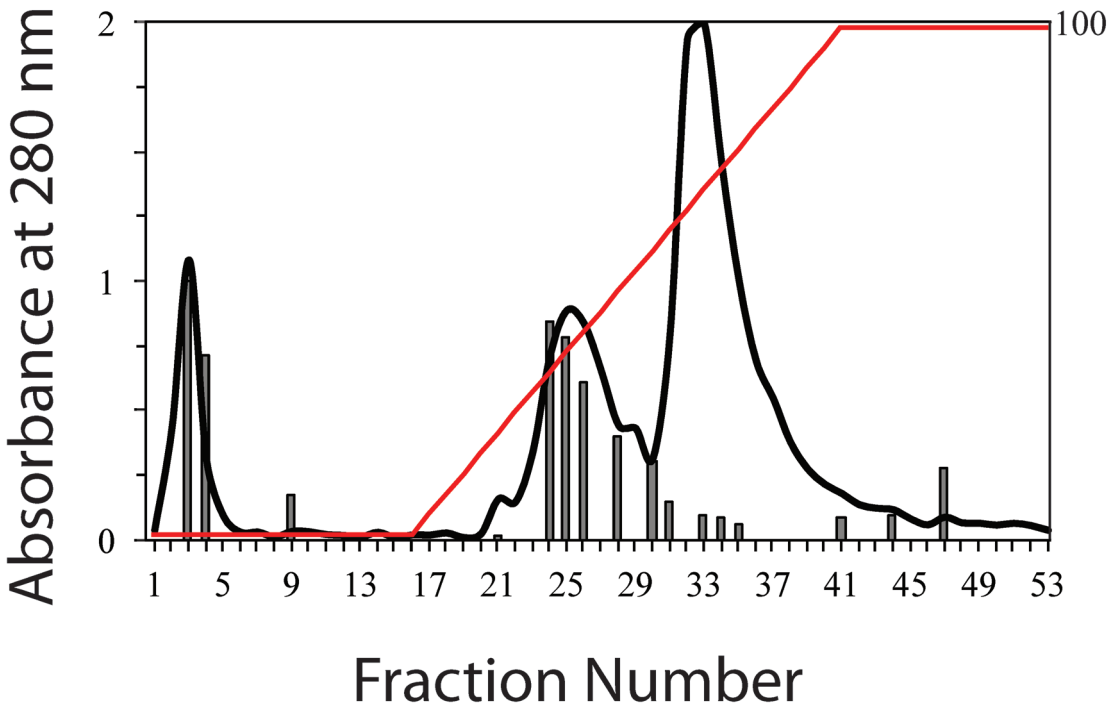

Figure 6. Protein elution pattern (black line) and elution buffer percentage (red line) overlaid with hSHMT activity (grey bar) Protein elution pattern was determined by measuring the absorbance at 280 nm for each fraction. The hSHMT activity was measured by a coupled assay with L-*allo*-threonine and ADH.

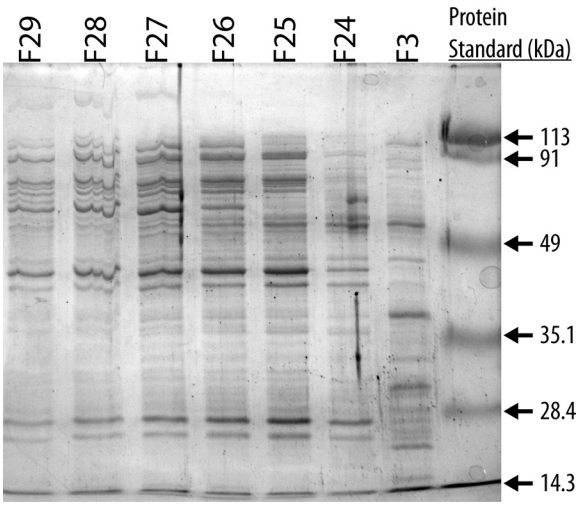

Figure 7. Fractions were analyzed by SDS-PAGE using a 5% stacking and a 12% resolving polyacrylamide gel at 200–220 V and visualized by coomassie blue staining. All samples were reduced with DTT.

with fractions 4 and 5 containing large amounts of hSHMT. Traces of hSHMT were also detected in fractions 41, 44, and 47 during the isocratic flow of 100% elution buffer. In the SDS-PAGE analysis of fractions 24–29 and fraction 3 (Figure 7), all the fractions were found to contain multiple bands; all of these fractions also contained a 53 kDa band that corresponded to hSHMT. The anion-exchange FPLC resulted in the partial purification of hSHMT.

Ni^{+2}-NTA affinity-chromatography

The Ni^{+2}-NTA affinity-chromatography of hSHMT was performed by applying crude lysate from *E. coli* directly to the column, the unbound (flow-through), washes 1–3, and eluted fractions 1–3 were analyzed by SDS-PAGE. The results (Figure 8) show multiple bands in each of the eluted fractions. The Ni^{+2}-NTA affinity-chromatography did not purify the protein. The highest MW band was 53 kDa, and it was visible in the unbound protein lane, elute 1, and elute 2. This is important because the 53 kDa band corresponds to hSHMT without a polyhistidine affinity tag and a large amount of this protein did not bind to the column. The enzyme assay was used to analyze wash 1, and eluted fractions

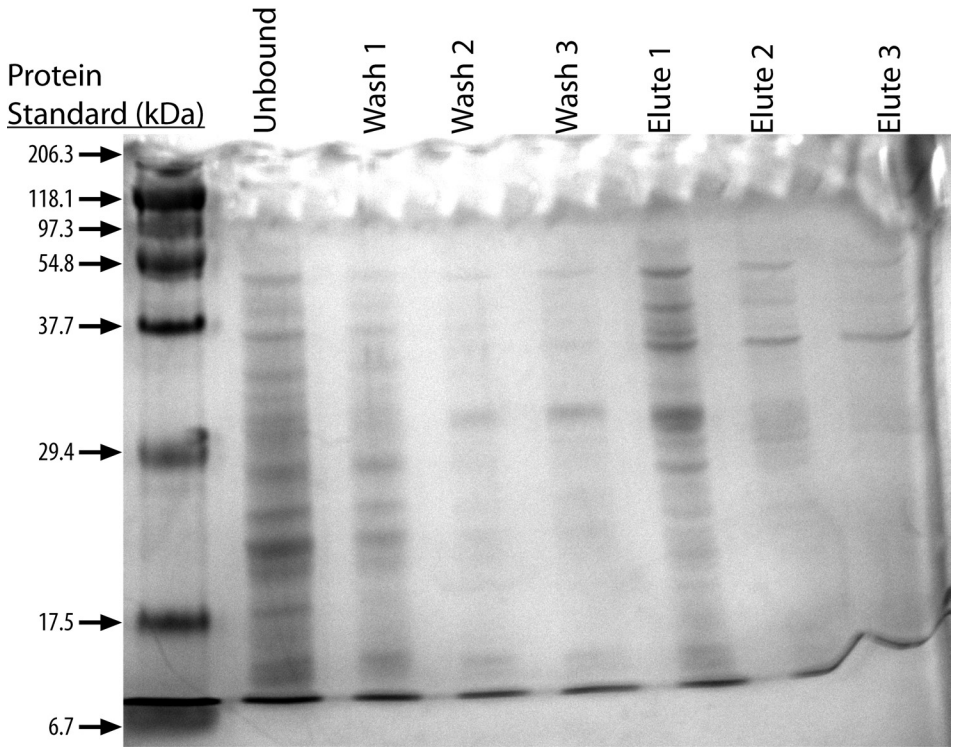

Figure 8. Fractions from the Ni^{+2}-NTA affinity-purification of hSHMT were subjected to SDS-PAGE in a 5% stacking and 12% resolving polyacrylamide gel at 170–220 V and visualized with coomassie blue staining. All samples were adjusted to contain equal protein concentrations, and reduced with DTT.

1–3. The results identified elution fraction 1 and wash 1 to contain active hSHMT; elution fraction 1 to contain more SHMT than wash 1 (results not shown).

Subcloning of hSHMT cDNA into pET151/D-TOPO

The PCR amplification of hSHMT cDNA resulted in two bands when analyzed by electrophoresis in a 1% agarose gel (Figure 9). One band was approximately 1,500 bp and the other band was approximately 1,000 bp. The 1,500 bp band was gel purified using a QIAprep kit and no band was visible when 5 mL of 4.0 ng μL^{-1} gel-purified DNA was analyzed by agarose gel electrophoresis (results not shown). Nevertheless, the gel-purified DNA fragment was subcloned into a pET151/D-TOPO vector and 100 μL of transformed cells were plated. This resulted in 14 isolated colonies on plate 1, and 8 isolated colonies on plate 2.

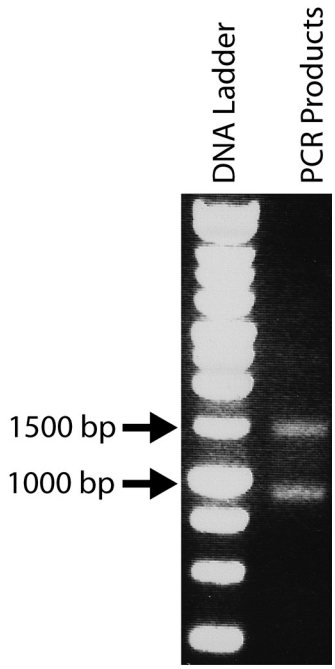

Figure 9. PCR amplification of hSHMT cDNA with *PfuTurbo* DNA polymerase was subjected to electrophoresis in a 1% agarose gel containing EtBr with 1 × TAE buffer at 90 V and visualized under UV light.

Colony Screening

Plasmid DNA was extracted from 4 colonies on plate 1 (P1C1, P1C2, P1C5, P1C7), and from 2 colonies on plate 2 (P2CA, P2CI). Plasmid DNA labeled P1C2, P1C5, P1C7, P2C2 was restricted with *Nde*I and *Hin*dIII and analyzed by electrophoresis on a 1% agarose gel (Figure 10). The fragment sizes for P1C2, P1C5, P1C7, P2C2 were calculated to be 6445 bp, 6956 bp, 6801 bp, 5313 bp, respectively.

In figure 11, the *Kpn*I and *Sac*I restriction of P1C1, P1C7, P2CA, P2CI resulted in single fragments, and the calculated sizes were 5982 bp, 6090 bp, 6145 bp, 5466 bp, respectively. P1C2 was restricted with *Nde*I and *Sac*I, *Nde*I and *Bam*HI, *Kpn*I and *Sac*I and the fragment size were calculated to be 5467 bp, 5667 bp, 5982 bp, respectively.

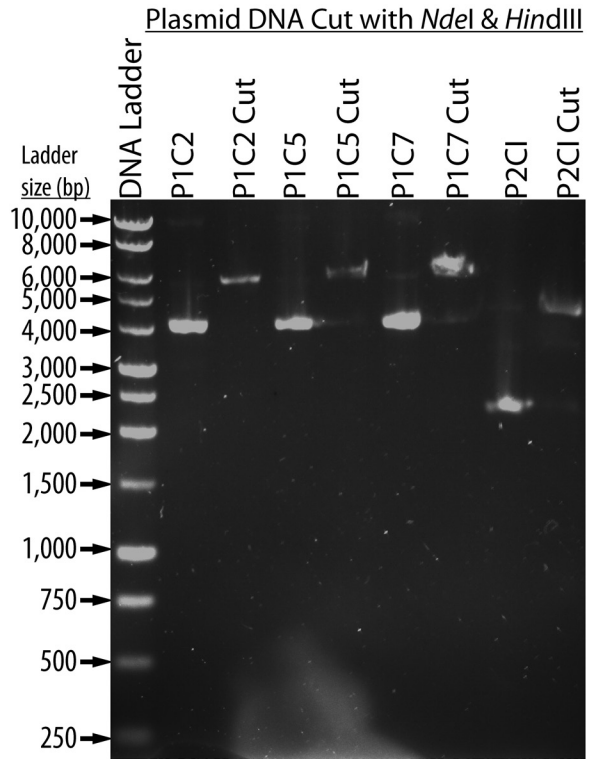

Figure 10. Plasmid DNA was restricted (cut) with *Nde*I and *Hin*dIII and subjected to electrophoresis in a 1% agarose gel with 1 × TAE buffer at 90 V. The DNA was visualized with EtBr staining under UV light.

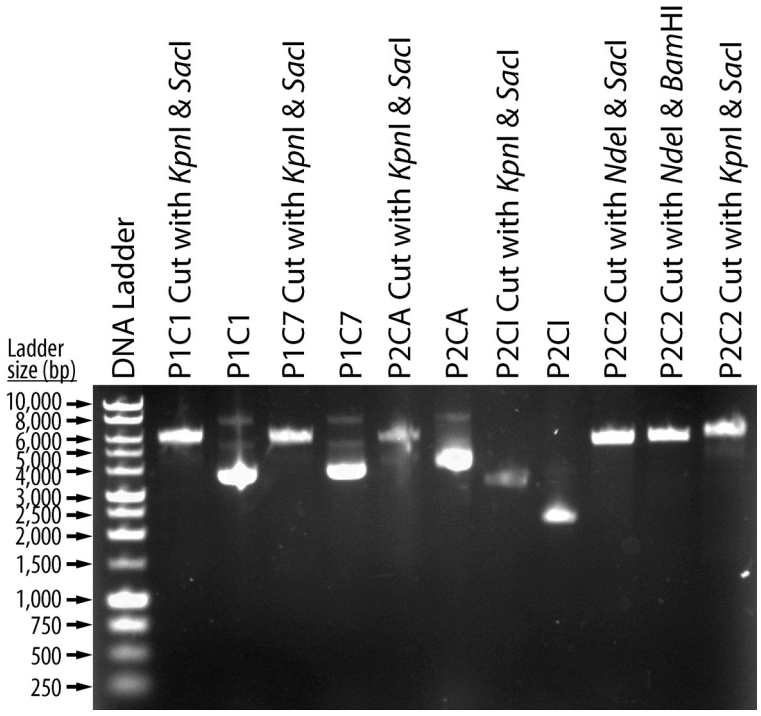

Figure 11. Plasmid DNA was restricted (cut) with either *Kpn*I and *Sac*I, *Nde*I and *Sac*I, *Nde*I and *Bam*HI, or *Nde*I and *Hin*dIII and subjected to electrophoresis in a 1% agarose gel with 1 × TAE buffer at 90 V. The DNA was visualized with EtBr staining under UV light.

PCR Analysis of Isolated Clones

Plasmid DNA was PCR amplified using hSHMT cDNA sequence specific primers to help screen colonies for the presence of hSHMT subcloned into a pET151/D-TOPO vector. The PCR products were analyzed by electrophoresis in a 1% agarose gel. The results in figure 12 show that the sequence specific primers did not amplify the hSHMT cDNA from P1C1. The control PCR amplification of circular pET151/D-TOPO resulted in a single band that was approximately 380 bp. In figure 13 the PCR amplification of plasmid DNA P1C1, P2CI, P1C7, P2CA resulted in 3 bands with fragment lengths around 9,000 bp, 3,500 bp, and 1,900 bp.

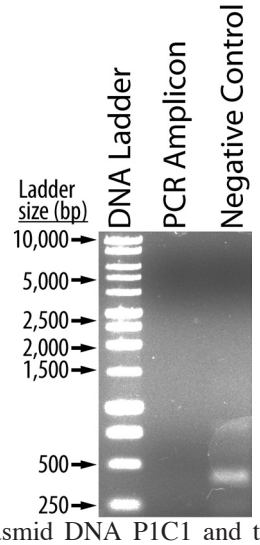

Figure 12. PCR amplification of plasmid DNA P1C1 and the negative control (pET151/D-TOPO plasmid) was performed using 5 U of Taq DNA polymerase, 3.0 mM $MgCl_2$, and with a primer annealing temperature of 55 °C. The samples were subjected to electorphoresis in a 1% agarose gel containing EtBr in 1 × TAE buffer at 90 V and visualized under UV light.

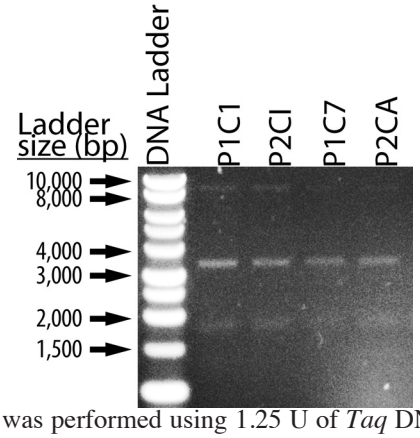

Figure 13. PCR amplification was performed using 1.25 U of *Taq* DNA polymerase, 2.0 mM $MgCl_2$, and with a primer annealing temperature of 60 °C. The samples were subjected to electorphoresis in a 1% agarose gel containing EtBr in 1 × TAE buffer at 90 V and visualized under UV light.

IV. DISCUSSION and CONCLUSION

The main goal for this project was to obtain pure human serine hydroxymethyltransferase with the ultimate goal to isolate possible inhibitors of SHMT. Human SHMT was overexpressed in *E. coli* and partially purified by selective ammonium sulfate fractionation and anion-exchange fast protein liquid chromatography.

It was expected that the purification of hSHMT from crude extract (lysate) by selective ammonium sulfate fractionation at 0–30% and 30–70% saturation, followed by subsequent anion-exchange fast protein liquid chromatography would result in significant purification of the protein as evidenced by a dominant band at 53 kDa when analyzed by SDS-PAGE. The FPLC elution profile was expected to have all of the hSHMT in a single well-resolved peak. The results obtained from anion-exchange FPLC and from the coupled assay with L-*allo*-threonine (Figure 6) did not show this. Instead, a large amount of hSHMT was found in the flow-through peak (F3, F4), and the bulk of the hSHMT was not detected in a single well-resolved peak; hSHMT was also detected in fractions 9, 41, 44, and 47. The SDS-PAGE results (Figure 7) showed multiple bands in addition to a 53 kDa band in F3, and F24–29. The detection of hSHMT in the flow-through peak is most likely the result of exceeding the binding capacity of the column. It is believed that this was caused by the binding of sheered nucleic acid fragments, which is supported by the strong absorbance at 260 nm relative to 280 nm in these fractions. The multiple bands observed in the SDS-PAGE analysis were the result of poor separation of hSHMT from *E. coli* proteins. It is unknown if the detection of hSHMT in F41, F44, and F47 by the coupled assay were false positives since these fractions were not analyzed by SDS-PAGE. However, fraction 9 is believed to be a false positive caused by contamination because it does not contain any protein, as indicated by a lack of absorbance at 280 nm. Based on these results, it was determined that our methods of overexpressing and purifying hSHMT

by selective ammonium sulfate fractionation and anion-exchange FPLC were unsuccessful in accomplishing our goal to obtain pure hSHMT.

After the unsuccessful purification of hSHMT by anion-exchange FPLC, the purification of hSHMT in a single step using Ni^{+2}-NTA affinity chromatography was evaluated. It was expected that the purification of hSHMT from *E. coli* lysate would result in a single band with a MW greater than 53 kDa when analyzed by SDS-PAGE. However, the obtained results were not as expected. Instead, multiple bands were observed in addition to a 53 kDa band in the eluted fraction as well as the unbound fraction. These multiple bands were the result of eluting the column before all non-specific bound proteins had been washed out of the column. The first wash and the three eluted fractions were analyzed with the coupled assay, and the results determined that wash 1 and elution 1 both contained active hSHMT (wash 2 and wash 3 were not analyzed). Taken together, these results support that hSHMT was not expressed as a fusion protein containing a polyhistidine affinity tag. Fusion proteins that contain polyhistidine affinity tags such as His_6 or His_{10} will commonly have additional amino acids in between the protein and the polyhistidine tag to act as a spacer as well as offer a cleavage site for removing the affinity tag after purification. Therefore, hSHMT containing a polyhistidine affinity tag would have a greater MW than native hSHMT without any affinity tag. The largest MW band in elute 1 was approximately 53 kDa, which indicates that it is native hSHMT (un-tagged).

The Ni^{+2}-NTA affinity-purification trials concluded that hSHMT was not expressed as a fusion protein containing a polyhistidine tag. This led to the decision to PCR amplify hSHMT cDNA from the arabinose-induced plasmid and subclone it into a pET151/D-TOPO (Invitrogen) vector for better over-expression and easier purification by Ni^{+2}-NTA affinity chromatography. Other researchers have reported that the overexpression and purification of human recombinant SHMT by Ni^{+2}-NTA affinity chromatography yielded up to 15 mg of pure hSHMT from 1 L of bacterial culture.[16]

In the subcloning process of hSHMT, the PCR amplification of hSHMT cDNA resulted in two bands; one that was approximately 1,500 bp and the other was approximately 1,000 bp. Different parameters were adjusted in order to optimize the PCR amplification that would result in a single band of approximately 1,500 bp. The primer annealing temperature was increased, the primer extension time was increased from 60 seconds to 90 seconds, and primer concentration was decreased. However, optimal PCR amplification conditions were not achieved by these adjustments, and the desired 1,500 bp band was gel-purified.

Colonies were screened for hSHMT cDNA by both restriction analysis and PCR amplification of isolated plasmid DNA. A single band was observed from the restriction analysis of all the colonies, which is not expected from the successful subcloning of hSHMT cDNA into pET151/D-TOPO. Fragment sizes of 5,760 bp and 1,554 bp, 5,980 bp and 1,244 bp, 5,652 bp and 1,572 bp, 6,295 bp and 929 bp were expected for the restriction of plasmid DNA with *Nde*I and *Hin*dIII, *Kpn*I and *Sac*I, *Nde*I and *Sac*I, *Nde*I and *Bam*HI. For the colony screening by PCR amplification, a discrete band of 1,464 bp was expected from colonies containing hSHMT cDNA. The results were not as expected; a 1,464 bp band was not observed for any of the analyzed colonies. Based on the results from the restriction analysis and PCR amplification of plasmid DNA isolated from transformed colonies, it can be concluded that the subcloning of hSHMT cDNA into pET151/D-TOPO was unsuccessful.

One possible explanation for this is that the pET151/D-TOPO vector had self ligated. The other possibility is that the PCR product was degraded resulting in a smaller fragment that was successfully ligated into the vector. The restriction of plasmid DNA obtained from P2CI in figures 10 and 11 show a significantly smaller fragment compared the rest of the colonies. This supports the possibility of degradation although the mechanism by which such degradation occurred is unclear.

In recent literature, zebrafish cytosolic serine hydroxymethyltransferase (zSHMT-1) was over-expressed in *E. coli* and successfully purified 14.8 fold to 95% purity.[17] The use of CM-Sephadex greatly simplified and significantly increased the efficiency of zSHMT-1 purification because most *E. coli* proteins, including *E. coli* SHMT, were unable to bind to cation exchange resins.[17] They were able to remove the bulk of *E. coli* proteins to achieve a 13.4 fold purification with 90% purity.[17] Previous work to purify SHMT from monkey liver also uses similar techniques to those used to purify zSHMT-1.[17,18] The purification steps for isolating SHMT from monkey liver involved ammonium sulfate precipitation (25–50% saturation), CM-Sephadex ion-exchange chromatography, Ultrogel AcA-34 gel chromatography, and the final step was either Blue Sepharose CL-6B affinity chromatography to achieve a degree of purification of 183, or Folate-AH-Sepharose 4B which gave a degree of purification of 189.[17] Our results are not comparable to these results that have been reported.

In this experiment, the coupled assay allowed us to detect active hSHMT throughout the purification steps by monitoring the decrease in absorbance at 340 nm. However this assay method was limited to the detection of active hSHMT only. Furthermore, the current control (DI water) does not account for background activity caused by any *E. coli* enzymes. One of the difficulties in this experiment was determining the presence of hSHMT by SDS-PAGE analysis since the majority of the proteins present in the all the fractions were *E. coli* proteins. In addition, the lack of a comparison of induced vs. un-induced *E. coli* lysate made it difficult to determine if the 53 kDa band was hSHMT or an *E. coli* protein.

The detection of hSHMT on a SDS-PAGE gel can be improved by performing a western blot using a specific antibody against hSHMT. This would also be able to detect inactive hSHMT, which is not possible with our current coupled assay. The enzyme assay could be improved by using the lysate from un-induced *E. coli* as a control. The use of cation-exchange chromatography (cation-exchange FPLC) may result in better purification

of hSHMT from *E. coli* proteins. In addition, the implementation of similar purification methods described, such as size-exclusion or affinity chromatography can be used to further purify hSHMT after anion-exchange FPLC.

In this project, hSHMT was overexpressed in *E. coli* and partially purified by selective ammonium sulfate fractionation followed by anion-exchange fast protein liquid chromatography. The active fractions were identified with the coupled assay using L-*allo*-threonine and ADH. The Ni^{+2}-NTA affinity purification trials concluded that hSHMT was not expressed as a fusion protein containing a polyhistidine affinity tag. The gene encoding for human SHMT was then cloned into a pET151/D-TOPO (Invitrogen) plasmid for better purification. A fraction of transformed colonies were screened for the cloned gene by PCR amplification and restriction enzyme digestion of isolated plasmid DNA. None of the colonies screened were found to contain the full-length hSHMT gene.

V. REFERENCES

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