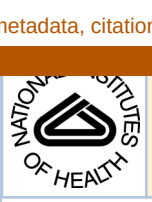

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Recombinant human sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) is expressed at high yield as an active homotetramer in baculovirus-infected insect cells

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Abstract

The sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) isoform is a promising contraceptive target because it is specific to male germ cells, essential for sperm motility and male fertility, and well suited to pharmacological inhibition. However, GAPDHS is difficult to isolate from native sources and recombinant expression frequently results in high production of insoluble enzyme. We chose to use the Bac-to-Bac baculovirus-insect cell system to express a His-tagged form of human GAPDHS (Hu his-GAPDHS) lacking the proline-rich N-terminal sequence. This recombinant Hu his-GAPDHS was successfully produced in *Spodoptera frugiperda* 9 (Sf9) cells by infection with recombinant virus as a soluble, enzymatically active form in high yield, >35mg/L culture. Biochemical characterization of the purified enzyme by mass spectrometry and size exclusion chromatography confirmed the presence of the tetrameric form. Further characterization by peptide ion matching mass spectrometry and Edman sequencing showed that unlike the mixed tetramer forms produced in bacterial expression systems, human his-GAPDHS expressed in baculovirus-infected insect cells is homotetrameric. The ability to express and purify active human GAPDHS as homotetramers in high amounts will greatly aid in drug discovery efforts targeting this enzyme for discovery of novel contraceptives and three compounds were identified as inhibitors of Hu his-GAPDHS from a pilot screen of 1120 FDA-approved compounds.

Keywords

GAPDHS; Baculovirus; insect cell; sperm; mass spectrometry; homotetramer

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Introduction

Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDHS; GenBank Accession ID: **NM_014364.4**), the sperm-specific isoform of human GAPDH [1–3], is under active study as a male contraceptive target [4–6]. Gene targeting studies in mice provided compelling evidence that GAPDHS is required for sperm function and male fertility in mice [6–7], and that males lacking this isozyme are infertile and produce sperm with very low ATP levels and no progressive motility [6]. In contrast to the somatic form of GAPDH, the sperm-specific GAPDHS possesses a unique proline-rich extension at the amino terminus which varies between species (a 72 amino acid extension in human [1], of which 42 residues comprise the proline-rich region (50% proline)[3]), and may mediate binding to the fibrous sheath in the sperm flagellum [1,3,8].

The ability to identify potential drugs targeting human GAPDHS has been hindered by the difficulties in producing sufficient soluble and active enzyme for study. Isolation of native GAPDHS from sperm has proved challenging due to its tight association with the fibrous sheath. The expression of recombinant GAPDH enzymes in *E. coli* has met with varied success, with high production of insoluble material frequently observed [9]. A recent study found that expression of rat GAPDHS, either as a full-length protein or a truncated protein lacking the proline-rich N-terminus, resulted in protein that was predominantly insoluble [5]. We have had similar difficulties obtaining high yields of soluble protein when expressing recombinant mouse or human GAPDHS in *E. coli.* Although native GAPDH enzymes typically function as homotetramers (molecular mass ~150 kDa), the expression of rat GAPDHS in *E. coli* resulted in mixed tetramers consisting of 1 subunit of rat GAPDHS and 3 subunits of *E. coli* GAPDH [5]. The generation of GAPDH heterotetrameric forms combining purified GAPDH dimers from two different species has been reported [10–11]. Recombinant forms of GAPDH enzymes from a number of human parasites have also been expressed in *E. coli* and shown to be tetrameric [12–16]. There are few reports on the expression of recombinant GAPDH enzymes in host systems other than *E. coli*, and expression of a GAPDH enzyme in *Pichia pastoris* yielded inactive enzyme [9]. The eukaryotic baculovirus-insect cell system has been used to produce soluble oligomeric forms of human GAPDH [17] and GAPDH from *Schistosoma mansoni* [9]. The *S. mansoni* GAPDH expressed in Sf9 cells consisted of both monomeric and tetrameric forms, although it was not determined whether the tetrameric form consisted of a single type or mixed subunits.

Herein, we describe the expression and biochemical characterization of the sperm-specific human GAPDHS protein in baculovirus-infected insect cells. In this system, the protein was highly expressed as a biologically active and soluble form. Extensive analysis by mass spectrometry and Edman sequencing demonstrated that the protein was a homotetrameric species. The availability of this form of GAPDHS will greatly facilitate ongoing efforts to identify novel inhibitors that may have potential as male contraceptives.

Materials and methods

Construction of the recombinant human GAPDHS baculovirus

Unless stated, all procedures were performed according to manufacturer's procedures (BactoBac™, Invitrogen). The full length gene for human sperm-specific gyceraldehyde-3-phosphate dehydrogenase (GAPDHS) was synthesized (GeneArt, Inc.). A truncated GAPDHS sequence lacking the proline-rich domain was generated by creating an EcoR1 restriction site upstream of residue 69 of the synthetic gene, and this region comprising residues 69–408 was subcloned in-frame into the EcoRI and XhoI sites of a phosphatase (CIP)-treated pFastBac HT baculovirus shuttle vector (Invitrogen). The resulting

recombinant gene encodes 30 amino acids at the N-terminus, comprising a hexahistidine tag, a spacer region and a TEV protease cleavage site plus the truncated GAPDHS gene (Fig. 1). The resulting clonal DNA was sequenced across the two flanking restriction sites to verify correct gene insertion.

The sequence-verified clone was transformed into DH10Bac, an *E. coli* strain carrying both a baculovirus genome-containing plasmid and a plasmid encoding a transposase enzyme. The GAPDHS gene was inserted via Tn7 transposable elements immediately downstream of the polyhedrin promoter in the baculoviral DNA. Positive white colonies from the lacZ DH10Bac strain were restreaked twice to ensure clonal recombinant virus. Mini-prep DNA from the resulting white colony isolates, bacmid DNA, was PCR verified for the presence of the GAPDHS insertion, and then used in the initial *Spodoptera frugiperda* (Sf9) transfection.

Sf9 cell maintenance

Spodoptera frugiperda (Sf9) cells (ATCC) adapted to HyQ SFX-Insect serum free medium (HyClone) supplemented with 2 mM L-glutamine and 1× Penicillin/Streptomycin were cultured at 27°C in either monolayer or suspension culture. Sf9 suspension cultures were maintained at a cell density of 0.5×10^6 to 3×10^6 cells/ml as 80 ml cultures in 250 ml shaker flasks shaken at 115 rpm in an ATR Multitron shaking incubator (ATR, Inc.)

Insect cell transfection and recombinant virus isolation

To generate viral particle formation, newly attached Sf9 cells in 6-well plates (9×10^5 cells/well) were transfected with the PCR-verified recombinant GAPDHS bacmid DNA (1–2 μg) in Cellfectin™ (Invitrogen) and incubated in unsupplemented Sf-900 II SF medium (Invitrogen for 5 h at 27°C. After aspiration of the transfection mixture, TNM-FH medium (HyClone) supplemented with 10% FBS, 2 mM L-glutamine and 1x Penicillin/Streptomycin was added. Conditioned medium was collected 5 d after transfection and the supernatant was reserved as P1 viral stock. P2 and P3 viral amplification was accomplished in 75 cm^2 T-flasks at a multiplicity of infection (MOI) of 0.1 assuming titers of 1×10^6 and 2×10^7 plaque forming units (pfu)/ml for P1 and P2 viral stocks, respectively. The resulting P3 viral stock was titered via the end-point dilution method [18], and determined to be 2×10^8 pfu/ml.

Expression optimization of recombinant Hu his-GAPDHS in Sf9 cells

Optimization of protein expression was performed in 6-well plates by varying multiplicity of infection (MOI, values of 1, 2, 5, 10 and 20) and time of expression (24, 48, 72 and 96 h). Cells were seeded at 2×10^6 cells/well, and baculovirus stock added in 2.5 ml supplemented (as described above) HyQ SFX-Insect medium. At regular time points, cells were washed with PBS, harvested, and protein expression in the conditioned medium and cell pellet assessed by SDS-PAGE.

Small scale expression and optimization of purification of Hu his-GAPDHS

Small scale expression and optimization of purification was done in 25 cm^2 T-flasks; 6×10^6 Sf9 cells were seeded per T-flask, infected at MOI 10 in supplemented HyQ SFX-Insect medium, and harvested 72 h post infection. Cells were washed with ice-cold PBS, harvested by centrifugation (233 × g for 10 min) and cells lysed with 25 mM Tris-HCl pH 8.0, 300mM NaCl, 10mM imidazole, 17% glycerol, 0.1% Triton X-100, 2.5U/ml Benzonase (Novagen), and 1 mM PMSF. After incubation on ice for 30 min at 4°C, the crude lysate was collected by centrifugation. All purification steps were performed at 4°C. Crude lysate was incubated with pre-equilibrated Ni^{2+}-NTA His-Bind resin (Novagen) for 1 h to allow binding of

recombinant GAPDHS. The resin was recovered by centrifugation at 233 × g for 1 min, washed five times with 5 resin volumes of Ni^{2+}-NTA binding buffer (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole and 1 mM PMSF) and eluted with 3 resin volumes of Ni^{2+}-NTA binding buffer containing 250 mM imidazole. Following the incubation, the sample was centrifuged and the supernatant taken as the purified protein fraction. Fractions were analyzed by SDS-PAGE.

Large scale production and purification of recombinant Hu his-GAPDHS

For large scale production, Sf9 suspension culture (600 ml of 1.4×10^6 cells/ml) in a 2 L shaker flask was grown in supplemented HyQ SFX-Insect medium, infected at an MOI of 5 and cultured at 27°C in a Multitron (ATR, Inc.) shaking incubator at 72 rpm. Cells were harvested 64 h post-infection and recovered from conditioned medium by centrifugation at 2000 × g for 20 min. Crude cell extracts were prepared by resuspending the cell pellet in ice-cold lysis buffer. After 2 h on ice, centrifugation at 1,000 × g for 10 min at 4°C was used to pellet cell debris and separate the crude lysate. The clarified, crude lysate was incubated with pre-equilibrated Ni^{2+}-NTA His-Bind resin (Novagen) at a ratio of 1 ml beads/100 ml lysate for 1 h at 4°C. After binding, the resin was recovered by centrifugation at 233 × g for 7 min, washed five times with 5 resin volumes of Ni^{2+}-NTA binding buffer and eluted with 3 resin volumes of Ni^{2+}-NTA binding buffer containing 175 mM imidazole. Fractions were aliquoted and stored at −80°C.

SDS-PAGE and Western blot analysis

Proteins were analyzed by SDS-PAGE (4–12% Bis-Tris gels, NuPAGE MOPS SDS running buffer, Invitrogen) and stained for protein with Coomassie Blue. Protein concentration was determined by Bradford assay (Biorad). For Western blotting, protein samples were separated by SDS-PAGE and electrophoretically transferred to PVDF at 30 V constant for 1 h using a Novex apparatus (Invitrogen). Membranes were incubated in TBS-T blocking buffer (Tris buffered saline, 0.1% Tween-20, 5% dry milk) at room temperature for 1 h. Blocked membranes were probed with either a GAPDHS-specific antibody (raised to residues 159–194 of mouse GAPDHS [8]) diluted 1:3000 in blocking buffer followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (1:5000, Pierce) or with a penta-his HRP conjugate (1:2000, Qiagen). Membranes were washed 3 times for 3 min each in TBS-T, visualized using SuperSignal West Dura Extended Duration Substrate chemiluminescent kit (Pierce) and detected using a Kodak Image Station 4000R.

Size exclusion chromatography

Protein size was determined by size exclusion chromatography at 15°C using an Akta FPLC system (GE Healthcare) fitted with a Superose 12 10/300 GL column. Potassium phosphate buffer (50 mM, pH 7.0) containing 150 mM NaCl served as the mobile phase at a flow rate of 0.5 ml min^{-1}. Proteins from low and high molecular weight calibration kits (GE Healthcare) were used to estimate apparent molecular size. K_{av} values were calculated as in [19].

Electroblotting and Edman sequencing

Proteins were separated by SDS-PAGE (4–12% Bis-Tris gels, NuPAGE MOPS SDS running buffer, Invitrogen) and then transferred in 100 mM CAPS pH 11 to sequencing grade PVDF (GE Water & Process Technologies) at 30 V constant for 1 hr. Membranes were stained with Coomassie Blue R-250 for 3 min and bands excised for tryptic peptide analysis. N-terminal Edman sequencing (7 cycles) was performed on separated peptides at the University of Texas protein chemistry core using a Procise cLc 494 protein sequencer (Applied Biosystems).

Whole mass measurement by MALDI-TOF mass spectrometry

Purified Hu his-GAPDHS was desalted using a C_4 ZipTip (Millipore Co.). The sample was deposited onto an α-cyano-4-hydroxycinnamic acid matrix prepared in an aqueous solvent containing 50% acetonitrile and 10 mM ammonium citrate. Protein mass was determined using a matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer (MS) (Applied Biosystems 4800) with a mass range setting of 10,000 to 50,000 amu. Bovine serum albumin served as the calibrant. Mass spectrometry experiments were performed at the University of North Carolina at Chapel Hill Michael Hooker Proteomics Center.

Peptide mass fingerprinting

Peptides were generated by in-gel tryptic digestion of a Coomassie Blue stained protein band excised from a 1D SDS-PAGE gel. Resulting peptides were analyzed using MALDI TOF/TOF MS (Applied Biosystems 4800) as previously described [20]. Observed peptide masses were compared to the NCBInr database using MASCOT MS/MS Ions search algorithm (Matrix Science: www.matrixscience.com). Mass spectrometry analysis was completed at the University of North Carolina at Chapel Hill Michael Hooker Proteomics Center.

Enzymatic assay for GAPDHS

The dehydrogenase activity of GAPDHS was measured kinetically by monitoring the accumulation of NADH at 340 nm [21] immediately following the addition of the substrate, DL-glyceraldehyde-3-phosphate (GAP) (Sigma G5251). One enzyme unit is defined as the amount of enzyme necessary for the formation of 1 μmol of 1,3-diphosphoglycerate min^{-1}. For K_m determination for GAP, the reaction mixture contained GAPDHS, 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, pH 8.9, 0.5 mM NAD and varying concentrations of GAP.

Pilot high throughput screen

The high throughput screen (HTS) assay is based on the standard colorimetric assay of GAPDH activity described above. All steps were carried out in 384-well plates at room temperature. The assay consisted of two 25 μl additions carried out using a Multidrop liquid dispenser (Thermo Scientific). The final assay (50 μl) contained 30 nM GAPDHS, 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, pH 8.9, 0.5 mM NAD and 0.5 mM GAP. Kinetic reads (at 30 sec intervals for 5 mins) of the plates were carried out on a Spectramax Plus 384 reader (Molecular Devices). The Prestwick collection (Prestwick Chemical Co.) containing 1,120 FDA approved compounds was prepared as 10 mM stocks in 100% DMSO. Compounds (0.5 μl) were pre-spotted into the 384-well plates using a Biomek NX workstation to give a final screening concentration of 10 μM. Inhibition data was analyzed in ActivityBase (IDBS Inc.) and using Excel.

Results

Generation of bacmid virus for recombinant human GAPDHS protein expression

We expressed a recombinant form of the human GAPDHS that lacks the proline-rich N-terminal domain (Fig. 1) in the eukaryotic bacmid-*Spodoptera frugiperda* insect cell expression system. GAPDHS lacking the proline-rich region has previously been shown to be active [5] and the variable length of this region between species [3] suggests that its predominant role is in localizing the enzyme to the fibrous sheath and is not critical for function [1]. Advantages of the baculovirus-insect cell system include high expression, correct folding, potential post-translational modifications and the production of biologically

active protein. The recombinant GAPDHS comprised residues 69 to 408 of the full-length enzyme (GenBank Accession ID: **NP_055179.1**) with an additional 30 amino acid residues for a cleavable hexahistidine tag fused onto the N-terminus (Hu his-GAPDHS) (Fig. 1). From the amino acid sequence for the recombinant Hu his-GAPDHS (Fig. 2; for simplification the first residue in recombinant Hu his-GAPDHS is referred to as number 1), a predicted mass of 40,779.41 kDa was calculated using the Swiss-Prot ExPASy Compute pI/Mw tool.

The synthetic gene for human GAPDHS was subcloned into the baculovirus shuttle vector pFastBac HT. The presence of insert was verified by restriction digest with EcoR1 and Xho1 which generated a band of ~1 kb that closely corresponded to the expected 1032 bp GAPDHS gene insert (data not shown). Sequencing across the insertion sites was also used to verify insertion. Due to the large size of the bacmid it is difficult to verify inserts by restriction digest and so PCR amplification of the bacmid DNA gene cassette insertion with the M13 forward and reverse primer pair was used to confirm the size of the insert, which yielded a band of ~3.5 kb corresponding closely to the predicted 3462 bp size (data not shown).

Expression and purification of recombinant human his-GAPDHS in Sf9 Cells

Optimization of expression in Sf9 cells was initially undertaken on a small batch scale in 6-well plates, assessing expression in Sf9 cells at different MOI (0, 1, 2, 5, 10 and 20) and time of harvest (48, 72, 96 h). Although expression of recombinant proteins in Sf9 cells does not typically result in high levels of secreted recombinant protein (Bac-to-Bac, Baculovirus Manual, Invitrogen), GAPDHS expression in both crude cell lysates (L) and conditioned media (M) was assessed by SDS-PAGE (Fig. 3A). At all MOI tested, Sf9 cells infected with purified recombinant human his-GAPDHS virus showed the presence of a protein with apparent molecular weight ~40 kDa (protein band highlighted with arrow) in cell lysates that was not present in mock transfected cells (MOI = 0) (Fig. 3A). Western blotting of the crude lysates with an anti-Histag antibody (Fig. 3B) indicated the presence of a band at ~40 kDa in all samples except the mock infected suggesting that this band represents the recombinant Hu his-GAPDHS. A band corresponding to Hu his-GAPDHS was not observed in the media fractions. After comparing expression levels using different incubation conditions, an MOI of 10 and 72 h post-infection harvest time were chosen for subsequent protein expression experiments.

Purification of Hu his-GAPDHS was first confirmed on a small batch scale in 25 cm^2 T-flasks (Fig. 3C). Sf9 cells were infected at an MOI of 10, and harvested 72 h post-infection. Cells were lysed and Hu his-GAPDHS purified from the crude extract over Ni^{2+}-NTA metal affinity resin as described in the materials and methods. Eluted Ni^{2+}-NTA affinity column fractions (Fig. 3C, lane 5) from Sf9 cells infected with GAPDHS virus contained a major band with an apparent molecular weight of ~40 kDa corresponding to Hu his-GAPDHS which was not present in the equivalent fraction from the mock transfection (not shown). A high degree of purification was achieved as visualized by Coomassie Blue staining of an SDS-PAGE gel and the lack of significant lower molecular weight protein bands suggests that proteolysis of GAPDHS was not an issue under the expression and purification conditions used. A preliminary activity assay of the purified protein indicated good activity (data not shown).

Large scale expression and purification of recombinant human his-GAPDHS protein in Sf9 suspension culture

To obtain sufficient purified recombinant human GAPDHS for characterization, a large-scale suspension culture of Sf9 cells (600 ml, 1.5×10^6 cells/ml) was infected with the

recombinant baculovirus carrying Hu his-GAPDHS. The optimal MOI of 10 and 72 h time of harvest conditions determined in the small-scale expression studies described above were first used in an initial 80 ml suspension culture expression experiment. However, cell viability at time of harvest was determined to be only 60.1% (data not shown). Hence, to minimize loss of cell viability and resulting loss of protein due to cell lysis, an MOI of 5 was used and cell number and cell viability monitored throughout the infection process to determine the optimal time of harvest as 64 h. These infection and culture conditions were used for the subsequent large scale expression studies. For the 600 ml culture, cells were collected, lysed and the Hu his-GAPDHS purified by one-step Ni^{2+}-NTA metal affinity chromatography using an optimized concentration of 175 mM imidazole to elute the Hu his-GAPDHS from the resin. High purity of Hu his-GAPDHS was achieved by this Ni^{2+}-NTA metal affinity step as assessed by Coomassie Blue-stained (Fig. 4A, lane 1) and silver-stained (Fig. 4A, lane 2) SDS-PAGE gels. Approximately 21 mg of Hu his-GAPDHS was purified from a 600 ml Sf9 culture giving a yield of 35 mg/L. The yield of ~35 mg/L from the large scale study is in line with those typically reported [22] wherein “high-level” is defined as >100 mg of recombinant protein per liter of infected cells. The expression level we observed in our large scale suspension culture studies was higher than in our preliminary small scale experiments and may be attributed to a number of potential factors including differences in cell culture conditions between the small and large scale cultures (MOI, time of harvest and aeration). Such differences led to lower viability of the cells at small scale and subsequent lower yield of recombinant Hu his-GAPDHS protein.

The Hu his-GAPDHS eluted from the Ni^{2+}-NTA metal affinity column was further analyzed by Western blotting with an anti-GAPDHS antibody [8] and an anti-Histag antibody. Since the anti-GAPDHS antibody does not cross-react with mouse, rat or *E. coli* GAPDH [8], it is unlikely that it cross-reacts with Sf9 GAPDH. On Western blots, both the anti-GAPDHS antibody (Fig. 4A, lane 3) and the anti-Histag antibody (Fig. 4A, lane 4) reacted with a ~40 kDa band. Those results, combined with the calculated size of Hu his-GAPDHS (40 kDa, see above) confirmed that the protein corresponding to this band was Hu his-GAPDHS.

Predicted amino acid sequence for native Spodoptera GAPDH

Extensive literature and database searching revealed no published protein sequence for Sf9 GAPDH. To identify the sequence of Sf9 GAPDH and hence its predicted molecular mass, a search of NCBI protein databases was performed using the amino acid sequence for human GAPDH (Accession ID: **NP_002037**) (corresponding to the mRNA sequence, Accession ID: **NM_002046**). The most significant alignment was a 125 amino acid sequence of *Spodoptera frugiperda* GAPDH corresponding to a 376 bp mRNA sequence (Accession ID: **EU372957.1**). The mRNA sequence was used to BLAST the SPODOBASE *Spodoptera* database of 79,148 sequences [23]. The SPODOBASE contains expressed sequence tags (ESTs) from independent cDNA libraries including from the Sf9 cell line. The best 61 sequences (including 99% identity matches) belonged to cluster SF9L02522, which itself is composed of 68 sequences and had been used to assemble a 1428 bp contig sequence, SF9L02522-Contig2 (Supplemental Table 1; Supplemental Figure 1). The longest open reading frame (ORF) from SF9L02522-Contig2 was 332 amino acids in length and had a deduced molecular weight of 35,602 kDa for *Spodoptera* GAPDH. The ORF sequence was then aligned against the human GAPDH protein sequence (335 amino acids) and revealed a sequence identity of 76.4% (256/335) and sequence similarity of 85.7% (287/335) (data not shown). This predicted Sf GAPDH sequence was also aligned with the Hu GAPDHS (excluding the FastBac tag) and had 67.6% identity (230/340) (see Fig. 2, light grey background) and 82.9% similarity (282/340) (see Fig. 2, dark grey background).

An estimation of *Spodoptera* GAPDH molecular weight as 37 kDa was reported previously from SDS-PAGE analysis [24]. Protein loading SDS-PAGE gel analysis was undertaken as

a first test for the presence of Sf9 GAPDH in the purified Hu his-GAPDHS preparation (Fig. 4B), and indicated the presence of only a single band with apparent molecular weight ~40 kDa. The 12% NuPAGE gel combined with the MOPS buffer system was chosen to give maximal separation in the 30–50 kDa range and no band was observed in the 35–37 kDa range that might indicate the presence of insect-derived GAPDH in the purified enzyme.

Molecular mass and size analysis of the recombinant Hu his-GAPDHS protein

Whole mass determination of purified Hu his-GAPDHS by MALDI-TOF gave an observed mass of 40,740.2 Da (Fig. 5) close to the predicted mass of 40,779 Da from the sequence of Hu his-GAPDHS. No other masses were observed that might correspond to Sf9 host-derived GAPDH and if present, we would expect at a minimum, a peak of ~1/3 intensity assuming comparable ionization efficiencies.

Size exclusion chromatography (SEC) was used to assess both the quality and molecular mass of Hu his-GAPDHS. Baculovirus-insect cell expressed purified Hu his-GAPDHS was applied to a Superose 12 column and eluted as a single symmetrical peak (Fig. 6A), suggesting the protein consists of a single well folded species. Comparison with known protein standards (Fig. 6B) yielded a molecular size of ~130 kDa, suggesting that the protein was still an intact oligomer. The size we observed for Hu his-GAPDHS is somewhat lower than might be expected for a tetramer (~160 kDa). Under the same conditions, human somatic GAPDH (from human erythrocytes, Sigma, G6019) yielded a molecular mass of ~140 kDa and further analysis of Hu his-GAPDHS in a different Tris buffer system indicated a molecular size of ~135 kDa (data not shown). Taken together with the difficulties in accurately assessing molecular size on Superose 12 [25], our SEC data suggests that Hu his-GAPDHS expressed in Sf9 cells is all in a tetrameric form.

Peptide sequencing of the recombinant Hu his-GAPDHS protein expressed in Sf9

Mass spectrometry and N-terminal Edman sequencing were used to assess the homogeneity among primary amino acid sequences in the purified Hu his-GAPDHS. For MS analysis, purified Hu his-GAPDHS was subjected to SDS-PAGE, the Coomassie Blue-stained band was excised from the gel and peptide mass fingerprinting carried out as described in materials and methods. Twenty two unique peptides were extracted and analyzed by peptide mass fingerprinting and MS/MS peptide fragment ion matching (Table 1). The observed masses of all the peptides matched (Δ<42 ppm) the calculated masses of the expected peptides resulting from tryptic cleavage of human GAPDHS and provided 65% coverage of the sequence (Table 3, shaded region). Other than methionine oxidation on some of the peptides, no other modifications were observed. Observed peptide masses were compared to the NCBInr database using MASCOT software [26] and five significant ($p< 0.05$) database matches were detected, all corresponding to human sperm-specific GAPDHS. The best database match (GenBank ID: **238537990**) had a probability based MOWSE score of 803 providing high statistical confirmation that all the identified peptides come from human GAPDHS.

For Edman sequencing, no sequence was obtained from the purified protein in solution, suggesting a blocked N-terminus. To obtain internal sequences, purified Hu his-GAPDHS was subjected to SDS-PAGE, transferred to a PVDF membrane and stained with Coomassie Blue as described in materials and methods. Peptides generated by tryptic digest of the band were separated by reversed-phase liquid chromatography and sequences determined by Edman sequencing. Sequences were obtained for 13 peptides and for all peptides there was a 100% sequence match to human GAPDHS and no match to *S. frugiperda* GAPDH (Table 2; Table 3, underlined sequences). Two peptides were sequenced by Edman that were not detected by MS/MS analysis, providing overall Hu his-GAPDHS coverage of 68.5% (Table

3). The absence of any peptides from insect-cell derived GAPDH indicates that the human GAPDHS expressed in the baculovirus-infected insect cells is produced as a homotetrameric form.

Enzymatic activity of purified Hu his-GAPDHS protein

The assay for NAD-dependent dehydrogenase activity using DL-glyceraldehyde-3-phosphate (GAP) as substrate indicated that the purified his-GAPDHS expressed in the baculovirus system was enzymatically active. The kinetic constants of the Hu his-GAPDHS were determined by measuring initial rates at various GAP concentrations in the presence of a constant saturating concentration (0.5 mM) of NAD. The K_m value measured at room temperature was 0.41 ± 0.06 mM and the specific activity (~28 U/mg) was in the range of that reported for the activity of commercial preparations of somatic GAPDH purified from human erythrocytes.

Pilot small molecule screen of Hu his-GAPDHS

Using the purified Hu his-GAPDHS expressed in the baculovirus-insect cell system, we carried out a pilot high throughput screen in 384-well format against the Prestwick collection, a purchased library containing 1,120 compounds, 90% of which are FDA-approved drugs. The assay was first validated using procedures previously described for enzyme assays [27]. The scatter plot for the Prestwick screen is shown in Fig. 7. Using a threshold of 30% inhibition (3 SD above mean), this screen had a hit rate of 0.3%.

Discussion

Using the baculovirus-insect cell expression system, we found that the sperm specific isoform of human glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) lacking the proline-rich N-terminus could be expressed as a soluble biologically active form with substantially higher yields than we have achieved in *E. coli*, and with specific activities comparable to GAPDH preparations that are commercially available. Previously, we and others have not been able to express large amounts of soluble GAPDHS in *E.coli* [5,8], even for constructs lacking the polyproline region [5] (D. Lamson, P. Danshina and K. P. Williams, unpublished data). The use of a His-tagged construct enabled a rapid, high-purity, one-step purification of Hu GAPDHS from the baculovirus-insect cell system as assessed by silver staining and mass spectrometry. The Hu his-GAPDHS was tetrameric as determined by size exclusion chromatography, consistent with previous reports that native GAPDH enzymes are typically tetramers [28–29].

The ability of GAPDH to form mixed tetramers has long been known from GAPDH subunit exchange studies in which heterotetrameric forms of GAPDH were generated from purified rabbit- and yeast-derived GAPDH enzyme [11]. Rat GAPDHS expressed in *E. coli* yielded heterotetramers consisting of 1 subunit of rat GAPDHS and 3 subunits of *E. coli* GAPDH [5]. Hence, we were interested to see if human GAPDHS expressed in the baculovirus-insect cell system would likewise yield a heterotetrameric form comprising Hu GAPDHS and insect-cell derived GAPDH, or if a homotetrameric form of human GAPDHS would be generated. To identify if the purified human GAPDHS expressed in the baculovirus-infected insect cell system were homo- or heterotetrameric, we first needed to know the molecular weight of GAPDH from *Spodoptera frugiperda*. Since a full-length sequence of *Spodoptera frugiperda* GAPDH was not available, the human GAPDH amino acid sequence was used to find an ortholog Sf9 GAPDH sequence which was then used to search the SPODOBASE Spodoptera EST database. A Sf9 GAPDH contig match was found which contained an ORF with 76.4% identity to human GAPDH and had a predicted mass of 35,602 Da. To our knowledge this is the first published sequence for *Spodoptera frugiperda* GAPDH. The

purified Hu his-GAPDHS exhibited a prominent band of ~40 kDa on Coomassie Blue stained gels and contained no band around ~35 kDa that might correspond to Sf GAPDH. In contrast, SDS-PAGE analysis of rat GAPDHS expressed in *E. coli* clearly showed the presence of two bands corresponding to bacterial and recombinant enzyme [5].

To confirm the absence of insect-derived GAPDH in our purified Hu his-GAPDHS enzyme preparation we undertook peptide mapping analysis by both peptide ion matching mass spectrometry and Edman sequencing. All peptides analyzed corresponded to human GAPDHS and no peptides were identified that matched those predicted for insect GAPDH. Hence, our data indicates that the Hu his-GAPDHS expressed in baculovirus-infected insect cells yields homotetramers in contrast to the mixed tetramers seen previously for *E. coli* expression.

In the baculovirus/insect system, mechanistic and titration effects may have contributed to the formation of GAPDHS homotetramers rather than mixed tetramers composed of Hu GAPDHS and Sf9-derived GAPDH. The dimerization domains of Sf9 GAPDH and human GAPDHS may differ such that mixed dimers/tetramers would not occur. The dimerization site between Hu His-GAPDHS and *E.coli* GAPDH (residues 149–314) share 85% sequence identity in this region and mixed tetramers were observed [5]. The same domains in Hu GAPDHS and Sf9 GAPDH share 80% sequence identity, which suggests that mixed tetramers are possible. We suggest that the primary factor influencing the homogeneity of homotetrameric GAPDHS is the high expression level achieved in the baculovirus-insect cell system and an intrinsic property of the system where viral takeover of the host gene expression system favors recombinant gene expression at the expense of host gene expression. Although GAPDH is a housekeeping gene expressed at relatively high levels, there may be conditions under which the Sf9 GAPDH concentration is sufficiently low to preclude mixed tetramer formation, particularly since host gene expression is typically impaired during the time of recombinant gene expression, with expression from 24–36 h post-infection being primarily viral-specific [18]. Previous studies that expressed recombinant GAPDH using the baculovirus-insect cell system were not characterized for homo- versus heterotetramer content [9,17].

To demonstrate the feasibility of using purified Hu his-GAPDHS to identify enzyme inhibitors that may serve as preliminary leads for contraceptive agents, we screened the Prestwick library, a collection of structurally diverse FDA-approved compounds. Three inhibitors of Hu his-GAPDHS enzyme activity were identified and will be detailed in a forthcoming screening publication. The availability of this active homotetrameric form of human GAPDHS produced in the baculovirus-insect cell system in relatively large quantity will greatly facilitate ongoing efforts to use both high throughput screening of large compound collections and structure-based drug design approaches to identify novel inhibitors as potential male contraceptives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

GAPDHS	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic

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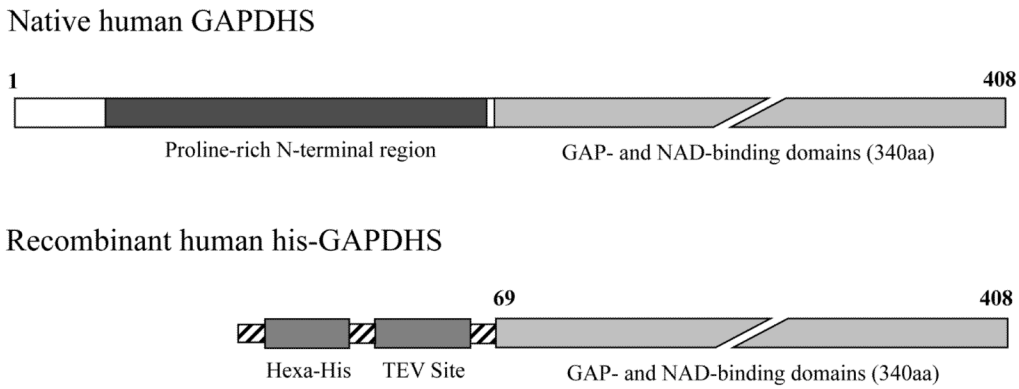

Figure 1.

Schematic diagram of full-length human GAPDHS and the recombinant human his-GAPDHS expressed in insect cells. The sperm isozyme possesses a proline-rich domain at the N-terminus that is not present in the somatic GAPDH isozyme. In human GAPDHS, this extension comprises a 19 amino acid conserved sequence, 3 amino acid intervening proline cysteine region and a 42 amino acid proline-rich region (50% proline) from residues 27–67 [3]. Numbering in this figure refers to the sequence for human sperm-specific gyceraldehyde-3-phosphate dehydrogenase (GAPDHS) (GenBank Accession ID: **NP_055179.1**). For the form of GAPDHS expressed in insect cells herein, the N-terminal extension containing the proline-rich region was deleted (residues 1–68) and replaced by the 30 amino acid FastBac vector sequence which included a hexahistidine purification tag and TEV protease cleavage sequence.

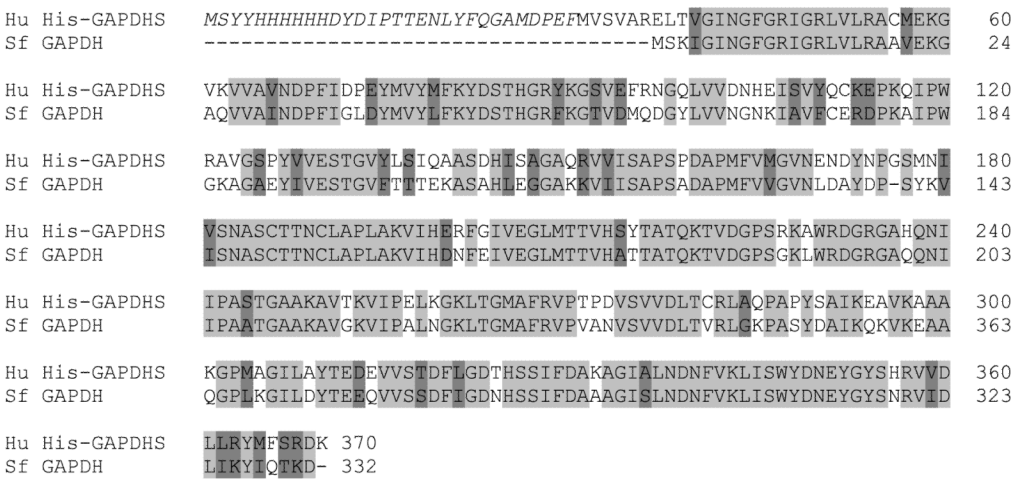

Figure 2.

Sequence alignment between recombinant human his-GAPDHS and predicted *Spodoptera frugiperda* GAPDH. For simplification, residue numbering begins at 1 for the recombinant his-GAPDHS. The 30 residue FastBac tag is shown italicized. The sequence for *Spodoptera frugiperda* (Sf) GAPDH was from SF9L02522-Contig2, a contig assembled from the SPODOBASE EST database (see supplemental Fig. 1 and supplemental Table 1). Regions of identity are shown with a light grey background and regions of similarity with a dark grey background.

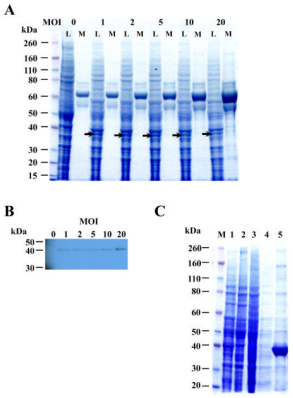

Figure 3.

Optimization of recombinant human his-GAPDHS expression in Sf9 cells. **A**. MOI analysis of his-GAPDHS expression. Sf9 cells were infected with recombinant GAPDHS virus at the indicated MOI and samples of media (M) and cell lysates (L) taken 96 h post-infection were analyzed by SDS-PAGE. Protein bands resulting from infection with the recombinant virus carrying the gene for GAPDHS are indicated by arrows. **B**. Western blot analysis of cell lysates at the indicated MOI from experiment **A** above using a penta-his HRP conjugated antibody. **C**. Small scale expression in 25 cm^2 T-flask of Hu his-GAPDHS in Sf9 cells and Ni-NTA affinity purification. Lane M, Novex prestained protein markers; lane 1, cell lysate from recombinant virus infected S9 cells, lane 2, mock infected Sf9 cells, lane 3, Ni-NTA resin unbound fraction, lane 4, Ni-NTA resin wash fraction, lane 5, Ni-NTA resin eluted fraction showing a major band at ~40 kDa corresponding to human his-GAPDHS.

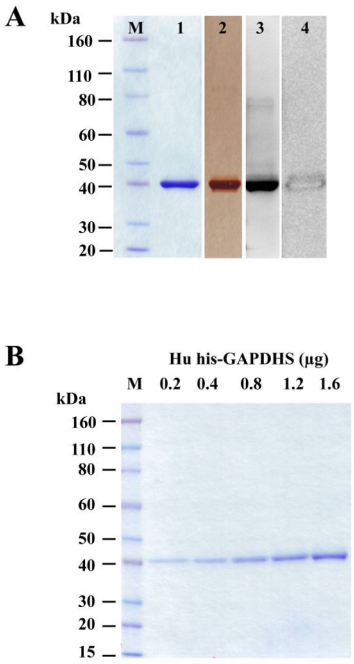

Figure 4.

SDS-PAGE/Western blot analysis of human his-GAPDHS purified by Ni^{2+}-NTA affinity chromatography. **A**. Coomassie-Blue stained gel, lane M, Novex prestained protein markers; lane 1, Hu his-GAPDHS, lane 2, Silver-stained gel of Hu his-GAPDHS, lane 3, Western blot of Hu his-GAPDHS with polyclonal antibody raised to residues 159–194 of mouse GAPDHS [8], lane 4, Western blot of Hu his-GAPDHS with penta-his HRP conjugated antibody. **B**. Coomassie-Blue stained gel; lane M, Novex prestained protein markers and indicated amounts of purified Hu his-GAPDHS.

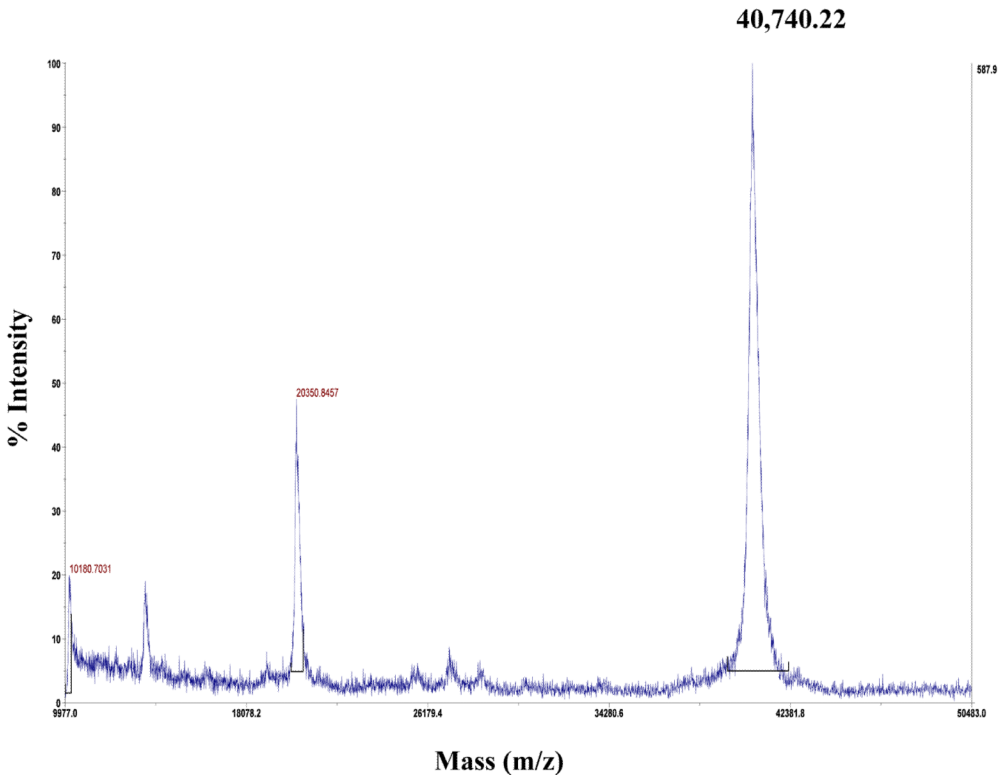

Figure 5.
MALDI-TOF MS spectra of purified recombinant human GAPDHS. MALDI-TOF analysis was performed using α-cyano-4-hydroxycinnamic acid as the matrix. The peaks at 40,740.22, 20,350.85 and 10,180.70 are single-, double-, and quadruple-charge species.

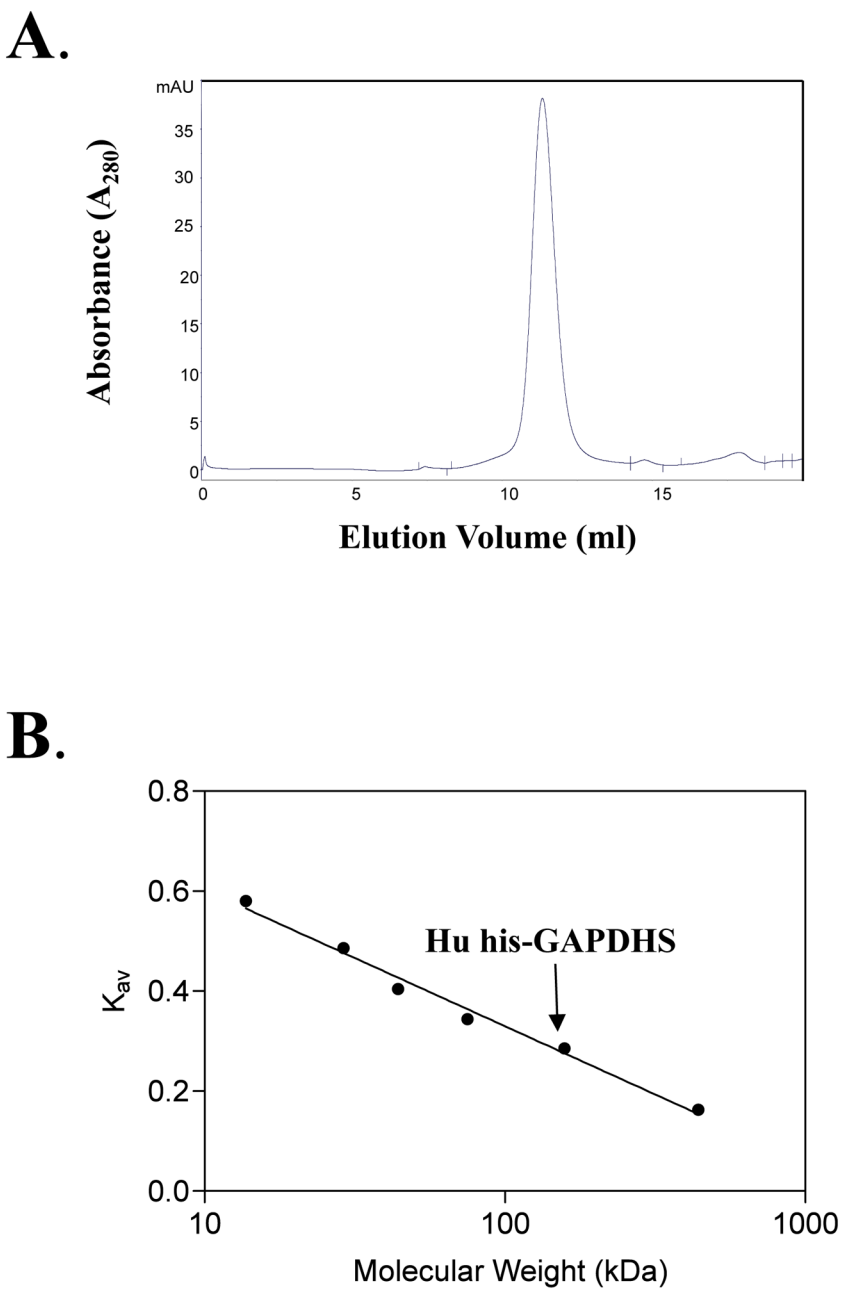

Figure 6.

Size exclusion chromatography of purified human his-GAPDHS. **A**. Chromatogram of purified Hu his-GAPDHS loaded on a Superose 12 column at 0.5 ml/min in 50 mM potassium phosphate pH 7.0, 150 mM NaCl buffer. **B**. A calibration curve was created using the elution volumes (V_e) of the following standard proteins; ferritin (M_r 440,000), aldolase (M_r 158,000), conalbumin (M_r 75,000), ovalbumin (M_r 44,000), carbonic anhydrase (M_r 29,000), ribonuclease A (M_r 13,700). K_{av} values were calculated [19] using a void volume (V_o) of 7.4 ml and included volume of 19.9 ml (V_i). The molecular size of recombinant human his-GAPDHS was estimated to be ~130,000 (arrow).

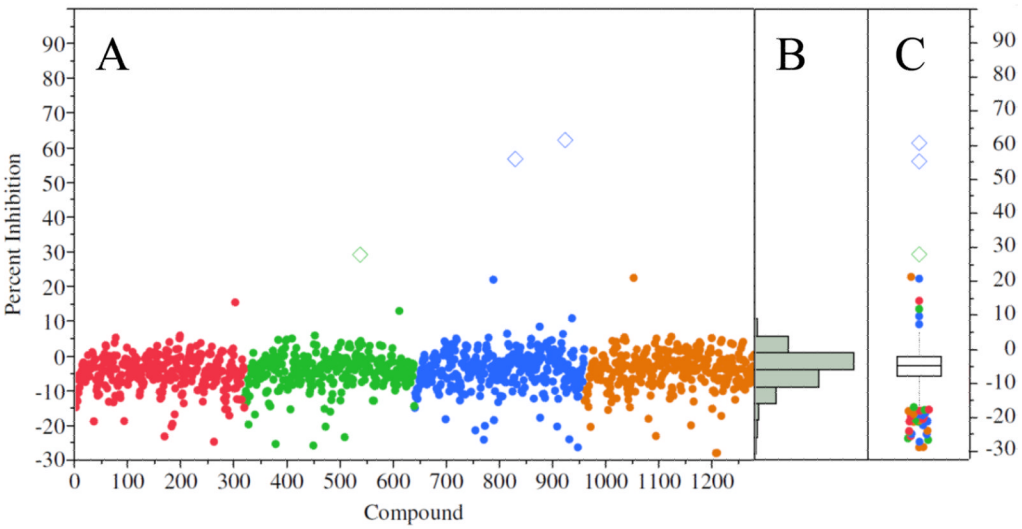

Figure 7.

Pilot high throughput screen using Hu his-GAPDHS enzyme. The Prestwick compound collection was pre-spotted (0.5 μl) in 384-well plates and the Hu his-GAPDHS enzyme assay carried out in a final volume of 50 μl as described in materials and methods. **A**. Scatterplot showing percent inhibition for compounds screened and with hits shown as diamonds above 30% inhibition. **B**. Histogram of screening data showing a normal distribution and, **C**. Box plot representing quantiles and outliers.

Table 1

Identified recombinant human GAPDHS peptide masses by MS/MS peptide fragment ion matching[a].

M_r				Peptide	
m/z obs.	obs.[b]	calc.[c]	Δ (ppm)[d]	Sequence[e]	start end in rhis GAPDHS [f]
1162.6166	1161.6093	1161.6142	−4.22	R.ELTVGINGFGR.I	37–47
2277.0889	2276.0816	2276.0898	−3.57	K.VVAVNDPFIDPEYMVYMFK.Y	63–81
2293.0803	2292.0730	2292.0847	−5.08	K.VVAVNDPFIDPEYMVYMFK.Y + Oxidation (M)	63–81
2309.0728	2308.0655	2308.0796	−6.09	K.VVAVNDPFIDPEYMVYMFK.Y + 2 Oxidation (M)	63–81
3093.4053	3092.3980	3092.4412	−13.97	K.VVAVNDPFIDPEYMVYMFKYDSTHGR.Y[g]	63–88
835.3660	834.3587	834.3620	−3.96	K.YDSTHGR.Y	82–88
985.5052	984.4979	984.5029	−5.02	R.YKGSVEFR.N[g]	89–96
1945.8625	1944.8552	1944.9364	−41.71	R.NGQLVVDNHEISVYQCK.E	97–113
3033.5149	3032.5076	3032.5203	−4.19	R.AVGSPYVVESTGVYLSIQAASDHISAGAQR.V	122–151
2099.0051	2097.9978	2098.0405	−20.35	R.FGIVEGLMTTVHSYTATQK.T + Oxidation (M)	202–220
731.3844	730.3771	730.3610	22.1	K.TVDGPSR.K	221–227
795.4175	794.4102	794.4109	−0.84	K.LTGMAFR.V	262–268
1500.7762	1499.7689	1499.7654	2.34	R.VPTPDVSVVDLTCR.L	269–282
2640.3911	2639.3838	2639.3993	−5.85	R.VPTPDVSVVDLTCRLAQPAPYSAIK.E[g]	269–293
1158.6409	1157.6336	1157.6444	−9.33	R.LAQPAPYSAIK.E	283–293
3627.6895	3626.6822	3626.7450	−17.32	K.AAAKGPMAGILAYTEDEVVSTDFLGDTHSSIFDAK.A[g]	298–332
3302.4893	3301.4820	3301.5337	−15.64	K.GPMAGILAYTEDEVVSTDFLGDTHSSIFDAK.A + Ox (M)	302–332
1161.6193	1160.6120	1160.6190	−5.97	K.AGIALNDNFVK.L	333–343
2945.4275	2944.4202	2944.4144	1.99	K.AGIALNDNFVKLISWYDNEYGYSHR.V[g]	333–357
2498.2083	2497.2010	2497.2390	−15.20	K.LISWYDNEYGYSHRVVDLLR.Y[g]	344–363
714.4549	713.4476	713.4436	5.68	R.VVDLLR.Y	358–363
703.3295	702.3222	702.3159	8.98	R.YMFSR.D	364–368

[a] Sf9 expressed Hu GAPDHS was extracted from a gel slice and trypsinized prior to entering a MALDI TOF/TOF MS. Observed sequences were compared to the NCBInr database using MASCOT (Matrix Science (26)). The best and most significant match (score of 803) was to chain A, crystal structure of human sperm-specific glyceraldehyde-3- phosphate dehydrogenase (GAPDHS) complex with NAD^+ and phosphate (accession number gi 238537990). Probability of this match occurring at random: 10^{-80}. Expect-value: 1.1e–075.

[b] observed mass of the neutral fragment (m/z obs. minus proton mass (1.0073))

[c] calculated mass of the peptide sequence

[d] proportional difference between the calculated and observed mass (M_r obs. minus M_r calc. divided by M_r obs.)

[e] observed peptide sequence bracketed by the adjacent amino acid residues found in the matching peptide sequence of the matching protein

[f] amino acid residue locations in the matching protein that match the observed peptide sequence, residue numbers correspond to the recombinant Hu his-GAPDHS sequence (Fig. 2)

[g] indicates a missed trypsin cleavage site present in the peptide sequence

Table 2

Edman sequencing of tryptic peptides from recombinant human his-GAPDHS

peptide sequence	sequence location[a]
ELTVGI	37–43
VVAVNDP	63–69
AVGSPYV	122–128
VVISAPS	152–158
FGIV?[b] GL	202–208
VIPEL	274–278
VPTPDVS	269–275
LAQPA?Y	283–289
GPMAGIL	302–308
AGIALND	333–339
VVDLLR	358–363
YMFSR	374–378

[a] residue numbers correspond to the recombinant human his-GAPDHS sequence (Fig. 2)

[b] ? question mark is used as a place holder to indicate insufficient resolution in the data to identify the amino acid residue at this location in the sequence.

Table 3

Summary of peptide mapping results for recombinant Hu GAPDHS. Shaded regions correspond to those peptides detected by MS/MS analysis of the trypsin digested protein that match human GAPDHS (see Table 1). Underlined regions show peptides detected by Edman sequencing (see Table 2). Trypsin cleaved sites (R and K) are shown italicized. Amino acid sequence for the FastBac hexahistidine purification tag was excluded to aid data interpretation.

31	M	V	S	V	A	R	E	L	T	V	G	I	N	G	F	G	R	I	G	R	L	V	L	R	A	C	M	E	K	G	60
61	V	*K*	V	V	A	V	N	D	P	F	I	D	P	E	Y	M	V	Y	M	F	*K*	Y	D	S	T	H	G	*R*	Y	*K*	90
91	G	S	V	E	F	*R*	N	G	O	Q	L	V	V	D	N	H	E	I	S	V	Q	C	*K*	E	P	K	Q	I	P	W	120
121	*R*	A	V	G	S	P	Y	V	V	E	S	T	G	V	Y	L	S	I	Q	A	A	S	D	H	I	S	A	G	A	Q	150
151	*R*	V	V	I	S	A	P	S	P	D	A	P	M	F	V	M	G	V	N	E	N	D	Y	N	P	G	S	M	N	I	180
181	V	S	N	A	S	C	T	T	N	C	L	A	P	L	A	K	V	I	H	E	*R*	F	G	I	V	E	G	L	M	T	210
211	T	V	H	S	Y	T	A	T	Q	*K*	T	V	D	G	P	S	*R*	K	A	W	R	D	G	R	G	A	H	Q	N	I	240
241	I	P	A	S	T	G	A	A	K	A	V	T	*K*	V	I	P	E	L	K	G	*K*	L	T	G	M	A	F	*R*	V	P	270
271	T	P	D	V	S	V	V	D	L	T	C	*R*	L	A	Q	P	A	P	Y	S	A	I	*K*	E	A	V	*K*	A	A	A	300
301	*K*	G	P	M	A	G	I	L	A	Y	T	E	D	E	V	V	S	T	D	F	L	G	D	T	H	S	S	I	F	D	330
331	A	*K*	A	G	I	A	L	N	D	N	F	V	*K*	L	I	S	W	Y	D	N	E	Y	G	Y	S	H	*R*	V	V	D	360
361	L	L	*R*	Y	M	F	S	*R*	D	K																					370