Published in final edited form as:

Protein Expr Purif. 2007 July ; 54(1): 126–133.

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF BOVINE DEOXYHYPUSINE HYDROXYLASE cDNA AND HOMOLOGS

Jenq-Kuen Huang¹, Yalun Cui¹, Chieh-Hua Chen¹, Denae Clampitt¹, Chi-Tsai Lin², and Lisa Wen^{1,*}

1 Department of Chemistry, Western Illinois University, 1 University Circle, Macomb, IL 61455-1390

2 Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan 202

Abstract

Deoxyhypusine hydroxylase is the second of the two enzymes that catalyzes the maturation of eukaryotic initiation factor 5A (eIF5A). The mature eIF5A is the only known protein in eukaryotic cells that contains the unusual amino acid hypusine (N^ε-(4-amino-2(R)-hydroxybutyl)lysine). Synthesis of hypusine is essential for the function of eIF5A in eukaryotic cell proliferation and survival. Here we describe the cloning and characterization of bovine deoxyhypusine hydroxylase cDNA and its homologs. The deduced bovine deoxyhypusine hydroxylase protein is 87% identical to human enzyme and 45% identical to yeast enzyme. The overexpressed enzyme showed activity in catalyzing the hydroxylation of the deoxyhypusine residue in the eIF5A intermediate. An amino acid substitution from Glu 57 to Gly located at one of the four conserved His-Glu (HE) pairs, the potential metal coordination sites, resulted in severe reduction of deoxyhypusine hydroxylase activity. A deletion at the HEAT-repeats 1–3 resulted in complete losses of deoxyhypusine hydroxylase activity.

Keywords

deoxyhypusine hydroxylase; eIF5A; hypusine; HEAT-repeat-containing; cDNA cloning

INTRODUCTION

Eukaryotic initiation factor 5A (eIF5A) is a protein found in all eukaryotic cells and archaea [for reviews see 1–4] and is the only known protein that contains the unusual amino acid hypusine (N^ε-(4-amino-2(R)-hydroxybutyl)lysine). The synthesis of hypusine within eIF5A is a two-step posttranslational modification. The first step is catalyzed by deoxyhypusine synthase (DHS, EC 2.5.1.46) which mediates the NAD-dependent transfer of the butylamino moiety of spermidine to the ε-amino group of a specific lysine residue (lys50 in human), generating deoxyhypusine. The second step involves the hydroxylation of carbon 9 by deoxyhypusine hydroxylase (DOHH, EC 1.14.99.29), generating hypusine (Fig. 1). Hypusine

*Corresponding author: Dr. Lisa Wen, One University Circle, Department of Chemistry, Western Illinois University, Macomb, IL 61455, Tel.: 309-298-1667, Fax: 309-298-2180, E-mail: L-wen@wiu.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

is produced soon after the translation of eIF5A mRNA. This modification is essential for the function of eIF5A in cell proliferation and survival.

High concentrations of eIF5A have been found in tumor cells [5,6], and it also acts as a cofactor of the Rev transactivator protein in HIV-1-infected cells [7,8]. It has been suggested that posttranslational modifications of eIF5A could be a suitable target for anticancer therapy [9, 10] and antiviral therapy [11,12]. In mammalian cells, inhibitors of either DHS, e.g., spermidine analogs [12–16] or DOHH, e.g., metal chelating inhibitors [17–19] have been shown to have antiproliferative effects. A detailed knowledge of the structure and properties of DHS or DOHH should contribute to creating more specific inhibitors, which in turn may be used to better control hyperproliferative diseases. The crystal structure of human DHS (20) and the predicted structure of hDOHH (21) were reported. However, the x-ray crystallography of DOHH remains to be done.

Many studies have been done on the structural and functional aspects of eIF5A and DHS. cDNAs for eIF5A have been cloned from humans [22], bovine [23], chick embryo [24], tomato [25], lepidopteran insect [26], *Neurospora* [27], and its gene in human [28]. cDNAs for DHS have been cloned from humans [29,30], bovine [23], tobacco [31], *Neurospora crassa* [32], and its gene in yeast [33]. Very recently, yeast DOHH gene has been cloned and the homolog of human DOHH (hDOHH) gene identified [21]. The recombinant enzyme was characterized as an Fe(II)-dependent HEAT-repeat metalloenzyme [21,34]. HEAT-repeat-containing proteins named for human huntingtin (**H**), elongation factor 3 (**E**), a subunit of protein phosphatase 2A (**A**), and the target of rapamycin (**TOR**), commonly mediate protein-protein interactions [35]. They are involved in nucleocytoplasmic transport, vacuolar transport, and regulation of cytoskeletal organization.

Our laboratory has been interested in the eIF5A modifying enzymes from bovine. We have previously reported cloning and characterization of bovine eIF5A and DHS [23]. We reported that bovine DHS possesses considerably higher activity than the human enzyme in catalyzing the synthesis of eIF5A intermediate [36]. Our previous efforts in partial purification of bovine testis DOHH by an adenosine-5'-monophosphate (5'-AMP)- Sepharose affinity chromatography revealed multiple forms of DOHH [37]. In an effort toward understanding different forms of bovine DOHH (bDOHH), we started to clone and analyze bDOHH at the cDNA level. We report molecular cloning and characterization of bDOHH cDNA and non-enzymatic HEAT-repeat-containing clones.

MATERIALS AND METHODS

Materials

E. coli strains, JM109 was used as hosts. Terrific broth (1.2% Bacto tryptone, 2.4% yeast extract, 1.6% glycerol, 72 mM K₂HPO₄, and 17 mM KH₂PO₄, pH 7.2) (38) or LB broth supplemented with 100 g/mL ampicillin was used to culture selected clones. PCR ready first-strand bovine brain cDNA was from BioChain Institute. FidelityTaq™ PCR master mix was from USB Corporation. Alkaline phosphatase calf intestine and *Bam*HI were from Promega. Oligonucleotides were synthesized by Integrated DNA Technology. [¹⁴C]spermidine (N-(3-aminopropyl)-[1,4-¹⁴C]tetramethylene-1,4-diamine) (112 mCi/mmol), [α -³²P]dCTP (~ 6000 Ci/mmol), and pGEX 5' sequencing primer were from Amersham Biosciences. Glutathione (reduced form), glutathione-agarose, human plasma thrombin, and dimedone were from Sigma-Aldrich. The hDOHH DNA clone (MGC-4293) was from American Type Culture Collection. T4 DNA ligase and NEBlot kit were from New England Biolabs.

Amplification of bDOHH cDNA and homologs by PCR and Southern Blotting

The PCR mixture consisted of 1 L of the PCR ready first-strand bovine brain cDNA template, 25 M each of the gene-specific forward (5' ATGGTGACGGAGCAGGAAGTG) and reverse (5' CTAGGAGAGGGGTGAGCGCA) primers, and FidelityTM PCR master mix in a total volume of 25 L. The gradient PCR program consisted of pre-denaturation at 94°C for 3 min; 35 cycles of denaturation (94°C, 45 sec), annealing (50°C ± 10°C, 45 sec), and primer extension (68°C, 2 min); and a final 10 min primer extension at 68°C. An aliquot of each PCR product was analyzed on a 1% agarose gel. A Southern blot was prepared from the gel by capillary transfer [38] to a sheet of Zeta nylon membrane (Bio-Rad) and probed with the ³²P-dCTP-labeled hDOHH DNA. The hDOHH DNA was amplified by PCR from the MGC-4293 clone using two gene specific primers [21], and the desired fragment gel purified. The probe was prepared using a NEBlot kit which uses random sequence 8-mers as primers for *in vitro* DNA synthesis by the Klenow DNA polymerase fragment with the purified hDOHH cDNA insert as template. Hybridization was carried out for 16 hours at 50°C in a solution containing 50% formamide, 2X SSC (300 mM sodium chloride, 30 mM sodium citrate), 10% dextran sulfate, 1% SDS, 0.05 mg/mL heat-denatured sheared herring sperm DNA, and approximately 1x10⁶ cpm/mL of ³²P-dCTP-labeled hDOHH DNA probe. The posthybridization wash was done in 2X SSC/1% SDS 3 times at room temperature and then washed once at 50°C in 0.1X SSC/1% SDS. The wet membrane under Saran Wrap was exposed to Kodak Biomax MR-1 autoradiography film at -70 °C.

Cloning of bDOHH cDNA and homologs

The PCR was repeated using the same gene specific primers except that *Bam*HI site was appended to the 5' end of each primer to facilitate cloning. The PCR products were extracted with phenol/chloroform, chloroform/isoamylalcohol, and precipitated with ethanol [38]. The sample was digested with *Bam*HI. The digested products were separated on a 1% agarose gel and the desired bands were sliced out of agarose and gel purified, and then cloned into pGEX-2T (cut with *Bam*HI and dephosphorylated) and transformed into JM109 competent cells. The transformants were selected by ampicillin followed by Quick Screening as described before [23]. The selected recombinant plasmids were isolated using rapid boiling method [38] with slight modification. Briefly, cultured cell pellet was resuspended in 300 L of STET/lysozyme [8% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% (v/v) Triton X-100, and 200 g lysozyme]. The suspension was heated at 100°C for 2 min, cooled at 4°C for 5 min, and then centrifuged at 14,000x g for 15 min. Plasmids in the supernatant were precipitated by addition of 1 volume of isopropanol. The tube was incubated at -20°C for 5 min and then centrifuged at 14,000x g for 10 min at 4°C. The pellet was rinsed once with 70% ethanol, air-dried, and resuspended in 50 L of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA,). The orientation of the insert in the recombinant plasmids was checked by PCR using the pGEX 5' sequencing primer and the gene specific reverse primer. PCR products should only obtain from clones harboring an insert in the correct orientation. DNA sequence of both strands of selected clones was then determined by Laragen Inc. The DNA sequence was translated using the translate tool at the ExPASy (Expert Protein Analysis System) proteomics server (<http://www.expasy.ch/>). The identity of the cDNA clones were verified by comparing the DNA sequence and the inferred amino acid sequence in various nucleic acid and protein data banks using the BLAST(Basic Local Alignment Search Tool).

Overexpression and purification of bDOHH protein and homologs

Selected bDOHH homolog cDNA were expressed in *E. coli* strain JM109. Individual clone was grown in 100 mL terrific broth containing ampicillin (100 g/mL) at 37°C to A₆₀₀ of 0.5 to 0.6 before addition of IPTG to a final concentration of 0.5 mM. Cells were harvested 4 hours after IPTG induction. The individual recombinant GST fusion protein was purified using a

Glutathione Agarose Affinity column (2 mL) according to the instruction manual (Sigma). The GST carrier was cleaved by digestion with thrombin. The free glutathione was removed from the digestion product by ultrafiltration with several washes using PBS. The GST carrier was then removed by Glutathione Agarose affinity column chromatography.

Overexpression and purification of hDOHH protein

The hDOHH DNA was amplified by PCR from the MGC-4293 clone using two gene specific primers appended with *Bam*HI recognition sequence [21]. The desired fragment was gel purified and subcloned into pGEX2T. Overexpression and purification of hDOHH protein were done using the same procedures for the recombinant bovine proteins as described above.

DOHH Activity assay

DOHH substrate, eIF5A([¹⁴C]Dhp) (deoxyhypusine form of eIF5A), was prepared using functional recombinant bovine DHS which is more active (>20 fold) than the human DHS [36]. The DOHH activity of the cell-free extract of selected bovine cDNA clones or of the purified recombinant proteins (with GST tag removed) was carried out according to Csonga *et al* [39] with some modifications as described by Burgess [37]. Each reaction included 5–20 L of the cell-free extract (20–80 g protein) or 5 g of purified recombinant bovine or human proteins, 50 mM sodium phosphate buffer pH 7.4, 1 mM NAD, 1 mM DTT, and approximately 20,000 dpm eIF5A([¹⁴C]Dhp) in a total volume of 600 L. The reaction was carried out at 37° C for 3 hours. DOHH converted eIF5A([¹⁴C]Dhp) to eIF5A([¹⁴C]Hpu) (hypusine form of eIF5A). Once eIF5A([¹⁴C]Hpu) was formed, the oxidative cleavage of the hypusyl residue yielded eIF5A(Lys), formaldehyde, and β-aminopropionaldehyde. Following oxidative cleavage, the radioactively labeled aldehydes were converted with dimedone into toluene-soluble compounds, and extracted into the organic phase [39]. The radioactivity was measured using a liquid scintillation counter (Packard Tri-Carb 2900 RT).

RESULTS AND DISCUSSION

A. Isolation and characterization of cDNA clones encoding bDOHH and its homologs

We initially screened the bovine testis cDNA library that was used previously to isolate bovine eIF5A and DHS cDNA [23] for DOHH clones using hDOHH cDNA as a probe. However, the process was unsuccessful as several false positive clones were isolated. We then used the published hDOHH protein sequence (accession no NP_112594) to search the non-redundant database using BLAST. A predicted *Bos taurus* (bovine) protein contains similar to HEAT-like (PBS lyase) repeat (accession # XP_604893) was retrieved which showed 80% identity to the human protein. The PBS stands for phycobilisomes which are macromolecular complexes involved in harvesting light energy by Cyanobacteria and red algae (40). The lyase enzymes (41) are components of the complexes that contain a short bi-helical repeat related to HEAT-repeat proteins.

Two gene-specific primers were synthesized according to the end sequences of bovine HEAT-like (PBS lyase) repeat (accession # XP_604893) to amplify the coding sequence in a pool of bovine brain cDNA. Fig. 2A shows the PCR amplified fragments using bovine brain cDNA as template. A major fragment of approximate 900 bp was amplified when the annealing temperature was set at 54.4°C or 59.7 °C. No PCR product was detected by ethidium bromide staining at the annealing temperature of 49.3°C. The amplified fragment (~900 bp) showed strong hybridization with hDOHH cDNA probe (Fig. 2B), suggesting that the amplified fragment is DOHH homolog. It is interesting to note that two distinct hybridizing fragments (~900 bp and ~600 bp) were detected in the lane where PCR was carried out at 49.3 °C (bands were not detectable by ethidium bromide staining). The results suggest the possible presence

of different forms of bDOHH cDNAs or homologs differing in size. The existence of multiple forms of bovine testis DOHH protein was discovered recently in our lab [37].

The PCR fragments were gel purified and amplified again using gene specific primers with *Bam*HI recognition sequence appended. The fragments were subcloned into pGEX-2T. Four clones #20, #20N, #36, and #68 (please note that clones 20 and 20N are two entirely different clones) appeared to contain DNA insert in the correct orientation were sequenced. DNA sequencing results showed that clones #20N (GenBank accession no. [DQ990881](#)), 36 (GenBank accession no. [DQ990882](#)), and 68 (GenBank accession no. [DQ990883](#)) (all are 912 bp in length) contain nearly identical insert sequences; clone #20 (GenBank accession no. [DQ990884](#)) is 657 bp in length. Fig. 3 shows the nucleotide sequence of clone 20N and its deduced amino acid sequence as well as the differences with clones 36 and 68. The deduced protein consists of 303 amino acid residues with a predicted molecular mass of 33 kDa and pI of 4.87 (clone 20N or 36) or 4.91 (clone 68).

Comparison the nucleotide sequence of clone #20N with that of hDOHH cDNA in the coding region (912bp vs. 909 bp) showed 86 % identity, arising from a total of 131 nucleotide substitutions and 3 nucleotides insertion (alignment not shown). The deduced amino acid sequences shared 87% identity, arising from 36 amino acid substitutions and one insertion (Fig. 4). DOHH has recently been shown to be highly conserved in all eukaryotes from fungi to human [21]. The deduced amino acid sequences of bovine clone #20N shared 45% identity with the yeast enzyme.

A search of homologous sequence to the deduced amino acid sequence of clone #20N in the non-redundant database, we found a *Bos taurus* (bovine) protein of unknown function (accession no. AAI20352) has the highest score sharing >99% identity, with only one amino acid substitution (Ser 266 to Pro). The definition of the sequence of AAI20352 was subsequently updated from protein of unknown function to similar to HEAT-like (PBS lyase) repeat containing. Fig. 4 shows the alignment of the deduced amino acid sequence of putative bovine HEAT-repeat containing clones #20, #20N, bovine protein of similar to HEAT-like (bHEAT like, accession no. AAI20352), human DOHH (bDOHH), and yeast DOHH (yDOHH). Clone #20 is missing a stretch of 85 amino acid residues at positions 12–96 when aligned with that of clone #20N. The overlapping regions have nearly identical sequences except for the F289 in clone 20N is changed to I204 in clone 20. The different length clones may have arisen from alternative splicing. It is very likely that the different size clones represent the two hybridizing fragments seen in Fig. 2B.

Park *et al* [21] constructed a 3-D homology model of hDOHH using the available crystal structure of a HEAT-repeat protein (the *E. coli* protein YibA (PDB: 1OYZ)) as a template. The model shows a structure consisting of eight HEAT-repeats in a symmetrical dyad of four HEAT motifs (a pair of α -helices). The eight HEAT-repeats are indicated in Fig. 4. The four His-Glu (HE) pairs (boxed), the two potential metal coordination sites, were strictly conserved.

B. Expression, purification, and functional assay of recombinant bDOHH, and homologs

We overexpressed GST fusion proteins of clones 20, 20N and 68 under the same cultivation conditions. After induction with IPTG, high level expression of a 62 kDa GST fusion protein in bovine clones 20N and 68 (Fig. 5A shows the expression of clone 20N only); and a high level expression of 51 kDa GST-fusion protein in clone 20 (Fig. 5B) were observed in JM109 cells. In another preparation, we overexpressed and purified the GST fusion proteins and the corresponding non-fusion proteins with GST carriers removed from bovine clones 20, 20N, 68, and hDOHH. The GST fusion proteins and non-fusion proteins were purified to near homogeneity as seen in Fig. 5C.

The recombinant proteins were tested for DOHH activity. *E. coli* cell-free extract of clone 20N expressing the bovine DOHH homolog gene displayed strong DOHH activity, confirming that clone 20N indeed encodes DOHH enzyme (Fig. 6). The increase in the amount of GST-DOHH protein in IPTG-induced cells correlated with the increase in DOHH enzyme activity (Fig. 6). The purified protein of clone 20N also showed strong DOHH activity; compatible with human DOHH. Clone 68 differs from clone 20N at one amino acid substitution at Glu 57 -->Gly (one of the His-Glu pair); very little DOHH activity was detected in crude proteins or purified protein of clone 68. The results confirmed the importance of His-Glu pairs for DOHH activity [21, 34]. The clone 20 encodes is a smaller protein (51-kDa protein with 218 amino acid residues) missing HEAT-repeat motifs 1–3 (including the first two His-Glu pairs). Such a deletion resulted in complete loss of DOHH activity (Fig. 6). It is possible that the expressed protein of clone 20 plays a role in protein-protein interactions. It has been reported that the majority of HEAT-repeat domains known to date are noncatalytic proteins with roles in protein-protein interactions [21,35]. The further investigations of the presence of multiple forms of DOHH by Northern blotting and Western blotting are underway.

As stated in the Introduction, the synthesis of hypusine, and therefore of mature eIF5A, is important for its biological activity. In view of the importance of hypusine in cell proliferation and HIV-1 replication, its occurrence in a single protein, and the specificities of the enzymes involved in its biosynthesis, both DHS and DOHH are regarded as potential targets for antiproliferative and antiretroviral therapy. Therefore, a detailed knowledge of the structure and properties of DHS and DOHH should contribute to achieving specific inhibitors of these enzymes, and perhaps the control of hyperproliferative diseases. The crystal structures of the human DHS complex with its cofactor NAD⁺ or with inhibitor has been determined [20,42]. However, the x-ray crystal structure of DOHH is not yet available, although a 3-D model of hDOHH has been reported. The availability of the cDNA and functional recombinant protein of bDOHH will allow studies of structure-function analysis, interactions with substrate, and drug design of specific inhibitors.

Acknowledgements

This work was supported by grants from National Institutes of Health 1R15 GM60266-01A1, and University Research Council at Western Illinois University.

References

1. Park MH, Colff E, Folk JE. Is hypusine essential for eukaryotic cell proliferation? Trends Biochem Sci 1993;18:475–479. [PubMed: 8108861]
2. Park MH, Lee YB, Joe YA. Hypusine is essential for eukaryotic cell proliferation. Biol Signals 1997;6:115–123. [PubMed: 9285094]
3. Chen KY, Liu AYC. Hypusine formation on eukaryotic initiation factor 5A: biochemistry and function. Biol Signals 1997;6:104–165.
4. Park MH. The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem 2006;139:161–169. [PubMed: 16452303]
5. Chen ZP, Chen KY. Marked elevation of hypusine formation activity on eukaryotic initiation factor 5A in v-HA-RAS transformed mouse NIH3T3 cells. Cancer Lett 1997;115:235–241. [PubMed: 9149130]
6. Caraglia M, Passeggio A, Beninati S, Leardi A, Nicolini L, Improta S, Pinto A, Bianco AR, Tagliaferri P, Abbruzzese A. Interferon α 2 recombinant and epidermal growth factor modulate proliferation and hypusine synthesis in human epidermoid cancer KB cells. Biochem J 1997;324:737–741. [PubMed: 9210396]

7. Bevec D, Jaksche H, Oft M, Wöhl T, Himmelsbach M, Pacher A, Schebesta M, Koettnitz K, Dobrovnik M, Csonga R, Lottspeich F, Hauber J. Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF5A. *Science* 1996;271:1858–1860. [PubMed: 8596953]
8. Rosorius O, Reichart B, ratzer F, Heger P, Dabauvalle MC, Hauber J. Nuclear pore localization and nucleocytoplasmic transport of eIF-5A: evidence for direct interaction with the export receptor CRM1. *J Cell Sci* 1999;112(Pt 14):2369–2380. [PubMed: 10381392]
9. Caraglia M, Tagliaferri P, Budillon A, Abbruzzese A. Post-translational modifications of eukaryotic initiation factor-5A (eIF-5A) as a new target for anti-cancer therapy. *Adv Exp Med Biol* 1999;472:187–198. [PubMed: 10736626]
10. Caraglia M, Marra M, Giuberti G, D'Alessandro AM, Baldi A, Tassone P, Venuta S, Tagliaferri P, Abbruzzese A. The eukaryotic initiation factor 5A is involved in the regulation of proliferation and apoptosis induced by interferon- α and EGF in human cancer cells. *J Biochem* 2003;133:757–765. [PubMed: 12869532]
11. Junker U, Bevec D, Barske C, Kalfoglou C, Escaich S, Dobrovnik M, Hauber J, Bohnlein E. Intercellular expression of cellular eIF5A mutants inhibits HIV-1 replication in human T cells: a feasibility study. *Hum Gene Ther* 1996;15:1861–1869. [PubMed: 8894678]
12. Hauber I, Bevec D, Heukeshoven J, Krätzer F, Choidas A, Harrer T, Hauber J. Identification of cellular deoxyhypusine synthase as a novel target for antiretroviral therapy. *J Clin Invest* 2005;115:76–85. [PubMed: 15630446]
13. Jakus J, Wolff EC, Park MH, Folk JE. Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. Effective inhibition by bis- and mono-guanylated diamines and polyamines. *J Biol Chem* 1993;268:13151–13159. [PubMed: 8514754]
14. Park MH, Wolff EC, Lee YB, Folk JE. Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines. *J Biol Chem* 1994;269:27827–27832. [PubMed: 7961711]
15. Lee YB, Park MH, Folk JE. Diamine and triamine analogs and derivatives as inhibitors of deoxyhypusine synthase: synthesis and biological activity. *J Med Chem* 1995;38:3053–3061. [PubMed: 7636868]
16. Lu J, Chen ZP, Yan YP, Knapp S, Schugar H, Chen KY. Aminohexanoic hydroxamate is a potent inducer of the differentiation of mouse neuroblastoma cells. *Cancer Letters* 2000;160:59–66. [PubMed: 11098085]
17. McCaffrey TA, Pomerantz KB, Sanborn TA, Spokojny AM, Du B, Park MH, Folk JE, Lamberg A, Kivirikko KI, Falcone DJ, Mehta SB, Hanauske-Abel HM. Specific inhibition of eIF5A and collagen hydroxylation by a single agent. Antiproliferative and fibrosuppressive effects on smooth muscle cells from human coronary arteries. *J Clin Invest* 1995;95:446–455. [PubMed: 7860726]
18. Hanauske-Abel HM, Park MH, Hanauske AR, Popowicz AM, Lalande M, Folk JE. Inhibition of the G1-S transition of the cell cycle by inhibitors of deoxyhypusine hydroxylation. *Biochim Biophys Acta* 1994;1221:115–124. [PubMed: 8148388]
19. Andrus L, Szabo P, Grady RW, Hanauske AR, Huima-Byron T, Slowinska B, Zagulska S, Hanauske-Abel HM. Antiretroviral effects of deoxyhypusyl hydroxylase inhibitors, a hypusine-dependent host cell mechanism for replication of human immunodeficiency virus type 1 (HIV-1). *Biochem Pharmacol* 1998;55:1807–1818. [PubMed: 9714299]
20. Umland TC, Wolff EC, Park MH, Davies DR. A new crystal structure of deoxyhypusine synthase reveals the configuration of the active enzyme and of an enzyme.NAD. inhibitor ternary complex. *J Biol Chem* 2004;279:28697–28705. [PubMed: 15100216]
21. Park JH, Aravind L, Wolff EC, Kaevel J, Kim YS, Park MH. Molecular cloning, expression, and structural prediction of DOHH: A HEAT-repeat-containing metalloenzyme. *Proc Natl Acad Sci USA* 2006;103:51–56. [PubMed: 16371467]
22. Smit-McBride Z, Dever TE, Hershey JW, Merrick WC. Sequence determination and cDNA cloning of eukaryotic initiation factor 4D, the hypusine-containing protein. *J Biol Chem* 1989;264:1578–1583. [PubMed: 2492279]
23. Huang JK, Tsai S, Huang GH, Sershon VC, Alley AM, Wen L. Molecular cloning of bovine eIF5A and deoxyhypusine synthase cDNA. *DNA Sequence* 2004;15:26–32. [PubMed: 15354351]

24. Rinaudo MS, Joe YA, Park MH. Cloning and sequencing of a chick embryo c DNA encoding the 20-kDa hypusine-containing protein, eIF-5A. *Gene* 1993;137:303–307. [PubMed: 7916728]
25. Wang TW, Lu L, Wang D, Thompson JE. Isolation and characterization of senescence-induced cDNAs encoding deoxyhypusine synthase and eucaryotic translation initiation factor 5A from tomato. *J Biol Chem* 2001;276:17541–17549. [PubMed: 11278418]
26. van Oers MM, van Marwijk M, Kwa MS, Vlak JM, Thomas AA. Cloning and analysis of cDNAs encoding the hypusine-containing protein eIF5A of two lepidopteran insect species. *Insect Mol Biol* 1999;8:531–538. [PubMed: 10620048]
27. Tao Y, Chen KY. PCR-based cloning of the full-length *neurospora* eukaryotic initiation factor 5A cDNA: polyhistidine-tagging and overexpression for protein affinity binding. *Biochem J* 1994;302(Pt 2):517–525. [PubMed: 8093005]
28. Koettnitz K, Kappel B, Baumruker T, Hauber J, Bevec D. The genomic structure encoding human initiation factor eIF-5A. *Gene* 1994;144:249–252. [PubMed: 7545941]
29. Joe YA, Wolff EC, Park MH. Cloning and expression of human deoxyhypusine synthase cDNA. Structure-function studies with the recombinant enzyme and mutant proteins. *J Biol Chem* 1995;270:22386–22392. [PubMed: 7673224]
30. Tao Y, Chen KY. Molecular cloning and functional expression of human deoxyhypusine synthase cDNA based on expressed sequence tag information. *Biochem J* 1996;315:429–434. [PubMed: 8615810]
31. Ober D, Hartmann T. Deoxyhypusine synthase from tobacco. cDNA isolation, characterization, and bacterial expression of an enzyme with extended substrate specificity. *J Biol Chem* 1999;274:32040–32047. [PubMed: 10542236]
32. Tao Y, Chen KY. Molecular cloning and functional expression of *Neurospora* deoxyhypusine synthase cDNA and identification of yeast deoxyhypusine synthase cDNA. *J Biol Chem* 1995;270:23984–23987. [PubMed: 7592594]
33. Kang KR, Wolff EC, Park MH, Folk JE, Chung SI. Identification of YHR068w in *Saccharomyces cerevisiae* chromosome VIII as a gene for deoxyhypusine synthase. Expression and characterization of the enzyme. *J Biol Chem* 1995;270:18408–18412. [PubMed: 7629166]
34. Kim YS, Kang KR, Wolff EC, Bell JK, McPhie P, Park MH. Deoxyhypusine hydroxylase is an Fe (II)-dependent, HEAT-repeat enzyme, identification of amino acid residues critical for Fe(II) binding and catalysis. *J Biol Chem* 2006;281:13217–13225. [PubMed: 16533814]
35. Andrade MA, Petosa C, O'Donoghue SI, Muller CW, Bork P. Comparison of ARM and HEAT protein repeats. *J Mol Biol* 2001;309:1–18. [PubMed: 11491282]
36. Huang JK, Tsai S, Huang GH, Gowda PG, Walzer AM, Wen L. Higher activity of recombinant bovine deoxyhypusine synthase vs. human deoxyhypusine synthase. *Protein Expr Purif* 2004;35:32–38. [PubMed: 15039063]
37. Burgess, ME. Partial purification and characterization of bovine deoxyhypusine hydroxylase, MS thesis. Western Illinois University; Apr. 2006
38. Sambrook, J.; Russell, DW. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 2001.
39. Csonga R, Ettmayer P, Auer M, Eckerskorn C, Eder J, Klier H. Evaluation of the metal ion requirement of the human deoxyhypusine hydroxylase from HeLa cells using a novel enzyme assay. *FEBS Lett* 1996;380:209–214. [PubMed: 8601426]
40. Fairchild C, Glazer A. Oligomeric structure, enzyme kinetics, and substrate specificity of the phycocyanin alpha subunit phycocyanobilin lyase. *J Biol Chem* 1994;269:8686–8694. [PubMed: 8132596]
41. Zhao KH, Deng MG, Zheng M, Zhou M, Parbel A, Storf M, Meyer M, Strohmam B, Scheer H. Novel activity of a phycobiliprotein lyase: both the attachment of phycocyanobilin and the isomerization to phycoviolobilin are catalyzed by the proteins PecE and PecF encoded by the phycoerythrocyanin operon. *FEBS Lett* 2000;469:9–13. [PubMed: 10708746]
42. Liao DI, Wolff EC, Park MH, Davies DR. Crystal structure of the NAD complex of human deoxyhypusine synthase: an enzyme with a ball and chain mechanism for blocking the active site. *Structure* 1998;6:23–32. [PubMed: 9493264]

Fig 1.
Posttranslational modification of eIF5A.

Fig 2.

Analysis of bovine DOHH homologous sequences amplified by PCR. Panel A. Agarose gel (1%) of PCR products. Lanes from left to right: 200 bp DNA ladder; PCR positive control using β -actin primers and bovine brain cDNA template; PCR using gene-specific primers and bovine brain cDNA template, annealing temperature at 49.3°C, 54.4°C, or 59.7°C. Panel B. Southern blot of the gel in panel A. The probe used was [32 P]-hDOHH DNA.

ATGGTGACGGAGCAGGAAGTGGAGGCAGTGGGGCAGACGCTGGTTGACCCCGGGCAGCCC 60
 M V T E Q E V E A V G Q T L V D P G Q P 20
 CTGCAGGCCCCGCTTCCGGGCACCTGTTCACTGCGTGGGCTCGGGGGTCCAGTGGCCATC 120
 L Q A R F R A L F T L R G L G G P V A I 40
 TCCTGGATCAGCCGGGCTTCGATGATGACTCAGCCCTGCTCAAGCATGAGCTGGCCTAC 180
 S W I S R A F D D D S A L L K H E L A Y 60
 (G) --#68
 TGCCTGGGGCCAAATGCAGGACCGACGGGCCATCCCCGTGCTGCTGGACGTGCTGCGGGAC 240
 C L G Q M Q D R R A I P V L L D V L R D 80
 ACCCGCCAGGAGCCCATGGTGCGCCATGAGGCAGGGGAGGCCCTGGGGGCCATTGGGGAC 300
 T R Q E P M V R H E A G E A L G A I G D 100
 CCAGAGGTGCTGGAGATCCTGAAGCAGTACTCTACTGACCCTGTTGTTGAGGTGGCTGAG 360
 P E V L E I L K Q Y S T D P V V E V A E 120
 ACATGCCAGCTGGCTGTCCGGCGGCTGGAGTGGCTGCAGCAGCATGGTGGAGAGTCAGCG 420
 T C Q L A V R R L E W L Q Q H G G E S A 140
 GTCCGGGGGCCCTACCTCTCTGTGGACCCAGCCCCGCGCTGAGGAGCGCGACCTGGGG 480
 V R G P Y L S V D P A P P A E E R D L G 160
 CAGCTGCGGGAGGCACTACTGGACGAGGCCCGGCCGCTCTTCGACCGATACCGAGCCATG 540
 Q L R E A L L D E A R P L F D R Y R A M 180
 TTTGCCCTGCGCGATGCTGGAGGCAAGGAGGCTGCCCTGGCGCTGGCCGAAGGCCTGCGC 600
 F A L R D A G G K E A A L A L A E G L R 200
 TGCGGCAGTGCCCTCTTCCGCCATGAGATCGGCTACGTTCTGGGCCAGATGCAGCACGAG 660
 C G S A L F R H E I G Y V L G Q M Q H E 220
 GCGGCAGTGCCCCAACTGGCGGCAGCCCTGGCGCAGCCCACTGAGAACCCCATGGTACGA 720
 A A V P Q L A A A L A Q P T E N P M V R 240
 CACGAGTGTGCCGAGGCCCTGGGGGCCATCGCCCGGCTGCCTGCCTGGCCGCCCTGCGG 780
 H E C A E A L G A I A R P A C L A A L R 260
 GCCCACGTGGCCGACTCTGAGCGTGTGGTGGCGGAGAGCTGCGAGGTGGCCCTGGACATG 840
 A H V A D S E R V V R E S C E V A L D M 280
 TACGAGTATGAGACGGGGTCGACCTTCCAATATGCAGACGGGCTGGAGCGGCTGCGCTCA 900
 Y E Y E T G S T F Q Y A D G L E R L R S 300
 CCCCTCTCCTAG 912
 P L S < 303

Fig 3.

Nucleotide and deduced amino acid sequences of bovine DOHH clone 20N (GenBank accession no. [DQ990881](#)) and clone 36 (GenBank accession no. [DQ990882](#)), and clone 68 (GenBank accession no. [DQ990883](#)). Clone 20N and clone 36 are different in one nucleotide substitution, but the amino acid sequences are identical. Clones 20N differ from clone 68 in one amino acid substitution (Glu57 --> Gly) caused by a one nucleotide difference.

Fig 4.

Alignment of the deduced amino acid sequence of putative bovine DOHH clones 20, 20N, predicted *Bos taurus* protein contains similar to HEAT-like (PBS lyase) repeat (bHEAT-like) (accession no. AAI20352), the hDOHH (accession no. NP_112594), and yeast DOHH (yDOHH) (accession no. NP_012604). The eight HEAT- repeats are indicated and the conserved HE (His Glu) pairs are boxed. (*) Indicated identical amino acids in all sequences. (:) Indicated conservative replacements.

Fig 5.

Expression and purification of bovine HEAT-repeat containing proteins and hDOHH. The HEAT-repeat-containing proteins were expressed in JM109 cells as described in Materials and Methods. (A) 10% SDS-PAGE of proteins from clone 20N. Lane 1: size marker, lane 2: cell lysate from uninduced cells (~15 g proteins), lane 3: cell lysate from IPTG-induced cells (~33 g proteins), lane 4: cell-free extract from IPTG-induced cells (~7 g proteins), lane 5: purified GST fusion protein. (B) 10% SDS-PAGE of proteins from clone 20. Lane 1: size marker, lane 2: cell lysate from uninduced cells, lane 3: cell lysate from IPTG-induced cells, lane 4: purified GST fusion protein. (C) Lanes 1 & 10 : size marker, lane 2: purified GST-hDOHH fusion protein, lane 3: purified non-fusion hDOHH protein, lane 4: purified GST-bovine protein from

clone 20, lane 5: purified bovine non-fusion protein from clone 20, lane 6: purified GST-bovine DOHH fusion protein from clone 20N, lane 7: purified bovine non-fusion DOHH protein from clone 20N, lane 8: purified GST-bovine fusion protein from clone 68, lane 9: purified bovine non-fusion protein from clone 68.

Fig 6. DOHH activity determination of bovine GST fusion proteins and purified non-fusion proteins IPTG induced cell-free crude extracts from clones 20, 20N and 68 were assayed for DOHH activity as described in the Materials and Methods. The assays were done with 20 g and 80 g total protein. The purified non-fusion proteins of human DOHH and bovine clones 20N, 68, and 20 were assayed with 5 g pure protein. Negative control included BSA.