行政院國家科學委員會專題研究計畫　成果報告

以斑馬魚模式確認新穎的酵素之催化特性及生理功能

計畫類別：個別型計畫
計畫編號：NSC91-2313-B-040-003-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：中山醫學大學營養學系

計畫主持人：劉德中

計畫參與人員：黃銓珍

報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 92 年 10 月 31 日

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一、ABSTRACT

In this study, by using BLAST (Basic Local Alignment Search Tool), a novel 16 α,17 β dihydroxysteroid Dehydrogenase(HSD) of zebra fish (NCBI accession number A1477544) and human(NCBI accession number NP_115679) were found from NCBI (National Center for Biotechnology Information) nucleotide database. The novel 16 α,17 β dihydroxysteroid Dehydrogenase(HSD) cDNA of zebra fish was successfully amplified from the 0-7 days old zebra fish embryo cDNA library by using the combination of PCR and 3 'rapid-amplification of cDNA ends (3 'RACE) methods. The cDNA has a continuous open reading frame of 1251 bps encoding a protein of 416 amino acids with a calculated molecular mass of 45,546.33 D.

The novel 16 α,17 β dihydroxysteroid Dehydrogenase(HSD) cDNA of human was successfully amplified from the human hepG2 cDNA library by using the PCR method. The cDNA has a open reading frame of 1038 bps encoding a protein of 345 amino acids with a calculated molecular mass of 37,320.22 D.

Both zebrafish and human enzyme homologues were cloned and expressed with a 6-histidine tag for specific purification. The purified recombinant proteins have a relative molecular weight of 46,000 and 38,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The purification of recombinant HSDs expressed in E. Using estriol and NAD^+ as well as estriol and $NADP^+$ as substrates, the specific activity of Ni^{2+} column purified human HSD were 10.74 and 10.73 U/mg protein respectively. While the specific activity of zebrafisf HSD were 3.16 and 3.71 U/mg protein.

Therefore, we termed these novel enzymes 16 α,17 β dihydroxysteroid Dehydrogenase.

Key words : National Center for Biotechnology Information, 16 α,17 β dihydroxysteroid Dehydrogenase.

二、Introduction：

Short-chain dehydrogenases:reductases (SDR) are defined to have distinct, common sequence motifs but constitute a functionally heterogenous superfamily of enzymes[1,2]. The functional assignments of these forms can be grouped into three main categories, which are i)enzymes involved in intermediary metabolism, ii)enzymes participating in lipid hormone and mediator metabolism, and iii)open reading frames (ORFs) of unknown enzymatic function[3,4].

Among them, the second SDR enzyme group constitutes presently the largest fraction and is characterized by its involvement in lipid mediator and to some extent in xenobiotic carbonyl metabolism [5].

Enzymatic activities of this group comprise various hydroxysteroid dehydrogenases (HSDs), prostaglandin dehydrogenases and retinoid metabolizing

enzymes. Several of these enzymes display overlapping substrate specificities. In this fashion, several 17β-hydroxysteroid dehydrogenases also mediate 3α- or 20β-hydroxysteroid dehydrogenase activities[6], microsomal 11β-HSD1 is involved in xenobiotic carbonyl metabolism [5,7], or cytosolic carbonyl reductase (CR), participates in prostaglandin, steroid and xenobiotic metabolism [8].

Due to their substrate specificities and reactions catalyzed, SDR members of this category are involved in essential functions in vertebrate physiology and development. Accordingly, 3β-hydroxysteroid dehydrogenases:Δ4–5-isomerases are indispensable in the biosynthesis of all classes of steroid hormones and bile acids. 11β-hydroxysteroid dehydrogenases catalyze the cell-type specific 'switch' between glucocorticoid receptor binding cortisol and its non-binding metabolite cortisone. Similarly, 17β-hydroxysteroid dehydrogenase isoforms catalyze the conversion of androgens and estrogens, again representing a cellular 'switch' mechanism [6,9]. Several SDR-type retinol and retinal dehydrogenases are identified, thus indicating a role of SDR enzymes in retinoic acid dependent developmental processes and in the visual process [10].

At present, all SDR enzymes, which found in public domain databases, contain parts of the highly conserved but still variable nucleotide binding and active site sequence motifs TGxxxGxG and catalytic triad of S-Y-K residues. The strictly conserved and ascribed a crucial enzymatic function Tyr residue in SDR enzymes in general is supported also by chemical modifications, site-directed mutagenesis, and an active site position in those tertiary structures that have been characterized. A lysine residue four residues downstream is also largely conserved[11].
Two SDR enzymes established by X-ray crystallography show a one-domain subunit with seven to eight B-strands. Conformational patterns are highly similar, except for variations in the C-terminal parts[1,12].

Additional structures occur in the family with extended chains. Some of the SDR molecules are known under more than one name, and one of the enzymes has been shown to be susceptible to native, chemical modification, producing reduced Schiff base adducts with pyruvate and other metabolic keto derivatives. Most SDR enzymes are dimers and tetramers. In those analyzed, the area of major subunit contacts involves two long α-helices (αE, αF) in similar and apparently strong subunit interactions. Future possibilities include verification of the proposed reaction mechanism and tracing of additional relationships, perhaps also with other protein families. Short-chain dehydrogenases illustrate the value of comparisons and diversified research in generating unexpected discoveries[1,13,14].

In this studies, by searching the NCBI sequence database, collect of all known DNA sequences, we find a novel enzyme of zebrafish, as notation: similar to

5'-adcohol dehydrogenase, its NCBI accession number is A1477544, which contained 632bp in length with a possible 5'-met residues but shortage of stop codin. By using this partial nucleotide sequence proceeds Translated BLAST Searches (http://www.ncbi.nlm.nih.gov/BLAST/) against nr data bases(blastx), we find a human homoloque cDNA with deduced 345 amino acid (NP_115679 or NM_032303). By using NM _032303 search against Unigene data bases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene)，we find this gene (UniGene Cluster Hs.47986 Homo sapiens) had been expressed in H.sapiens , M.musculus , R.norvegicus , and C.elegans.

Gene-card(NP_115679) survey showed that MGC10940 expression in normal human tissues (such as bone marrow, spleen, brain, heart, skeletal muscle, liver, pancrease, prostate, kidney, lung) based on quantifying ESTs from various tissues in Unigene clusters (http://www.rzpd.de/cgi-bin/cards/carddisp?MGC10940).

The enzymes aligned with alcohol dehydrogenase(data not shown), showed no significant similarity among human and other species. Searching with Phi Blast, these novel enzyme had two domains, i.e. which are short-chain alcohol dehydrogenase and steroid carrier protein 2 domain (adh_short, scp2).

Following the expression, purification, and kinetic studies, the enzymes can catalize the reduction of the substrates, estriol and 5-androstene 3β,16α,17β-triol, in the present of NAD^+/$NADP^+$. And, we named these enzyme 16α,17β dihydroxysteroid Dehydrogenase.

三、Methods

3.1.NCBI search

By searching the NCBI sequence database, collect of all known DNA sequences, a novel enzyme of zebrafish was found, its NCBI accession number was A1477544. By using this partial nucleotide sequence to perform standard tBLASTn and gapped tBLASTn searches of various genome data bases through the NCBI Blast search engine (http://www.ncbi.nlm.nih.gov/BLAST/) , we find a human homoloque cDNA(NM_03230).

3.2. PCR and 3' RACE (Rapid amplification of cDNA ends) PCR

The 3' end of zebrafish HSD was amplified by 3' RACE [15]from the chromosome of 0-7 days of embryo zebra fish and described as followed. The chromosome of 0-7 days of embryo zebra fish was prepared using the Marathon kit (Clontech). Two nested primers were designed based on the known sequences obtained from zebra fish partial cDNA clones. They are：

Nest1: 5' CTCAGGGGAACATACCTGACGTCTAAA 3';

Nest2: 5' CACACGGCTTACACCATTTGCAAGTAT 3'.

It is containing nt 633–1251 of the full-length zebra fish 16α, 17β hydroxysteroid

dehydrogenase cDNA. Using mRNA prepared from 0-7 days of embryo zebrafish, first-strand cDNA synthesis was performed using Nest1. PCR amplification of full-length coding region cDNA was carried out using Nest2 and a poly(T) primer, following an initial incubation at 96℃ for 5 min. PCR conditions were 96℃ for 30 s, 65℃ for 30 s and 72℃ for 2 min for40 cycles, followed by extension at 72℃for 15 min. A product of 615 bp was obtained. Strain JM109 was transformed with the composite plasmids(pGEM-T easy vector systems(promega product))[17] and plated onto LB solid media supplemented with Ampicilline(200mg/mL). As a result, the clone displaying the insertion fragment was identified by PCR(used Nest2 and oligo-T as primers).

3.3. Construction of an Expression Plasmid for hydroxysteroid dehydrogenase

Plasmid PQE, containing the phage T5 promotor and lac repressor, was previously isolated from a genomic library constructed in the vector λ-ZapII (Stratagene, La Jolla, CA)[16]. In a PCR reaction, SalI、BamHI and HindIII restriction sites(restriction sites are in bold lettering) were introduced at the 5' and 3' gene ends, respectively. The following primers were used (Invitrogen Custom Primers):

sense strand:

5'-AATGTCGACATGCTGCAGAACACAGGGAA-3' (Zebrafish)

5'-AATGGATCCATGTTACCCAACACCGGGAG-3' (Human)

antisense strand:

5'-CGCAAGCTTCAGTTTAGACTTCATCATG-3' (Zebrafish)

5'-CGGAAGCTTCAGTCTGGCATTCATCATCTGATT-3' (Human)

were synthesized by Invitrogen Custom Primers and used for subcloning of the HSD gene by polymerase chain reaction (PCR). Using Taq polymerase (Takara), two primer sets and the 0-7 days of embryo zebrafish and HepG2 cDNA as templates separately, the resulting PCR DNAs of 1.3(Zebrafish) and 1.0(Human)-kb length were recovered and ligated into the SalI-HindIII or BamHI--HindIII sites of plasmid PQE31 or PQE9 to yield plasmids PQE31-H-HSD(for human) and PQE9-Z-HSD(for zebrafish), respectively. To express the HSD gene in E. coli, plasmids PQE31-H-HSD and PQE9-Z-HSD were transformed into E. coli strain M15 to obtain recombinant strains M15/ PQE31-H-HSD and M15/ PQE9-Z-HSD, respectively[18]. The nucleotide sequence of cloned DNA was determined using an Perkin-Elmer ABI PRISM^{TM} 377 DNA synthesizer(Perkin-Elmer, CA).

3.4. Expression and Purification of hydroxysteroid dehydrogenase

Histidinetagged clones derived from both PQE31-H-HSD and PQE9-Z-HSD were expressed in E. coli strain strain M15. Cells were cotransfected with the histidine-tagged clones and the Kanamycin-resistant pDM1.1 plasmid encoding lac repressor. Cotransfected cells were cultured in LB medium at 37 until the culture

reached an A_{600} of 0.6–0.7 at which time isopropyl-β-D-thiogalactoside was added to a final concentration of 200 μg/ml. The IPTG-induced cells were further grown overnight at 30 to prevent sequestering of the hydroxysteroid dehydrogenase in inclusion bodies. Cells were harvested by centrifugation and the supernatant was discarded. The cell pellet was suspended in 50 mM Na_2HPO_4/NaH_2PO_4 buffer (including 0.25mM EDTA) pH 7.4, in 5-10% of the original culture volume. The IPTG-induced cells were harvested and disrupted with the French Press. Subsequently, All centrifugation steps and sodium phosphate buffers were at 4 ℃(17). Lysates were centrifuged at 10,000 g, and the supernatant was immediately loaded onto Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) previously equilibrated in the same lysis buffer. 2 ml of resin was used with 250 ml of culture. The extract was passed through the column, and the resin was washed with three bed volumes of column buffer (50 mM NaPO4, pH 7.4, 0.25 mM EDTA). Histidine-tagged protein was then eluted with 2ml of column buffer which containing 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100mM imidazole stepwisely and monitored by measuring absorbance at 280 nm. Fractions containing eluted protein were combined. The enzyme was stored in aliquots at -20℃ before enzyme activity analysis.

3.5. protein concentration

Protein concentration was measured by the Bio-Rad assay method exactly according to the manufacturer's protocol using bovin serum albumin as standard. The protein concentration was determined by measuring absorbance at 595 nm[19].

3.6. SDS-PAGE

SDS-PAGE was performed on a 12% gel according to the method of Laemmli [20].

3.7.Specific activity Assay

HSD assays, unless otherwise stated, were performed spectrophotometrically, at room temperature, in 1 ml reactions containing 50 mM NaPO4, pH 7.4, 1.5 mM NAD^+ or $NADP^+$, and
1–10 μg enzyme protein. Reactions were initiated by the addition of β-hydroxyacid substrate, and absorbance at 340 nm was monitored to measure the rate of NADH production. Spectrophotometric measurements were recorded on a Hitachi spectrophotometer and the specific activity was performed as unit/mg protein.

四、RESULTS

Cloning and Expression of Human and zebra fish hydroxysteroid dehydrogenase

The partial sequence of zebrafish HSD was originally derived from NCBI EST data bank, access number AI477544 and , which contained the N-terminal portion of the HSD(nucleotide number 1 to 632). The 3'-sequence from 633 to 1251 was obtained from 3'RACE PCR. The human(access number NP_115679 and) and zebra

fish hydroxysteroid dehydrogenase encoded 345 and 416-amino acid residue proteins with a calculated molecular mass of 37,320.22 and 45,546.33 D respectively (see Fig.1.1& Fig.1.2).

```
5'-ATG TTA CCC AAC ACC GGG AGG CTG GCA GGA TGT ACA GTT TTT ATC   45
   Met Leu Pro Asn Thr Gly Arg Leu Ala Gly Cys Thr Val Phe Ile
   ACA GGT GCA AGC CGT GGC ATT GGC AAA GCT ATT GCA TTG AAA GCA   90
   Thr Gly Ala Ser Arg Gly Ile Gly Lys Ala Ile Ala Leu Lys Ala
   GCA AAG GAT GGA GCA AAT ATT GTT ATT GCT GCA AAG ACC GCC CAG   135
   Lys Ala Asp Gly Ala Asn Ile Val Ile Ala Ala Lys Thr Ala Gln
   CCA CAT CCA AAA CTT CTA GGC ACA ATC TAT ACT GCT GCT GAA GAA   180
   Pro His Pro Lys Leu Leu Gly Thr Ile Tyr Thr Ala Ala Glu Glu
   ATT GAA GCA GTT GGA GGA AAG GCC TTG CCA TGT ATT GTT GAT GTG   225
   Ile Glu Ala Val Gly Gly Lys Ala Leu Pro Cys Ile Val Asp Val
   AGA GAT GAA CAG CAG ATC AGT GCT GCA GTG GAG AAA GCC ATC AAG   270
   Arg Asp Glu Gln Gln Ile Ser Ala Ala Val Glu Lys Ala Ile Lys
   AAA TTT GGA GCT TAT ACC ATT GCT AAG TAT GGT ATG TCT ATG TAT   315
   Lys Phe Gly Ala Tyr Thr Ile Ala Lys Tyr Gly Met Ser Met Tyr
   GTG CTT GGA ATG GCA GAA GAA TTT AAA GGT GAA ATT GCA GTC AAT   360
   Val Leu Gly Met Ala Glu Glu Phe Lys Gly Glu Ile Ala Val Asn
   GCA TTA TGG CCT AAA ACA GCC ATA CAC ACT GCT GCT ATG GAT ATG   405
   Ala Leu Trp Pro Lys Thr Ala Ile His Thr Ala Ala Met Asp Met
   CTG GGA GGA CCT GGT ATC GAA AGC CAG TGT AGA AAA GTT GAT ATC   450
   Leu Gly Gly Pro Gly Ile Glu Ser Gln Cys Arg Lys Val Asp Ile
   ATT GCA GAT GCA GCA TAT TCC ATT TTC CAA AAG CCA AAA AGT TTT   495
   Ile Ala Asp Ala Ala Tyr Ser Ile Phe Gln Lys Pro Lys Ser Phe
   ACT GGC AAC TTT GTC ATT GAT GAA AAT ATC TTA AAA GAA GAA GGA   540
   Thr Gly Asn Phe Val Ile Asp Glu Asn Ile Leu Lys Glu Glu Gly
   ATA GAA AAT TTT GAC GTT TAT GCA ATT AAA CCA GGT CAT CCT TTG   585
   Ile Glu Asn Phe Asp Val Tyr Ala Ile Lys Pro Gly His Pro Leu
   CAA CCA GAT TTC TTC TTA GAT GAA TAC CCA GAA GCA GTT AGC AAG   630
   Gln Pro Asp Phe Phe Leu Asp Glu Tyr Pro Glu Ala Val Ser Lys
   AAA GTG GAA TCA ACT GGT GCT GTT CCA GAA TTC AAA GAA GAG AAA   675
   Lys Val Glu Ser Thr Gly Ala Val Pro Glu Phe Lys Glu Glu Lys
   CTG CAG CTG CAA CCA AAA CCA CGT TCT GGA GCT GTG GAA GAA ACA   720
   Leu Gln Leu Gln Pro Lys Pro Arg Ser Gly Ala Val Glu Glu Thr
   TTT AGA ATT GTT AAG GAC TCT CTC AGT GAT GAT GTT GTT AAA GCC   765
   Phe Arg Ile Val Lys Asp Ser Leu Ser Asp Asp Val Val Lys Ala
   ACT CAA GCA ATC TAT CTG TTT GAA CTC TCC GGT GAA GAT GGT GGC   810
   Thr Gln Ala Ile Tyr Leu Phe Glu Leu Ser Gly Glu Asp Gly Gly
   ACG TGG TTT CTT GAT CTG AAA AGC AAG GGT GGG AAT GTC GGA TAT   855
   Thr Trp Phe Leu Asp Leu Lys Ser Lys Gly Gly Asn Val Gly Tyr
   GGA GAG CCT TCT GAT CAG GCA GAT GTG GTG ATG AGT ATG ACT ACT   900
   Gly Glu Pro Ser Asp Gln Ala Asp Val Val Met Ser Met Thr Thr
   GAT GAC TTT GTA AAA ATG TTT TCA GGG AAA CTA AAA CCA ACA ATG   945
   Asp Asp Phe Val Lys Met Phe Ser Gly Lys Leu Lys Pro Thr Met
   GCA TTC ATG TCA GGG AAA TTG AAG ATT AAA GGT AAC ATG GCC CTA   990
   Ala Phe Met Ser Gly Lys Leu Lys Ile Lys Gly Asn Met Ala Leu
   GCA ATC AAA TTG GAG AAG CTA ATG AAT CAG ATG AAT GCC AGA CTG   1035
   Ala Ile Lys Leu Glu Lys Leu Met Asn Gln Met Asn Ala Arg Leu
   TGA                                                          1038
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Fig.1.1.Nucleotide and deduced amino acid sequences of human hydroxysteroid dehydrogenase(H-HSD) cDNA. The nucleotide and deduced amino acid sequences of clone NM_03230 is shown. The numbering system is designed 1 both for the hypothetical starting ATG and Met. The stop codon is located at nt 1036 to 1038Entire coding region contained 345amino acids.

```
5'-ATG CTG CAG AAC ACA GGG AAG CTG GCA GGA TGC ACC ATT TTC ATC  45
   Met Leu Gln Asn Thr Gly Lys Leu Ala Gly Cys Thr Ile Phe Ile
   ACC GGA GCA AGT CGA GGT ATT GGC AAG GCC ATT GCT CTG AAA GCT  90
   Thr Gly Ala Ser Arg Gly Ile Gly Lys Ala Ile Ala Leu Lys Ala
   GCG CAG GAT GGA GCC AAT GTA GTC ATC GCT GCC AAA ACA GCT GAT  135
   Ala Gln Asp Gly Ala Asn Val Val Ile Ala Ala Lys Thr Ala Asp
   CCA CAT CCC AAA CTC CCC GGC ACC ATC TAC ACA GCT GCA GCA GAG  180
   Pro His Pro Lys Leu Pro Gly Thr Ile Tyr Thr Ala Ala Ala Glu
   ATT GAA GCA GCT GGA GGG AAA GCA CTG CCG TGT ATT GTG GAC GTC  225
   Ile Glu Ala Ala Gly Gly Lys Ala Leu Pro Cys Ile Val Asp Val
   CGT GAT GAG AAG CAG ATC AAT GAT GCT GTT GAA CAG GCT GTG GAG  270
   Arg Asp Glu Lys Gln Ile Asn Asp Ala Val Glu Gln Ala Val Glu
   AAG TTT GGA GGG ATT GAC ATA TTG GTC AAC AAT GCC AGT GCC ATC  315
   Lys Phe Gly Gly Ile Asp Ile Leu Val Asn Asn Ala Ser Ala Ile
   AAT TTA ACA GGG ACT CTT CAG ACT CCA ATG GAA AAG GCT GAC CTC  360
   Asn Leu Thr Gly Thr Leu Gln Thr Pro Met Glu Lys Ala Asp Leu
   ATG CTG GGC ATC AAT CTC AGG GGA ACA TAC CTG ACG TCT AAA CTG  405
   Met Leu Gly Ile Asn Leu Arg Gly Thr Tyr Leu Thr Ser Lys Leu
   TGC ATT CCA CAT CTT CTG AAG AGC AAA AAC CCT CAC ATA CTA AAC  450
   Cys Ile Pro His Leu Leu Lys Ser Lys Asn Pro His Ile Leu Asn
   CTC AGC CCA CCT CTC AAC CTT CAC CCC ATT TGG TTT AAA AAC CAC  495
   Leu Ser Pro Pro Leu Asn Leu His Pro Ile Trp Phe Lys Asn His
   ACC GGC TTA CAC CAT TGC AAA GTA TGG CAT GTC CAA TGT GTG ACT  540
   Thr Gly Leu His His Cys Lys Val Trp His Val Gln Cys Val Thr
   TGG GAA TGG CAG AAG AGT TCA CAG GAT CCA TTG CCG TTA ATG CCT  585
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Trp Glu Trp Gln Lys Ser Ser Gln Asp Pro Leu Pro Leu Met Pro
TAT GGC CAA AGA CAG CCA TTC AGA CGG TTG CCA TGG ACA TGT TGG  630
Tyr Gly Gln Arg Gln Pro Phe Arg Arg Leu Pro Trp Thr Cys Trp
GCG GTT CTG AGG TTG GCA AGC AGT GCA GGA AGG TGG AGA TCA TGG  675
Ala Val Leu Arg Leu Ala Ser Ser Ala Gly Arg Trp Arg Ser Trp
CTG ATG CAG CAT ACG CCA TCT TCA AAC AAC CCA CCA GCT TCA CCG  720
Leu Met Gln His Thr Pro Ser Ser Asn Asn Pro Pro Ala Ser Pro
GAC AGT TTG TTA TTG ATG AGG ACA TTC TCA AAA AGG AGG GCA TTA  765
Asp Ser Leu Leu Leu Met Arg Thr Phe Ser Lys Arg Arg Ala Leu
AAG ATT TTG ATG TTT ATG CTG TTG AGC CAG GTC ATC CAT TGC TTT  810
Lys Ile Leu Met Phe Met Leu Leu Ser Gln Val Ile His Cys Phe
CCT GAC TTT TTC TTG GAC GGC CAG CCT GAG GAT CTA GTC AAG CAT  855
Pro Asp Phe Phe Leu Asp Gly Gln Pro Glu Asp Leu Val Lys His
ATG GAG GCA CAT GGT GCC ACT CCG GCG TTC ACA ACT GCA AAA GCA  900
Met Glu Ala His Gly Ala Thr Pro Ala Phe Thr Thr Ala Lys Ala
GAT CCC GTT GCC GCA GGA CCA GTT TCT GAG ATG TTC AAT ACA ATC  945
Asp Pro Val Ala Ala Gly Pro Val Ser Glu Met Phe Asn Thr Ile
AGA GGA ATT ATC AGT CCA GAG ATG GTG AAA ACC ACA CAA GGA GTG  990
Arg Gly Ile Ile Ser Pro Glu Met Val Lys Thr Thr Gln Gly Val
TAC AAA TTT AAC TTA GCA GGC GAG CAT GCT GGA GTC TGG TAT CTT  1035
Tyr Lys Phe Asn Leu Ala Gly Glu His Ala Gly Val Trp Tyr Leu
GAC CTG AAG AAC GAT GCT GGA AGT GCT GGA AAT GGG GAA CCT CCT  1080
Asp Leu Lys Asn Asp Ala Gly Ser Ala Gly Asn Gly Glu Pro Pro
GTC AAA GCT GAT GTT GTC ATG TCG ATG GAC AGT GAG GAT TTT GTC  1125
Val Lys Ala Asp Val Val Met Ser Met Asp Ser Glu Asp Phe Val
AAG ATG TTT GGA GGG AAA TTA AAG CCA ACC ATG GCC TTC ATG TCT  1170
Lys Met Phe Gly Gly Lys Leu Lys Pro Thr Met Ala Phe Met Ser
GGA AAG CTG ACT ATT AAG GGT GAC ATG GCC CTT GTC ATC AAA CTG  1215
Gly Lys Leu Thr Ile Lys Gly Asp Met Ala Leu Val Ile Lys Leu
GAG AAG ATG ATG GCC ATG ATG AAG TCT AAA CTG TGA             1251
Glu Lys Met Met Ala Met Met Lys Ser Lys Leu ***
```

Fig.1.2.Nucleotide and deduced amino acid sequences of zebra fish hydroxysteroid dehydrogenase(Z-HSD) cDNA. The nucleotide and deduced amino acid sequences of clone A1477544
is shown. The numbering system is designed 1 both for the hypothetical starting ATG and Met. The stop codon is located at nt 1249 to 1251. Entire coding region contained 416 amino acids. The sequence from 633 to1251 was obtained from 3'RACE.

Sequence Analysis and Comparison

We have compared the amino acid sequences of H.sapiens(Access number NM_03230 and AK098721.1) , M.musculus(Access number NM_024255.1) , R.norvegicus(Access number XM_233942.1) , C.elegans(Access number NM_073411.1) and zebra fish(Access number AI477544) hydroxysteroid dehydrogenase. The comparison shows the regions that are highly conserved at TGxxxGxxxG(16-23th amino acid for both human and zebrafish) YxxxxK (156 ~ 161 th amino acid for human and 130 ~ 134 th amino acid for zebrafish) GxxxxxS(138 ~ 143 th amino acid for human and 128 ~ 133 th amino acid for zebrafish) between human and zebra fish(Fig.2.). The amino acid sequence deduced from the cDNA for zebra fish HSD did not show significant similarity with those of the human and the others, but it shared 27, 25, 24, and 45 % identity with H.sapiens , M.musculus , R.norvegicus , and C.elegans , respectively (see Fig.2.).

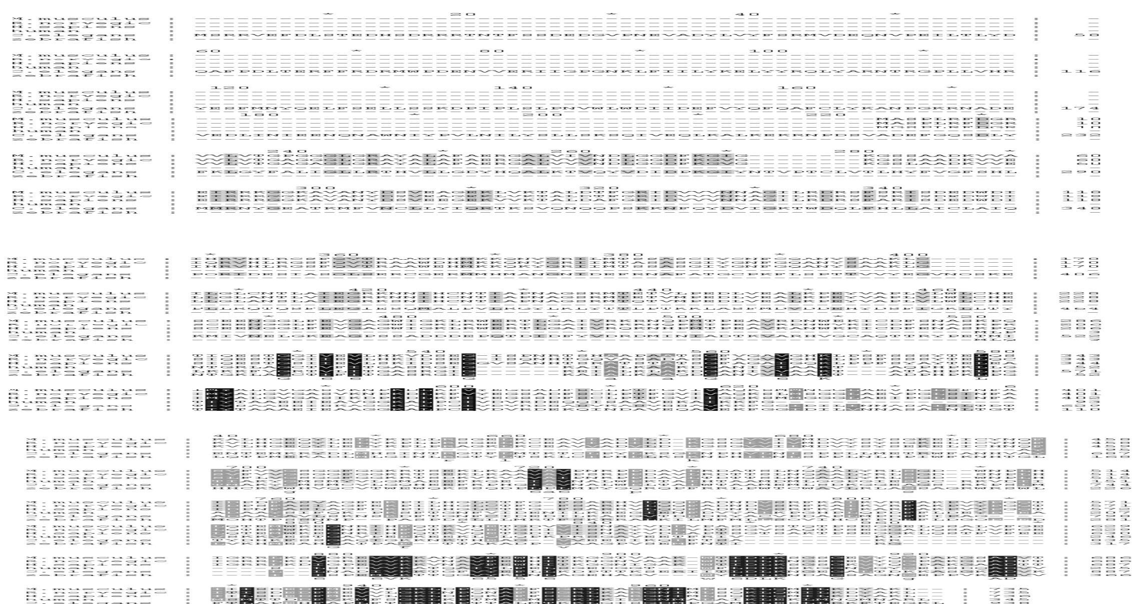


FIG.2.Amino acid sequence homology of β-hydroxyacid dehydrogenase homologues from H.sapiens(Access number NM_03230 and AK098721.1) , M.musculus(Access number NM_024255.1) , R.norvegicus(Access number XM_233942.1) , C.elegans(Access number NM_073411.1) and zebra fish(Access number AI477544) . Hypothetical protein sequences from EST data bases were aligned by the multi-alignment method(cluster and genedoc) using human β-hydroxyisobutyrate dehydrogenase amino acid sequence for pair-wise alignment.

Purification and activity assay of HSD

The purification of recombinant HSDs expressed in E. coli. The purification table for both human and zebrafish HSD showed in Table 1a and 1b. Using estriol and NAD^+ as well as estriol and $NADP^+$ as substrates, the specific activity of $Ni2^+$ column purified human HSD were 10.74 and 10.73 U/mg protein respectively. While the specific activity of zebrafisf HSD were 3.16 and 3.71 U/mg protein. SDS-polyacrylamide electrophoresis analysis for cell lysates and $Ni2^+$ affinity column purification fraction showed in Fig. 3a and 3b, lane 5. The estimated molecular mass of both human and zebrafish HSD were 38 and 45 kDa respectively.

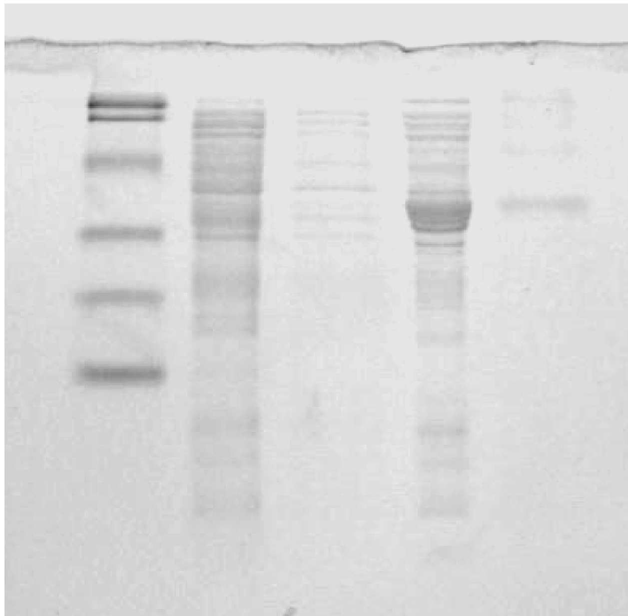


Fig.3a.Expression of Human HSD from E. coli as followed by SDS-polyacrylamide electrophoresis (12%) of whole cell extracts and Ni2^{+} affinity purification fraction. Lanes 1, marker proteins.Lanes 2,M15 strain(without PQE/HSD).lane3, M15 strain with PQE/HSD (without IPTG induced).lane-4, M15 strain used PQE/HSD and IPTG induced. lane-5,Ni^{2+} affinity column purified. Molecular weight standards used were phosphorylase b (113,000), albumin (92,000), ovalbumin (52,900), carbonic anhydrase (35,400), trypsin inhibitor (29,000), and lysozyme (21,500).

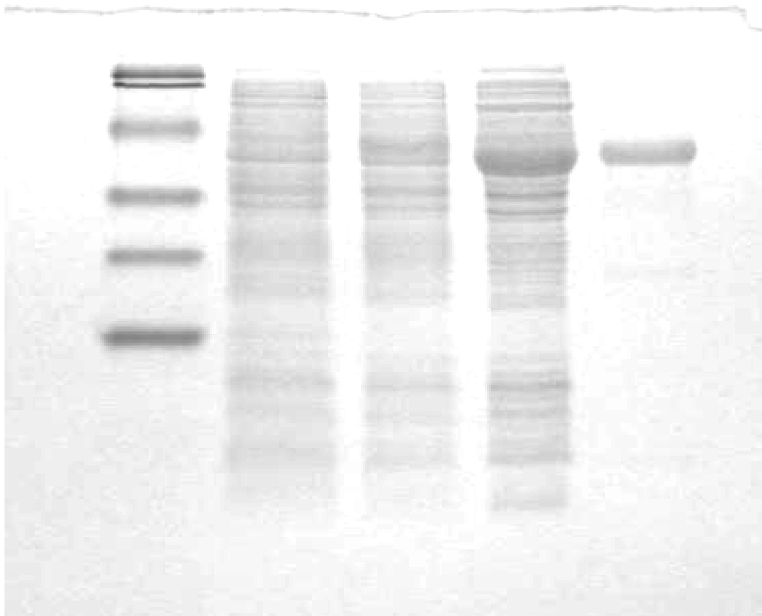


Fig.3b.Expression of zebra fish HSD from E. coli as followed by SDS-polyacrylamide electrophoresis (12%) of whole cell extracts and Ni2^{+} affinity purification fraction. Lanes 1, marker proteins.Lanes 2,M15 strain(without PQE/HSD).lane3, M15 strain with PQE/HSD (without IPTG induced).lane-4, M15 strain used PQE/HSD and IPTG induced. lane-5,Ni^{2+} affinity column purified. Molecular weight standards used were

phosphorylase b (113,000), albumin (92,000), ovalbumin (52,900), carbonic anhydrase (35,400), trypsin inhibitor (29,000), and lysozyme (21,500).

Table 1.：Purification and specific activity of human and zebra fish HSD

	Fraction	Protein (mg)	Specific Activity (unit/mg)	Recover of activity (%)
Zebrafish HSD (Estriol/NAD^+)*	supernatant	48.05	0.13	100
	Ni-column purify	1.73	3.16	42.9
Zebrafish HSD (Estriol/$NADP^+$)*	supernatant	48.05	0.17	100
	Ni-column purify	1.73	3.71	33.79
Human HSD (Estriol/NAD^+)	supernatant	28.56	0.19	100
	Ni-column purify	0.115	10.74	22.38
Human HSD (Estriol/$NADP^+$)	supernatant	28.56	0.18	100
	Ni-column purify	0.115	10.73	12.59

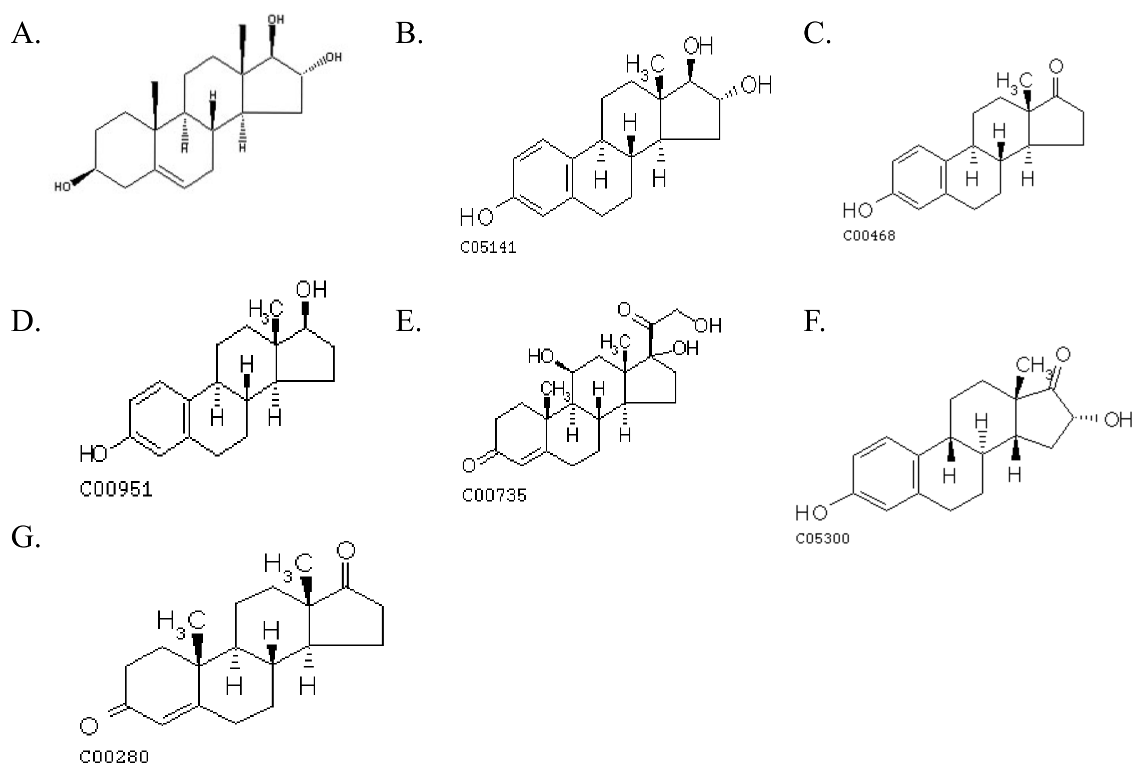
Table 2.：Purification and specific activity of human and zebra fish HSD

	Fraction	Protein (mg)	Specific Activity (unit/mg)	Recover of activity (%)
Zebrafish HSD (5-Androstene /NAD^+)*	supernatant	48.05	0.07	100
	Ni-column purify	1.73	1.75	95.58
Zebrafish HSD (5-Androstene /$NADP^+$)*	supernatant	48.05	0.06	100
	Ni-column purify	1.73	1.41	80.53
Human HSD 5-Androstene /NAD^+)	supernatant	28.56	0.30	100
	Ni-column purify	0.115	12.81	16.99
Human HSD (5-Androstene /$NADP^+$)	supernatant	28.56	0.21	100
	Ni-column purify	0.115	13.62	26.30

五、Discussion

In the sequence analysis both human and zebra fish protein the N-terminal portion showed the sequence homologous to the short chain dehydrogenasa domain(see Fig 4) with the common motif of TGxxxGxG (16-23th amino acid for both human and zebrafish), YxxxxK (156 ~ 161th amino acid for human and 130 ~ 134 th amino acid for zebrafish) and GxxxxxS(138 ~ 143 th amino acid for human and 128 ~ 133 th amino acid for zebrafish)(see fig 2). TGxxxGxG is the essential parts of the nucleotide cofactor binding region (Rossmann-fold). YxxxxK and GxxxxxS are the part of the active site[12]. This evident indicated that these novel enzyme may belongs to the short chain dehydrogenase/reductase family. Therefore we predicted NAD^+ and $NADP^+$ [13,14] are one of the possible substrate. Beside,

sequence alignment for phi blast also showed both enzyme had a steroid carrier protein domain(see Fig 4). Therefore, several steroid substrates were used for testing the novel enzymes activities, which are showed in table 2. Among them, the enzymes can reduce NAD^+ or $NADP^+$, as determined by the increasing of 340nm absorbance, only under the existence of estriol or 5-androstene-3 β, 16 α, 17 β-triol. The specific activities of human HSD for estriol and NAD^+ was10.74; for estriol and $NAPD^+$ was 10.73 U/mg protein, and the specific activity of human HSD for 5-androstene-3 β, 16 α, 17 β-triol and NAD^+ was 12.81; for 5-androstene-3 β, 16 α, 17 β-triol and $NADP^+$ was 13.62. The specific activities of zebrafish HSD for estriol and NAD^+ was 3.16; for estriol and $NAPD^+$ was 3.71 U/mg protein, and the specific activity of human HSD for 5-androstene-3 β, 16 α, 17 β-triol and NAD^+ was 1.75; for 5-androstene-3 β, 16 α, 17 β-triol and $NADP^+$ was 1.41. Both estriol and 5-androstene-3 β, 16 α, 17 β-triol had 16α and 17β hydroxy groups in common. Base on the results as described above, we termed the novel enzymes are 16α and 17β hydroxysteriod dehydrogenase.



	Molecular formula	molecular weight
A. 5-androstene-3 β, 16 α, 17 β-triol	$C_{19}H_{30}O_3$	306.4
B. Estriol	$C_{18}H_{24}O_3$	288.4
C. Estrone	$C_{18}H_{22}O_2$	270.4
D. Estradiol	$C_{18}H_{24}O_2$	272.4

E. Hydrocortisone	$C_{21}H_{30}O_5$	362.5
F.16alpha-Hydroxyestrone	$C_{18}H_{22}O_3$	286.4
G. 4-Androstene	$C_{19}H_{26}O_2$	286.4

Table 2.：The structure is predicted substrate of HSD

Zebrafish domain search

Human (NP_115679) domain search

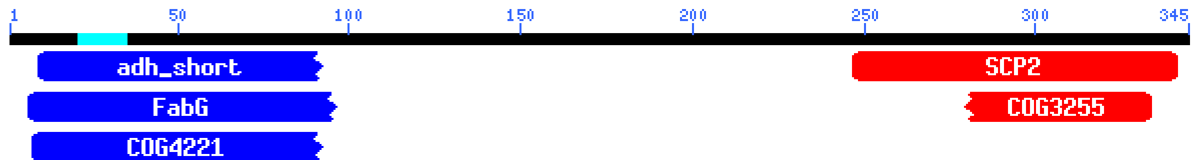


Fig 4.：The homologous domain of H-HSD and Z-HSD。The phi blast search showed both H-HSD and Z-HSD had N-terminal adh_short and C-terminal SCP2 domain.

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