

國立交通大學
生化工程研究所
碩士論文

人類醯亞胺水解酵素之分子
選殖表現及其功能性質探討

**Heterologous Expression, Purification and Functional
Characterization of Human Imidase**

研究生：廖家煒
指導教授：楊裕雄 教授

中華民國九十七年七月

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國立交通大學

生化工程研究所

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日期：民國 年 月 日

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人類醯亞胺水解酵素之分子選殖表現及其功能性質探討

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指導教授：楊裕雄 教授

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摘要

醯亞胺水解酵素(DHP, dihydropyrimidinase, EC 3.5.2.2)參與嘧啶的還原性分解途徑。人類醯亞胺水解酵素基因座落於染色體位置 8q22，含 1560 個鹼基所構成的開放讀碼框，編碼著由 519 個胺基酸所構成的多肽鏈。人類醯亞胺水解酵素屬於 cyclic amidohydrolase superfamily，其中包含了 dihydropyrimidinase、allantoinase、hydantoinase、dihydroorotase 等，皆參與嘌呤與嘧啶環的代謝。分析人類醯亞胺水解酵素的胺基酸序列，其與大鼠及小鼠分別有 90%及 88%的相似度。人類醯亞胺水解酵素缺乏症是一種體染色體隱性遺傳的疾病，不但可藉由檢測病患尿液中 dihydropyrimidine 含量來判斷是否患有醯亞胺水解酵素缺乏症，而且與許多臨床病徵如癲癇、畸形、心理及生理發育不完全有關。然而截至目前為止，在蛋白質層面，人類醯亞胺水解酵素仍有尚未被了解的生化特性及功能。為了分析人類醯亞胺水解酵素的特性，我們解決在大腸桿菌內表現人類醯亞胺水解酵素所遇到的蛋白質包涵體(Protein inclusion body)問題，在大腸桿菌內大量表現可溶並具活性的人類醯亞胺水解酵素，並進一步比較不同物種來源，在酵素動力學、pH 值及溫度對活性的影響、耐熱度、耐鹽度上的差異。除此之外我們還積極地建立一個能穩定生產人類醯亞胺水解酵素的平台，以便後續能夠從蛋白質層面來探討，在病理上如何因為單一個氨基酸突變而造成人類醯亞胺水解酵素失去活性，導致人類醯亞胺水解酵素缺乏症的發生。

Heterologous Expression, Purification and Functional Characterization of Human Imidase

Student: Chia-Wei Liao

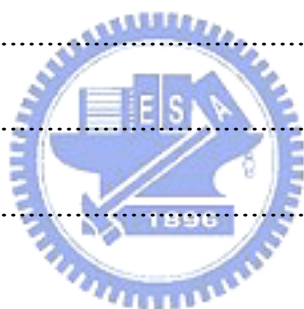
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ABSTRACT

Imidase participates in the reductive degradation pathway of pyrimidine. The location of *Homo sapiens* dihydropyrimidinase (*hDHP*, DPYS, NM_001385) gene is on the chromosome 8q22 and its cDNA clone contains 1560-bp open reading frames encoding polypeptides of 519 residues. *hDHP* belongs to the superfamily of cyclic amidohydrolase, including dihydropyrimidinase, allantoinase, hydantoinase, dihydroorotase, all of which are involved in the metabolism of purine and pyrimidine rings. The deduced amino acid sequence of *hDHP* shows 90% and 88% identity with that of rat and mouse DHP, respectively. *hDHP* deficiency is an autosomal recessive disorder characterized by dihydropyrimidinuria and associated with variable clinical phenotypes, such as seizures, mental retardation, growth retardation and dysmorphic features. However, little is known about the properties and biological functions of human imidase at protein level. In order to characterize the biochemical properties of human imidase, we expressed human imidase gene in *E. coli* and rendered the purified protein in soluble and active form. The characterizations in enzyme kinetics, the effect of pH and temperature to enzyme activity, thermostability and salt tolerance of human imidase and those from other species were compared. This provided a basis for the future preparation of imidase mutants to study the effect of single amino acid substitution on imidase that causes *hDHP* deficiency

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Abbreviation and Symbol

Abbreviation and Symbol	Full name
ϵ	Absorption (extinction) coefficient
A280	Absorption at 280 nm
A298	Absorption at 298 nm
Bis-Tris propane	1,3-bis[tris(hydroxymethyl) methylamino] propane
Tris-HCl	tris(hdroxymethyl) aminomethane hydrochloride
SDS	dodium dodecly sulfate
PAGE	polyacrylamide gel electrophoresis
<i>hdhp</i>	dihydropyrimidinase gene of <i>Homo sapien</i>
<i>hDHP</i>	dihydropyrimidinase of <i>Homo sapien</i>



Introduction

Imidase catalyzes the hydrolytic cleavage of imide bond. Imidase is also known as dihydropyrimidinase (EC 3.5.2.2), hydantoinase, dihydropyrimidine hydrase, and dihydropyrimidine amidohydrolase due to its broad substrate specificity.

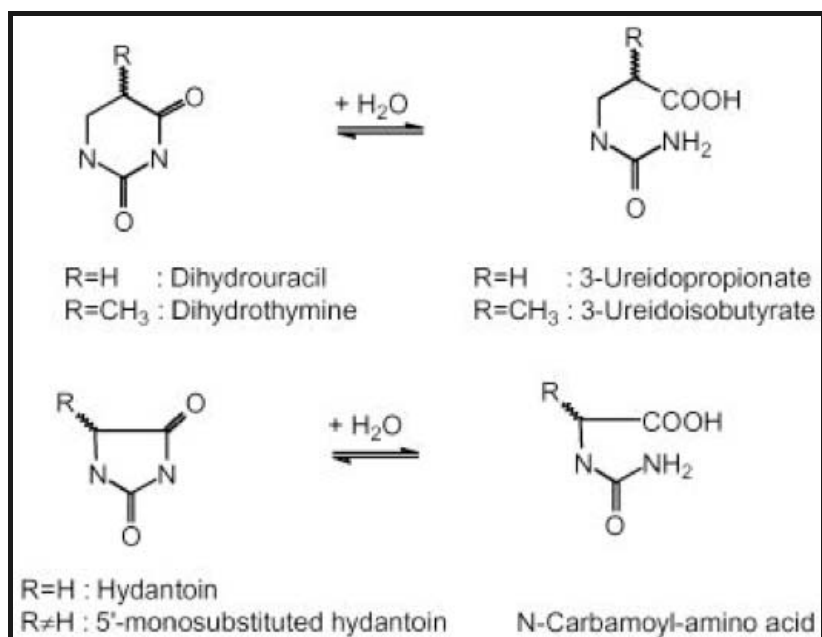


Figure.1 The typical reaction catalyzed by imidase

The first research of imidase activity in living organisms was found as far back as 1926. Gaebler and Keltch investigated the metabolism of hydantoin derivatives by detecting that the hydantoic acid was excreted after injection of hydantoin into dogs and called this kind of imidase as hydantoinase [1]. Afterward, many studies show that imidase activity could be found in some tissues of different plants and animals. Up to 1957, Wallach and Grisolia isolated imidase from calf liver and used dihydropyrimidine as nature substrate, so they called the imidase as dihydropyrimidinase. They proposed that dihydropyrimidinase is identical to

hydantoinase found by Gaebler and Keltch, and therefore imidase participates in the pyrimidine metabolism [2].

The cDNA clone encoding dihydropyrimidinase from human liver had been firstly isolated in 1996 [3]. The deduced amino acid sequence of human DHP show 90% and 88% identity with that of rat and mouse DHP, respectively (Appendix 3). The gene of *Homo sapiens* dihydropyrimidinase (*hDHP*, DPYS, NM_001385) locates on the chromosome 8q22 (Appendix 1) and its cDNA clone contains 1560-bp open reading frames encoding polypeptides of 519 residues (Appendix 2) [3]. While cloning the *hDHP* cDNA, there are three DHP related protein (DRP)-1, DRP-2 and DRP-3 have been found. All of them show high identity of amino acid sequence with *hDHP* (Appendix 3), but without imidase activity. DHP and DRPs display differential tissue distribution, i.e. human DHP in liver and kidney; human DRP-1 in brain; human DRP-2 ubiquitously expressed except for liver; human DRP-3 mainly in heart and skeletal muscle. The function of DRPs and their relationships with DHP in evolution are still not clear [3].

hDHP belongs to the superfamily of cyclic amidohydrolase, including dihydropyrimidinase, allantoinase, hydantoinase, dihydroorotase, all of which are involved in metabolism of purine and pyrimidine rings. In the biological system, pyrimidines are metabolized by either an oxidative or a reductive pathway. It is well-known that mammals, plants, and microorganisms utilize the reductive pathway for pyrimidine degradation.

Dihydropyrimidinase performs reversible hydrolytic ring-opening of dihydrouracil and dihydrothymine to *N*-carbamoyl- β -alanine and *N*-carbamoyl- β -aminoisobutyric acid, respectively. 3-ureidopropionase catalyzes the irreversible hydrolysis of *N*-carbamoyl- β -alanine and *N*-carbamoyl- β -aminoisobutyric acid to β -alanine, β -aminoisogutyric acid, ammonium ions, and carbon dioxide [4].

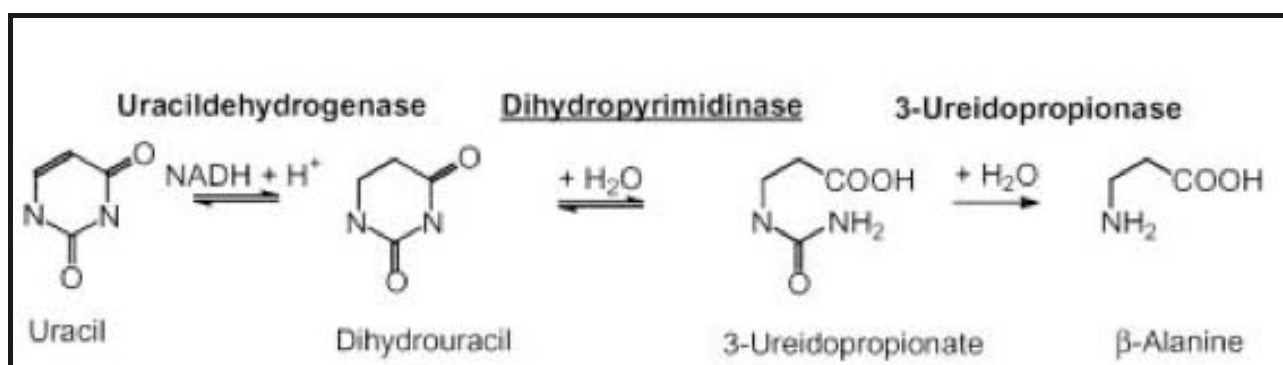


Figure 2. Reductive pathway of pyrimidine degradation

The reductive pyrimidine degradation pathways seem to be important especially in mammalian cell. In mammalian tissue, it is now established that the degradation of uracil via dihydrouracil and *N*-carbamoyl- β -alanine plays an important role in the synthesis of β -alanine and β -aminoisobutyric acid. β -alanine is a structural analogue of γ -aminobutyric acid and glycine, the important neurotransmitter in nerve system. Furthermore, β -aminoisobutyric acid has been shown to be a potential agonist of the glycine receptor [5]. Pathologically, the defect of dihydropyrimidine may cause the altered homeostasis of β -alanine in patients then accounted for the clinical symptom of DHP deficiency.

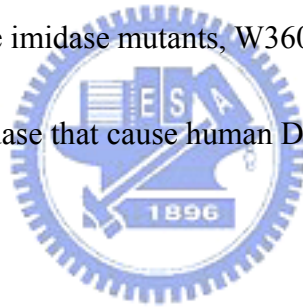
DHP deficiency (MIM 222748) is an autosomal recessive disorder that is characterized by dihydropyrimidinuria. To date, only nine cases of complete DHP deficiency have been reported. These patients reveal variable clinical phenotypes, such as seizures, mental retardation, growth retardation and dysmorphic features [6-15]. It is not a direct evidence to infer the relationship between the homeostasis of β -alanine and the apparent clinical abnormalities. DHP deficiency, therefore, is not only probably a necessary, but sufficient for the onset of a clinical phenotype.

Analysis of the genotype of some patients with DHP deficiency reveals the missense at the nucleotide location of open reading frame 1078, mutated from T to C (W360R) in exon 6 and a novel missense mutation 1235G to T (R412M) in exon 7 [15]. Analysis of the structural effect on the DHP mutations, W360 and R412, by structure modeling indicates that W360 and R412 are located on the periphery of *h*DHP rather distant from the catalytic centre. This excludes a direct effect of the amino acid exchanges on active site architecture and catalysis. Instead, the lack of residual activity for the point mutants is likely based on global effects of the exchanges on the protein structure [15]. However, all of these data are only based on bioinformatical prediction without the evidence at protein level.

According to the analysis of imidase structures, all imidases are metalloproteins with TIM-barrel architecture. All known mammalian imidases purified from the livers of bovine, calf, pig, and rat [11,16-19] are homotetrameric enzymes that contain four tightly bound Zn

atoms, and one Zn atom per subunit. The variations are found, however, in structure, metal content, substrate specificity and other requirement of similar enzyme from different organism.

Since then, little is known about the properties and biological functions of human imidase at protein level. In our experience, the bottleneck is the problem of protein inclusion bodies during expressing the *hdhp* gene in *E. coli*. In order to characterize the biochemical properties of human imidase, we will express human imidase gene in *E. coli* in soluble and active form. The differences in enzyme kinetics, the effect of pH and temperature to enzyme activity, thermostability and salt tolerance of human imidase and those from other species will be compared. We will also prepare imidase mutants, W360 and R412, to study the effect of single amino acid substitution on imidase that cause human DHP deficiency.



Experimental Procedures

Materials— cDNA of *hdhp* gene on pCMV-sports6 vector, obtained from human gene cDNA library in College of Biological Science and Technology, NCTU, pGEM-T Vector(Promega), modified *pET-43.1a(+)* vector(Novagena) modified by Dr. LE, BL21 (DE3)pLyS competent cell(genotype: *E. coli B F⁻ dcm ompT hsdS(r_B⁻ m_B⁻) gal λ(DE3)[pLysS Cam^r]*(Novagen), Sephacryl S-300 HR column(amersham pharmacia biotech), High Molecular Weight standard (code: 17-0041-01) Gel Filtration Calibration Kits(amersham pharmacia biotech).

Molecular cloning hdhp gene into the pET-43.1a(+) vector— The PCR products of cDNA of *hdhp* gene of *Homo sapien* on pCMV-sports6 vector was cloned into pGEM-T Vector (Promega), and the termini of amplified DNA was added the restriction sites of BamH I and Xho I with the pairing primers *hdhp*-F (the forward primer: 5'-CGGATCCATGGCGGC GCCCTCGCG-3') and *hdhp*-R (the reverse primer: 5'-CCTCGAGGGGGTG GGCCTGTTTCCTGG-3') by PCR. The DNA fragment containing the *hdhp* gene on pGEM-T vector was subcloned into the modified *pET-43.1a(+)* vector by restriction enzyme, BamH I & Xho I, and T4 DNA Ligase.

Bacterial expression, purification of hDHP— The flask culture (500 ml of LB medium containing 50 µg/ml ampicillin) of the BL21 (DE3)pLyS cell containing the construct of *hdhp* gene on the *pET-43.1a(+)* vector, IPTG-inducible expression vector was incubated for > 5

hours at 37°C in a shaking incubator until cells reach mid-log growth (A_{600} of 0.8-1.0). The culture was induced by adding 0.4mM IPTG and 1mM $CoCl_2$, then incubated at 20°C for 16 hours. The cells were harvested by centrifugation at 15000g for 30 min at 4°C. The cell pellet was resuspended in 20 ml of Histrap Column Buffer A (pH 8.0), consist of 50mM Tris, 500mM NaCl, 5mM Imidazole 10% Glycerol, and the cell was lysed by sonication with 550 Sonic Dismembrator (Fisher Scientific). The insoluble debris was removed by centrifugation at 30000g for 30 minutes at 4°C, if necessary, repeat this step again to ensure removing the insoluble debris completely. The sample should be filtered through a 0.45 mm filter before it is applied to the Histrap ion exchange column (Pharmacia), equilibrated by Histrap Column Buffer A. The column was washed with >1000 ml of Histrap Column Buffer B (pH 8.0), consist of 50mM Tris, 500mM NaCl, 50mM Imidazole, 10% Glycerol, until no material appears in the effluent. After changing the buffer in the column with Histrap Column Buffer A, applied 40 unit of bovine thrombin to column and incubate it for >16 hours at 4 °C to cleave the Nus-tag from the N-terminal of *hDHP*. The column was washed with 200 ml of Histrap Column Buffer B, then The bounded protein was eluted with 50 ml of Histrap Column Buffer C (pH 8.0), consist 50mM Tris, 500mM NaCl, 500mM Imidazole, 10% Glycerol. The product was concentrated with Amicon Ultra-15 50K NMWL device (MILLIPORE) by centrifugation at 4000g and exchanged the solvent with Histrap Buffer D (up to 100x).

In-gel-digestion and identification using MALDI-TOF— The spots of interest were excised and digested in gel with trypsin according to Shevchenko's method (Shevchenko et al., 1996). The digested sample was taken up and analyzed using MADI-TOF. The results were correlated with the sequence database using the NCBI and SwissProt database and analyzed by Mascot software.

Enzyme assays— A rapid Spectrophotometric assay was used as the standard assay. the decrease in absorbency at 298 nm was measured upon hydrolysis of phthalimide as the substrate at 25 °C. To start the reaction, the enzyme solution was added into 1 ml solution, containing 1 mM phthalimide and 100 mM Bis-Tri propane at pH 7.0. Under these conditions, a change in A_{298} of 2.26 represents the hydrolysis of 1 μ mol of the substrate. The hydrolysis of phthalimide was monitored with a UV/VIS spectrophotometer (Hitachi U3300).

Protein concentration determination— The protein concentration of enzyme solution was determined by A_{280} or BCA protein assay (Bio-rad) using bovine serum albumin as a standard. For the homogeneous imidase, 1 unit of A_{280} equals to 0.907 mg/ml imidase based on the BCA protein assay.

Kinetic constants determination— Measurement of kinetic constant of each substrate was performed by varying the concentration of one substrate, while keeping the other substrate at a fixed and near saturating concentration. The apparent K_m and V_{max} were determined using nonlinear regression by Sigmaplot 2001, V7.0 and Enzyme Kinetics Module, V1.1.

Temperature profile analysis— The specific activity of *hDHP* with phthalimide at given temperature was measured with a UV/VIS spectrophotometer. To start the reaction, the enzyme solution was added into 1 ml of solution containing 1 mM phthalimide and 100 mM Bis-Tri propane to process enzyme assay at pH 7.0 and given temperature.

pH profile analysis— The specific activity of *hDHP* with phthalimide at given pH was measured with a UV/VIS spectrophotometer. To start the reaction, the enzyme solution was added into 1 ml of solution containing 1 mM phthalimide and 100 mM Bis-Tri propane to process enzyme assay at given pH and 25°C

Thermostability analysis— The residual activity of *hDHP* with phthalimide was measured with a UV/VIS spectrophotometer. Prior to start the reaction, the enzyme solution was incubated in 1 ml of 100 mM Bis-Tri propane (pH 7.0) for time course at given temperature, then added 1mM phtalimide to process enzyme assay.

Salt tolerance analysis—The residual activity of *hDHP* with phthalimide was measured with a UV/VIS spectrophotometer. Prior to start the reaction, the enzyme solution was incubated in 1 ml of the given concentration urea and 100 mM Bis-Tri propane (pH 7) for time course at 25°C, then added 1mM phtalimide to process enzyme assay.

Size-exclusion chromatography and calibration curve preparation— Aliquots of 1 ml of various samples were applied on a Sephacryl S-300 HR column that was equilibrated with 20 mM Tris buffer pH 8.0 containing 150 mM NaCl. Protein were eluted with the same buffer at a

flow rate of 1.0 ml/min. Molecular weights were estimated against Gel Filtration Calibration Kits(amersham pharmacia biotech) consisted of aldolase(178 KDa), catalase(228 KDa), ferritin (437 KDa), thyroglobulin(692 KDa), Blue dextran(2000 KDa). Molecular weight of *h*DHP was estimated by gel filtration chromatography.



Result

Molecular Cloning of hdhp gene into the pET-43.1a(+) vector— we constructed *hdhp* gene on different vectors to screen an appropriated vector (Fig. 9, only show the *hdhp-pET-43.1a (+)* construct) to express soluble and active *hDHP*. We found *pET-43.1a (+)* vector can overexpress the soluble and active *hDHP*.

Bacterial Expression and Purification of hDHP—The *pET-43.1a(+)* vector provides the *hDHP* for fusion to Nus-tag and His-tag on N-terminus and C-terminus, respectively. The Nus-His-tagged fusion protein was expressed in the BL21 (DE3)pLyS cell and purified by HisTrap column as describe in “Experimental Procedure”. The enzyme was finally purified about 343.7-fold with 49% yield (Table I) and in a homogenous form according to the identifications of SDS-PAGE (Fig. 1) and MADI-TOF data (Fig. 2). These data display that we are the first one to not only purify *hDHP* in a homogenous and active form but also express the mammalian imidase in heterolous system. We analyzed the biochemical characterization to deduce the difference in enzyme kinetics, the effect of pH and temperature to enzyme activity, thermostability and salt tolerance of human imidase between from human and other species.

Substrate specificity and kinetic constants of the cobalt-amended hDHP with different substrates— A number of xenobiotic and physiological substrates were tested as substrates for *hDHP* (Table II). The specific activities of the different substrates of *hDHP* were determined with UV/VIS spectrophotometer as indicated under “Experimental Procedure”, and all the

concentration of substrates are 1 mM. The specific activities of *hDHP* with dihydrouracil, hydantoin, phthalimide, and maleimide are 0.37, 3.48, 24.49, and 29.72 $\mu\text{mol}/\text{min}/\text{mg}$, respectively. *hDHP* has higher specific activities with phthalimide, and maleimide in the condition. The kinetic constants of *hDHP* with phthalimide were determined experimentally by direct measurement with a spectrophotometer as indicated under “Experimental Procedures”. K_m , k_{cat} , and k_{cat}/K_m of *hDHP* with phthalimide are 0.65, 2615, and 4021 $\mu\text{mol}/\text{min}/\text{mg}$, respectively (Fig. 10).

The temperature and pH profile of hDHP— The temperature profile of *hDHP* is displayed by the specific activity of *hDHP* with phthalimide at given temperature measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” (Fig. 3). The specific activities of *hDHP* with phthalimide from 20 °C to 35 °C are all about 24.0 $\mu\text{mol}/\text{min}/\text{mg}$ without significant different. The pH profile of *hDHP* is displayed by the specific activity of *hDHP* with phthalimide at given pH measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” (Fig. 4). The specific activities of *hDHP* with phthalimide at pH6.5, pH7.0~7.5, 8.0~9.0, and 9.5 are about 14.2, 25.4, 31.2, and 24.5 $\mu\text{mol}/\text{min}/\text{mg}$, respectively. *hDHP* has higher specific activity at pH 8.0~9.0.

The thermostability of hDHP— The thermal stability of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” after treating at given temperature for time course

(Fig. 5). The residual activities of *hDHP* with phthalimide after treating at 30 °C for 60, 120, and 150 minutes are 98.53, 96.19, and 95.42 %, respectively. The residual activities of *hDHP* with phthalimide after treating at 37 °C for 60, 120, and 150 minutes are 71.54, 48.53, and 45.67 %, respectively. The residual activities of *hDHP* with phthalimide after treating at 50 °C for 60, 120, and 150 minutes are 15.66, 6.57, and 2.19 %, respectively. The residual activity of *hDHP* with phthalimide after treating at 60 °C for 15 minutes is 1.30 %. *hDHP* is much unstable above 37°C.

The effect of urea treatment of hDHP— The effect of urea treatment of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide and measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” after treating in given concentration of urea for time course (Fig. 6). The residual activity of *hDHP* with 0.1M, 1M, 2M, 3M, and 4M urea after treating for 15minutes are 70.01, 57.33, 25.87, 9.01, and 2.39%, respectively. The residual activity of *hDHP* with 0.1M, 1M, 2M, 3M, and 4M urea after treating for 30 minutes are 67.97, 49.34, 21.18, 8.76, and 0.71, respectively. After treating in different concentration of urea for 30 minutes, the residual activity of *hDHP* performed stable and didn't change.

Analysis of the quaternary structure of hDHP by gel filtration— The subunit size and native size of *hDHP* was estimated by its deduced amino acid sequence (A. 6) and gel filtration (Table V). The retention volume (V_e) and the parameter (K_{av}) were show as the Table IV. The

retention volume of the blue dextran 2000 was 37.28 ml and the K_{av} could be estimated by the equation: $K_{av} = (V_e - V_o) / (V_t - V_o)$. The calibration curve was plotted as the independent variable was $\log(M.W.)$ versus the dependent variable K_{av} as show in Fig. 8. The retention volume of *h*DHP and *a.r.*HYD were 54.31 ml and 60.40 ml, respectively (Table V). The corresponding molecular weight according to the retention time was shown in Fig. 8 and the calculated values were approximately equal to the theoretical molecular weight of *h*DHP and *a.r.*HYD (Table V). These data indicated *h*DHP and *a.r.*HYD are homopentamer and homotetramer, respectively.

Discussion

Since then, little is known about the properties and biological functions of human imidase at protein level, in our experience, the bottleneck is the problem of protein inclusion bodies during expressing the *hdhp* gene in *E. coli*. Here, the protein inclusion bodies mean the cytoplasmic aggregates of misfolded protein. This phenomenon was often founded during expressing the eukaryotic gene in heterologous system, especially in *E. coli*, and it was due to the internal microenvironment of *E. coli* may differ from that original source of the gene. A variety of methods have been published describing solving the problem of protein inclusion bodies. The choice of vector and expression host can significantly increase the activity and amount of target protein present in the soluble fraction. An appropriate vector can enhance solubility and/or folding by providing for fusion to a polypeptide that itself is highly soluble (e.g., NusA, GST, Trx), or providing for fusion to an enzyme that catalyzes disulfide bond formation (e.g., thioredoxin, DsbA, DsbC), or provide a signal sequence for translocation into the periplasmic space (pelB, DsbA, DsbC). In this study, we expressed the *hdhp* gene on the *pET-43.1a(+)* vector which provided for fusion to NusA (A. 4,5). Nus A, N utilization substance A, is the transcription factor of *E. coli* naturally. Nus A not only is high soluble but also can increase the solubility of the full-length fusion protein to solve the problem of protein inclusion bodies during expressing the *hdhp* gene in *E. coli*.

Imidase has been purified in a homogeneous form from *Pseudomonas* [20], *Bacillus* [21],

Agrobacterium [22], *Blastobacter* [23], *Arthrobacter* [24], bovine liver[16], calf liver[17], pig liver[18,25], and rat liver[11,19], and most purifications of these were conducted by three or four chromatography steps. We solved the problem of protein inclusion bodies during expressing the *hdhp* gene in *E. coli* and developed a time-and-effort-saving, inexpensive, high quality and quantity platform to express and purify homogenous *hDHP* with only one chromatography step for further biochemical characterization to deduce the difference of specific activity and structure of imidase between human and other species.

A number of xenobiotic and physiological compounds were tested as substrates for enzymes, and the specific activities of *hDHP* were compared with *a.r.*HYD (hydantoinase from *Agrobacterium radiobacter* NRRL B1) referred to the thesis of Yi-Rong Chen (Table II)[25]. The catalytic efficiencies of *hDHP* with the substrates are higher than that of *a.r.*HYD except dihydrouracil, the six-member ring substrate. The kinetic constants, K_m , K_{cat} , and K_{cat}/K_m , of *hDHP* with phthalimide as substrate are 0.65mM, 2615min⁻¹, and 4021mM⁻¹min⁻¹, respectively. Comparing with imidases from fish liver, pig liver, and *a.r.*HYD, *hDHP* performs better substrate affinity and catalytic efficiency (Table III).

The temperature profile of *hDHP* is displayed by the specific activity of *hDHP* with phthalimide at given temperature (Fig. 3). *hDHP* performs stable catalytic efficiency from 20 °C to 35 °C. It is to deserve to be mentioned the limit of enzyme assay that the substrate is degraded faster at the high temperature (>35 °C) than which catalyzed by *hDHP*. Therefore,

we can not measure the initial rate of *hDHP* activity with phthalimide at the high temperature and the specific activity at 40 °C is measured much lower than which at 35 °C. The pH profile of *hDHP* is displayed by the specific activity of *hDHP* with phthalimide at given pH measured (Fig. 4). *hDHP* performs stable catalytic efficiency from pH 8 to pH 9.

The thermal stability of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide after treating at given temperature for time course (Fig. 5). *hDHP* performs stable catalytic efficiency, the residual activity is 95.4% after treating at 30 °C for 150 minutes. The residual activities of *hDHP* are 45.8% and 2.2% after treating at 37 °C and 50 °C for 150 minutes, respectively. After treating at 60 °C for 15 minutes, the residual activity of *hDHP* is only 1.3%. Under the same treatment, 50 °C for 1 hour, the residual activities of *hDHP*, *a.r.HYD*, and fish imidase are about 13.6%, 93%, and 90%, respectively. So, *hDHP* is more thermolabile than *a.r.HYD* and fish imidase.

The effect of urea treatment of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide after treating in given concentration of urea for time course (Fig. 6). After treating in different concentration of urea for 30 minutes, the residual activity of *hDHP* performed stable and didn't change, so we can simply determine the stability of urea treatment of *hDHP* by measuring $[Urea]_{50\%}$, the concentration of urea at 50% residual activity of *hDHP* treated for 30 minutes (Fig. 7), for further comparing the difference of stability of *hDHP* mutants, W360 and

R412, with wild *hDHP* to study the effect of single amino acid substitution on *hDHP* that cause human DHP deficiency. The $[Urea]_{50\%}$ of wild *hDHP* is about 1 M.

The subunit size and native size of *hDHP* was estimated by its deduced amino acid sequence (A. 6) and gel filtration (Table V). This data indicated that the native form of *hDHP* is homopentameric different from other mammalian imidases, such as calf [17], pig (5) rat [19], yeast and slime mold DHPs [25] are homotetrameric.

In this study, it is the first one solved the problem of protein inclusion bodies during expressing the *hdhp* gene in *E. coli*, then developed a time-and-effort-saving, inexpensive, high quality and quantity platform to express and purify homogenous soluble and active *hDHP* with only one chromatography step. We have analyzed the complete biochemical characterization of *hDHP*. These results indicate that *hDHP* performs very unique biochemical characterization on specific activity, thermal stability, and the quaternary structure compared with the imidases from other species. It is worthy to know which reason caused it, maybe the sequence or structure, and its significance on evolution. We will resolve and clarify that in the future. On the other hand, we will prepare imidase mutants, W360 and R412, then analyzed the difference of the structure stability and other biochemical characterization of them to study the effect of single amino acid substitution on imidase that cause human DHP deficiency.

References

- [1] Gaebler OH, and Keltch AK (1926) On the metabolism of hydantoins and hydantoic acid. *J Bio, Chem* **70**:763-777.
- [2] D.P. Wallach and S. Grisolia (1957) The purification and properties of dihydropyrimidine hydase. *J Biol Chem.* **226**:277-288.
- [3] Hamajima N, Matsuda K, Sakata S, Tamaki N, Sasaki M, and Nonaka M (1996) A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. *Gene* **180**:157–163.
- [4] C. Wasternack (1980) Degradation of pyrimidines and pyrimidine and primidine analogs—path ways and mutual influences. *Pharmaco Ther.* **8**:629-651.
- [5] A.B.P. van Kuilenburg, A.E.M. Stroomer, H. van Lenthe, N.G.G.M. Abeling, and A.H. van Gennip (2004) New insights in dihydropyrimidinedehydrogenase deficiency: a pivotal role for betaaminoisobutyric acid? *Biochem. J.* **379**:119–124.
- [6] S. Sumi, M. Imaeda, K. Kidouchi, S. Ohba, N. Hamajima, K. Kodama, H. Togari, and Y. Wada (1998) Population and family studies of dihydropyrimidinuria: prevalence, inheritance mode, and risk of fluorouracil toxicity, *Am J Med Genet.* **78**:336–340.
- [7] M. Duran, P. Rovers, P.K. de Bree, C.H. Schreuder, H. Beukenhorst, L. Dorland, and R. Berger (1990) Dihydropyrimidinuria. *Lancet* **336**:817–818.
- [8] M. Duran, P. Rovers, P.K. de Bree, C.H. Schreuder, H. Beukenhorst, L. Dorland, and R. Berger (1991) Dihydropyrimidinuria: a new inborn error of pyrimidine metabolism. *J*

Inherit Metab Dis. **14**:367–370.

[9]M.J. Henderson, K. Ward, H.A. Simmonds, J.A. Duley, and P.M. Davies (1993)

Dihydropyrimidinase deficiency presenting in infancy with severe developmental delay. *J*

Inherit Metab Dis. **16**:574–576.

[10]C.W. Putman, J.J. Rotteveel, R.A. Wevers, A.H. van Gennip, J.A. Bakkeren, and R.A. De

Abreu (1997) Dihydropyrimidinase deficiency, a progressive neurological disorder?

Neuropediatrics **28**:106–110.

[11]Yang, Y.S., Ramaswamy, S. and Jakoby, W.B. (1993) Rat liver imidase. *J Biol Chem.*

268:10870-10875.

[12]S. Sumi, K. Kidouchi, K. Hayashi, S. Ohba, and Y. Wada (1996) Dihydropyrimidinuria

without clinical symptoms. *J Inherit Metab Dis.* **19**:701–702.



[13]S. Ohba, K. Kidouchi, S. Sumi, M. Imaeda, N. Takeda, H. Yoshizumi, A. Tatematsu, K.

Kodama, K. Yamanaka, M. Kobayashi, and Y. Wada (1994) Dihydropyrimidinuria: the

first case in Japan. *Adv Exp Med Biol.* **370**:383–386.

[14]N. Hamajima, M. Kouwaki, P. Vreken, K. Matsuda, S. Sumi, M. Imaeda, S. Ohba, K.

Kidouchi, M. Nonaka, M. Sasaki, N. Tamaki, Y. Endo, R.A. De Abreu, J. Rotteveel,

A.B.P. van Kuilenburg, A.H. van Gennip, H. Togari, and Y. Wada (1998)

Dihydropyrimidinase deficiency: structural organization, chromosomal localization, and

mutation analysis of the human dihydropyrimidinase gene. *Am J Hum Genet.* **63**:

717–726.

- [15] A.B.P. van Kuilenburg, J. Meijer, D. Dobritzsch, R. Meinsma, M. Duran, B. Lohkamp, L. Zoetekouw, N.G.G.M. Abeling, H.L.G. van Tinteren, and A.M. Bosch (2007) Clinical, biochemical and genetic findings in two siblings with a dihydropyrimidinase deficiency. *Mol Genetics and Metabolism* **91**:157-164.
- [16] K. P. Brooks, E.A. Jones, B.D. Kim, and E.B. Sander (1983) Bovine liver dihydropyrimidine amidohydrolase purification, properties and characterization as a zinc metalloenzyme. *Arch Biochem Biophys*. **226**:469-483.
- [17] Kautz, J. and Schnackerz, K.D. (1989) Purification and properties of 5,6-dihydropyrimidine amidohydrolase from calf liver. *Eur J Biochem*. **181**: 431-435.
- [18] T. M. Su and Y.S. Yang (2000) Identification, purification, and characterization of a thermophilic imidase from pig liver. *Protein Expr Purif*. **19**:289-297.
- [19] Kikugawa, M., Kaneko, M., Fujimoto-Sakata, S., Maeda, M., Kawasaki, K., Takagi, T. and Tamaki, N. (1994) Purification, characterization and inhibition of dihydropyrimidinase from rat liver. *Eur J Biochem*. **219**:393-399.
- [20] J. Ogawa, J.M. Kim, W. Nirdonoy, Y. Amano, H. Yamada, and S. Shimizu (1995) Purification and characterization of an ATP-dependent amidohydrolase, N-methylhydantoin amidohydrolase from *Pseudomonas putida* 77. *Eur J Biochem*. **229**: 284-290.

- [21]S.G. Lee, D.C. Lee and M.H. Sung (1994) Isolation of thermostable D-hydantoinase-producing thermophilic *Bacillus sp.SD-1*. *Biotech Lett.* **16**:461-466.
- [22]S.M. Runser and P.C. Meyer (1993) Purification and biochemical characterization of the hydantoin hydrolyzing enzyme from *Agrobacterium species* -A hydantoinase with no 5,6-dihydropyrimidine amidohydrolase activity. *Eur J Biochem.* **213**:1315-1324.
- [23]C.L. Soong, J. Ogawa, M. Honda, S. and Shimizu (2000) Cyclic-imide- hydrolyzing activit of D-hydantoinase from *Blastobacter sp. strain A17p-4*. *Appl Environ Microbiol.* **65**:1459-1462.
- [24]O. May, M Siemann, M. Pietzsch, M. Kiess, R. Mattes, and C. Sylatk (1998) Substrated-dependent enantioselectivity of a novel *hydantoinase from Arthrobacter aureescens* DSM 3745: purification and characterization as new member of cyclic amidases. *J Biotechnol.* **26**:1-13.
- [25]C.Y. Huang, Y.P. Chao and Y.S. Yang (2003) Purification of industrial hydantoinase in one step chromatography step without affinity tag. *Protein Expr. Purif.* **30**: 134-139
- [26]Gojkovic, Z., Rislund, L., Andersen, B., Sandrini, M. P. B., Cook, P. F., Schnackerz, K. D., and Piškur, J. (2003) Dihydropyrimidine amidohydrolases and dihydroorotases share the same origin and several enzymatic properties. *Nucleic Acids Res.* **31**:1683-1692.

Table I

Summary of purification of Nus-His-tagged recombinant hDHP from E. coli.

Step	Total Volume (ml)	Amount (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Yield (%)	Fold Purification
Extract	120	2777.8	203.7	0.07	100	1.0
Histrap Elute After Thrombin Digest ^a	2.5	4.0	101.7	25.21	49	343.7

^a. “Histrap Elute After Thombin Digest” represented the step of purification of hDHP with Histrap ion exchange column as described in “Experimental Procedure”.



Table II*Specific Activity of hDHP and a.r.HYD*

Substrate	Specific Activity(μ mol/min/mg)^b	
	<i>a.r.</i>HYD^a	<i>h</i>DHP
Dihydrouracil	5.08\pm0.06	0.37\pm0.00
Hydantoin	0.18\pm0.01	3.48\pm0.03
Phthalimide	0.76\pm0.00	24.49\pm0.50
Maleimide	4.7\pm0.1	29.72\pm0.54

^a. *a.r.*HYD represented *Agrobacterium radiobacter* hydantoinase. The specific activities of the different substrates of *a.r.*HYD were referred to the thesis of Yi-Rong Chen.

^b. The specific activities of the different substrates of *h*DHP were determined with UV/VIS spectrophotometer as indicated under “Experimental Procedure”, and all the concentration of substrates are 2 mM.

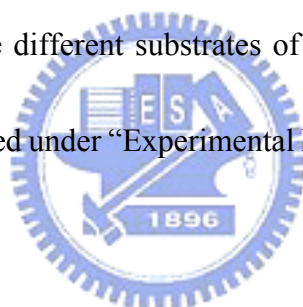


Table III

Kinetic constants of imidase from different sources with phthalimide

	k_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/k_m (mM ⁻¹ min ⁻¹)
<i>hDHP</i>	0.65 ± 0.07	2615 ± 75	4021
<i>a.r.HYD</i>	3.8 ± 1.8	40 ± 15	10
Fish liver	1.6 ± 0.1	1270 ± 80	790
Pig liver	0.8 ± 0.04	33 ± 1	41

^a *a.r.HYD* represented *Agrobacterium radiobacter* hydantoinase. The kinetic constants of imidases from fish (*Oreochromis niloticus*) liver, pig liver, and were *a.r.HYD* referred to the thesis of Cheng-Yang Huang

^b The kinetic constants of *hDHP* with phthalimide as substrate were determined with UV/VIS spectrophotometer as indicated under “Experimental Procedure”.

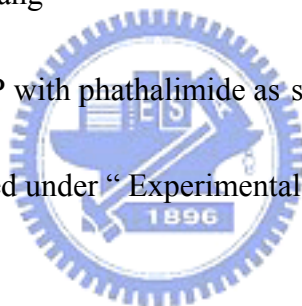


Table IV

Calibration curve determination of molecular weight on Sephacryl S-300 HR

	MW	log(MW)	V_e^a	K_{av}^b
Aldolase	178.00	2.25	60.36	0.28
Catalase	228.00	2.36	59.06	0.26
Ferritin	437.00	2.64	49.58	0.15
Thyroglobulin	692.00	2.84	40.06	0.03

^{a.} V_e represents the retention volume of eluted protein under the condition as indicated under “Experimental Procedures”.

^{b.} K_{av} was calculated by the equation: $K_{av} = (V_e - V_0) / (V_t - V_0)$. The V_t was the column volume as 120 ml and the V₀ was the retention volume of blue dextran 2000 (2000 KDa), whose retention volume was 37.28 ml.

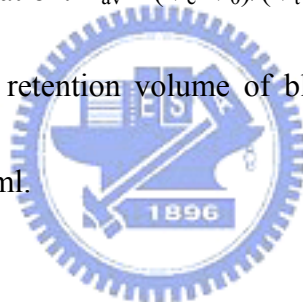


Table V

Physical and chemical parameters of hDHP

	Molecular weight ^a	Theoretical pI ^a	V_e ^b
<i>hDHP</i>	59439	6.58	54.31
<i>a.r.Hydantoinase</i>	49939	5.46	60.40

^{a.} The molecular weight and theoretical isoelectric point(pI) were determined by the ProtParam on ExPASy server (<http://tw.expasy.org/tools/protparam.html>).

^{b.} The V_e indicated the retention volume as described above and determined by Sephacryl S-300 HR.



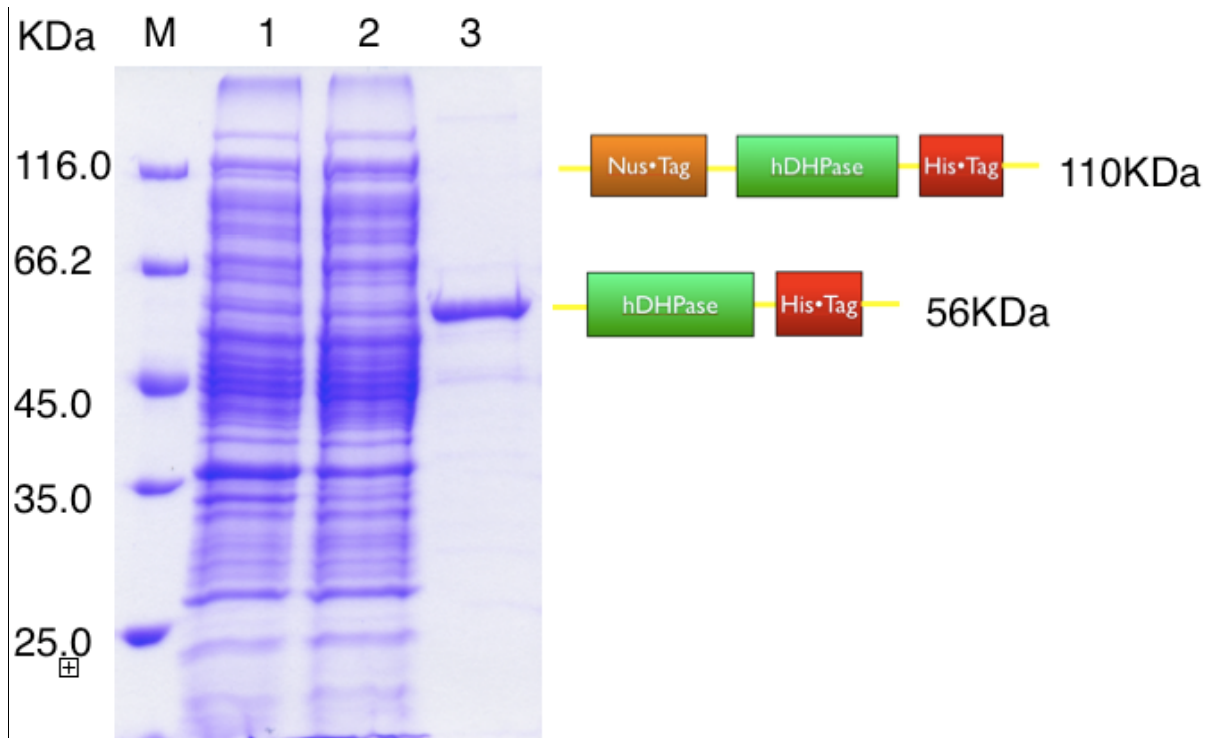


Figure 1. SDS-PAGE (10%) of purified recombinant *hDHP*. Lane 1, pellet; lane 2, supernatant; lane 3, Histrap elute after thrombin on-column digest. The steps of purification of *hDHP* with Histrap ion exchange column are described in “Experimental Procedure”

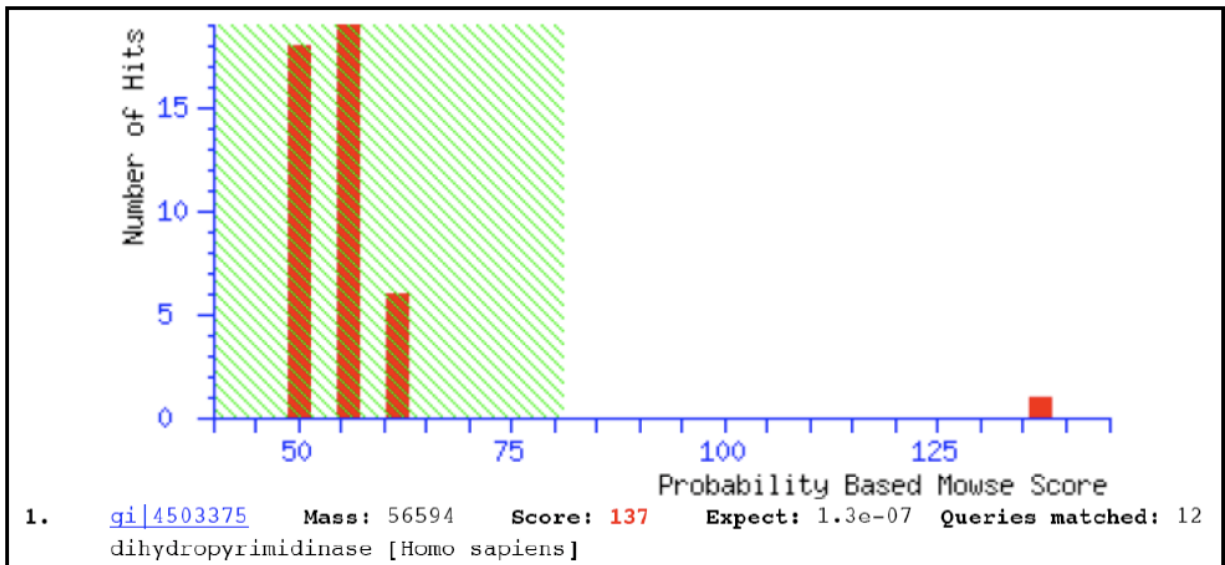


Figure 2. Purified recombinant *h*DHP analyzed by MALDI-TOF after trypsin-in-gel digestion. The purified recombinant *h*DHP on SDS-PAGE were excised and digested in gel with trypsin according to Shevchenko's method (Shevchenko et al., 1996). The digested sample was taken up and analyzed using MALDI-TOF. The results were correlated with the sequence database using the NCBI and SwissProt database and analyzed by Mascot software.

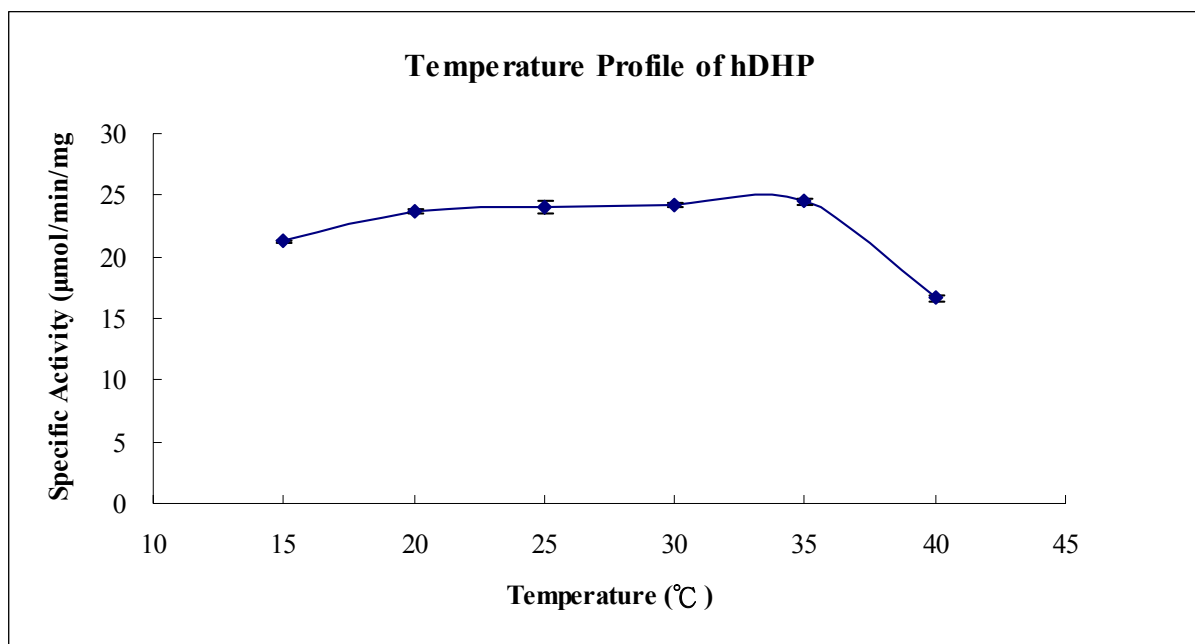


Figure 3. Temperature profile of *hDHP*. The temperature profile of *hDHP* is displayed by the

specific activity of *hDHP* with phthalimide at given temperature measured with a UV/VIS

spectrophotometer as indicated under “Experimental Procedure”.



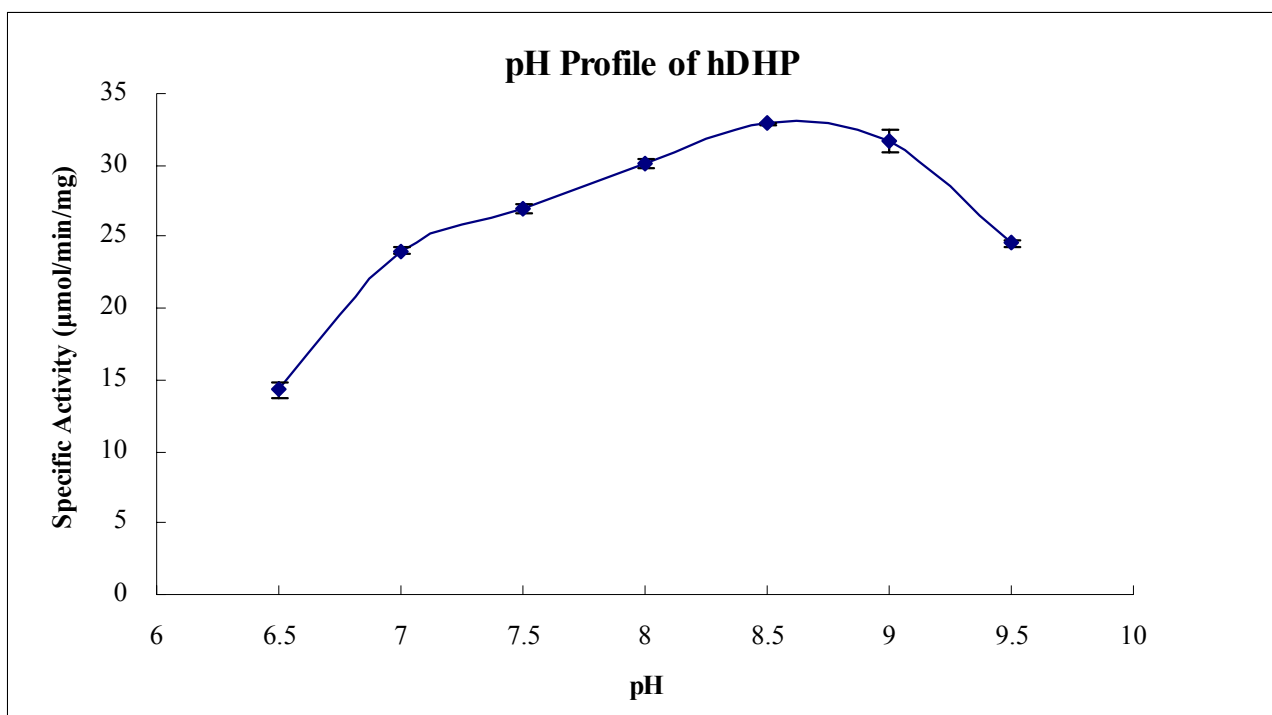
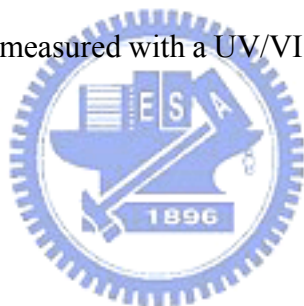


Figure 4. The pH profile of *hDHP*. The pH profile of *hDHP* is displayed by the specific activity of *hDHP* with phthalimide at given pH measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure”.



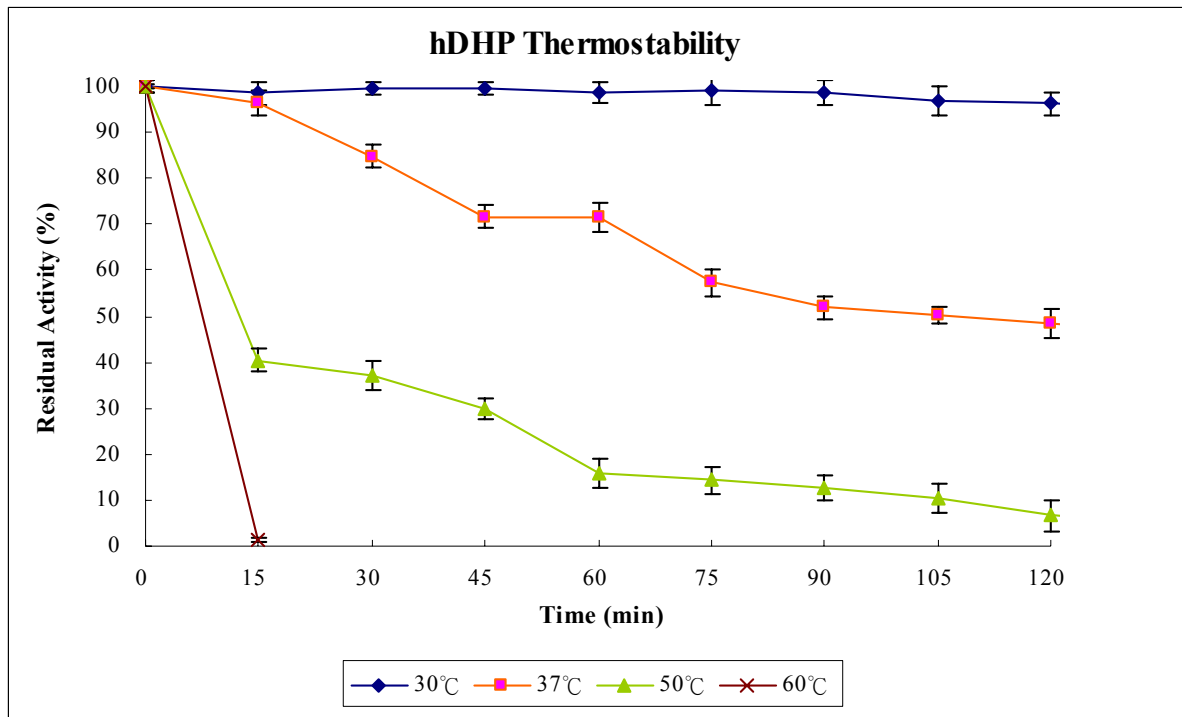


Figure 5. Thermal stability of *hDHP*. The thermal stability of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” after treating at given temperature for time course.

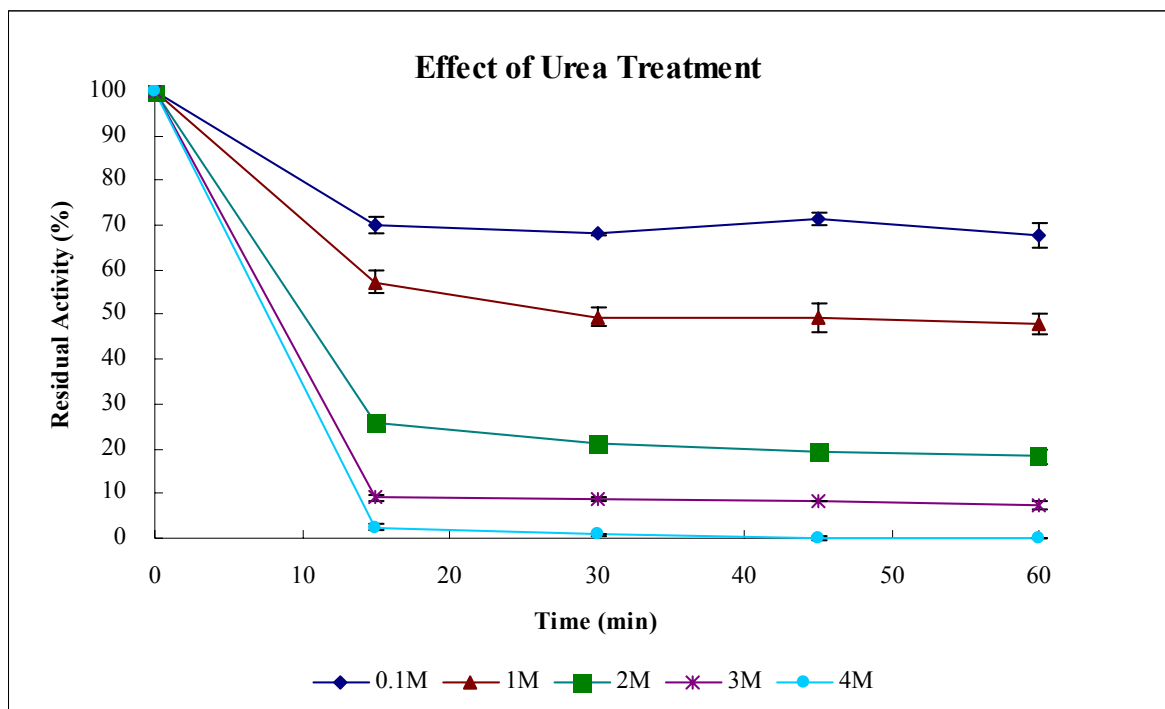


Figure 6. Effect of urea treatment of *hDHP*. The effect of urea treatment of *hDHP* is displayed by the residual activity of *hDHP* with phthalimide measured with a UV/VIS spectrophotometer as indicated under “Experimental Procedure” after treating in given concentration of urea for time course.

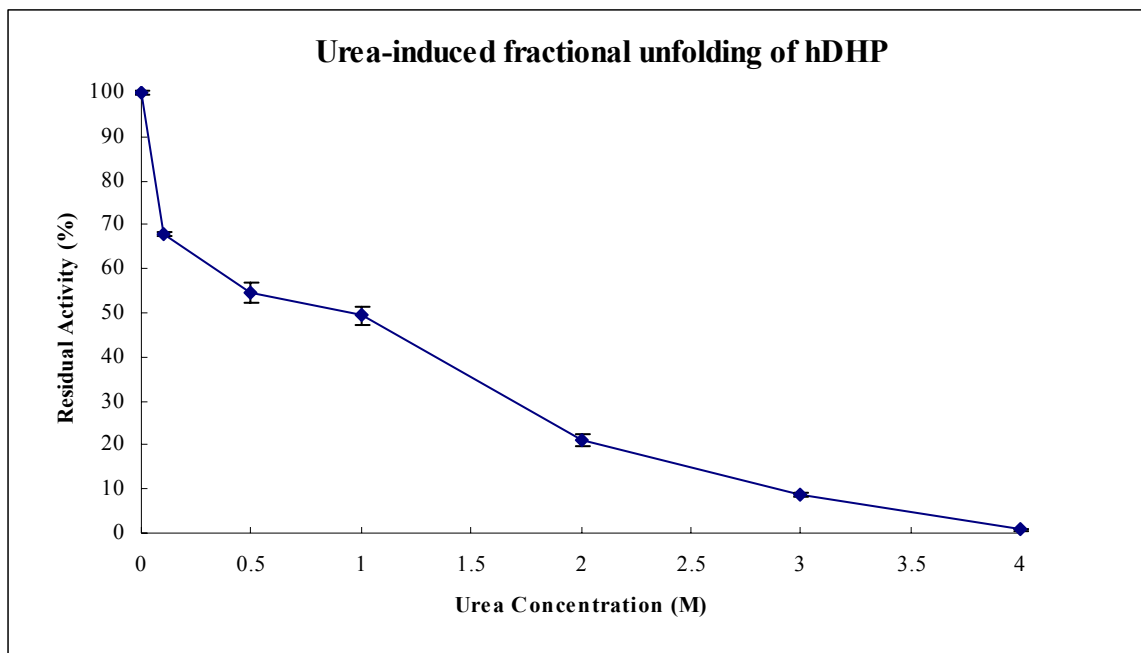


Figure 7. Urea-induced fractional unfolding of *hDHP*. Determine the stability of urea

treatment of *hDHP* by measuring $[Urea]_{50\%}$, the concentration of urea at 50% residual activity

of *hDHP* treated for 30 minutes.



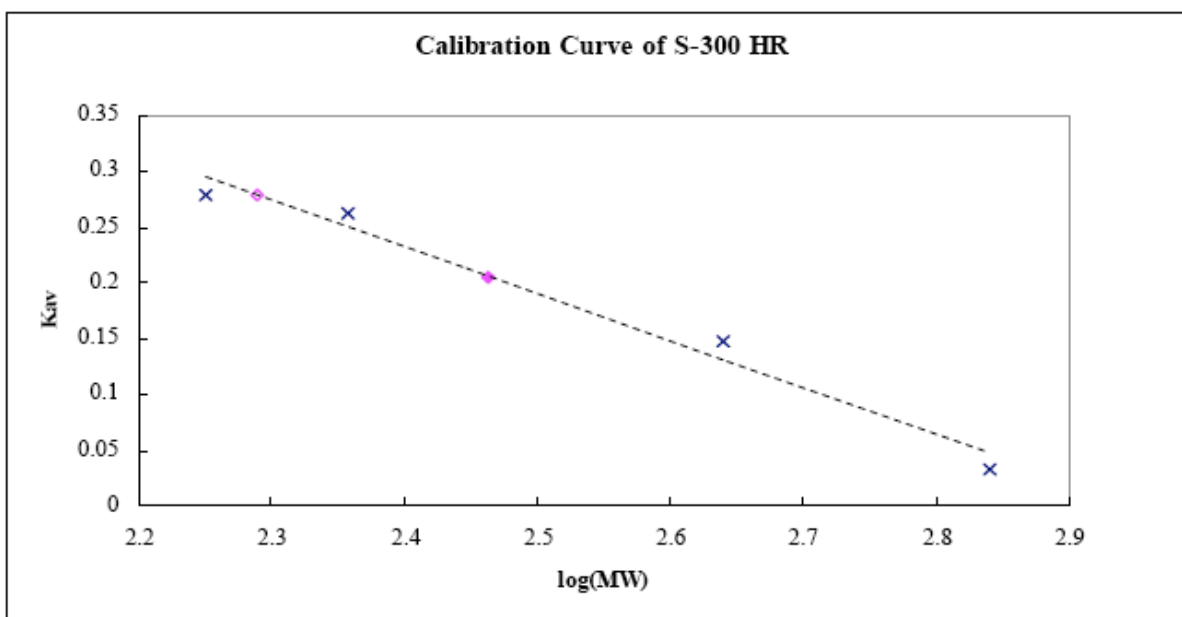


Figure 8. Calibration curve of Sephacryl S-300 HR. A molecular weight calibration curve, which defined the relationship between the elution volumes of a set standards and the logarithm of their respective molecular weights, was determined with Sephacryl S-200 HR. Calibration standard(crisscross) used were High Molecular Weight standard of Gel Filtration Calibration Kit, such as aldolase (178 KDa), catalase (228 KDa), ferritin (437 KDa), thyroglobulin (692 KDa), blue dextran (2000 KDa). The *h*DHP and *a.r.*HYD were closed and open diamonds, respectively. The K_{av} is obtained from equation $K_{av} = (V_e - V_o) / (V_t - V_o)$ as described above.



Model 3730
Version 3.2
KB.bcp
KB 1.2

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hOHPLin43a-1_Colidown
Lane 21

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Page 1 of 6
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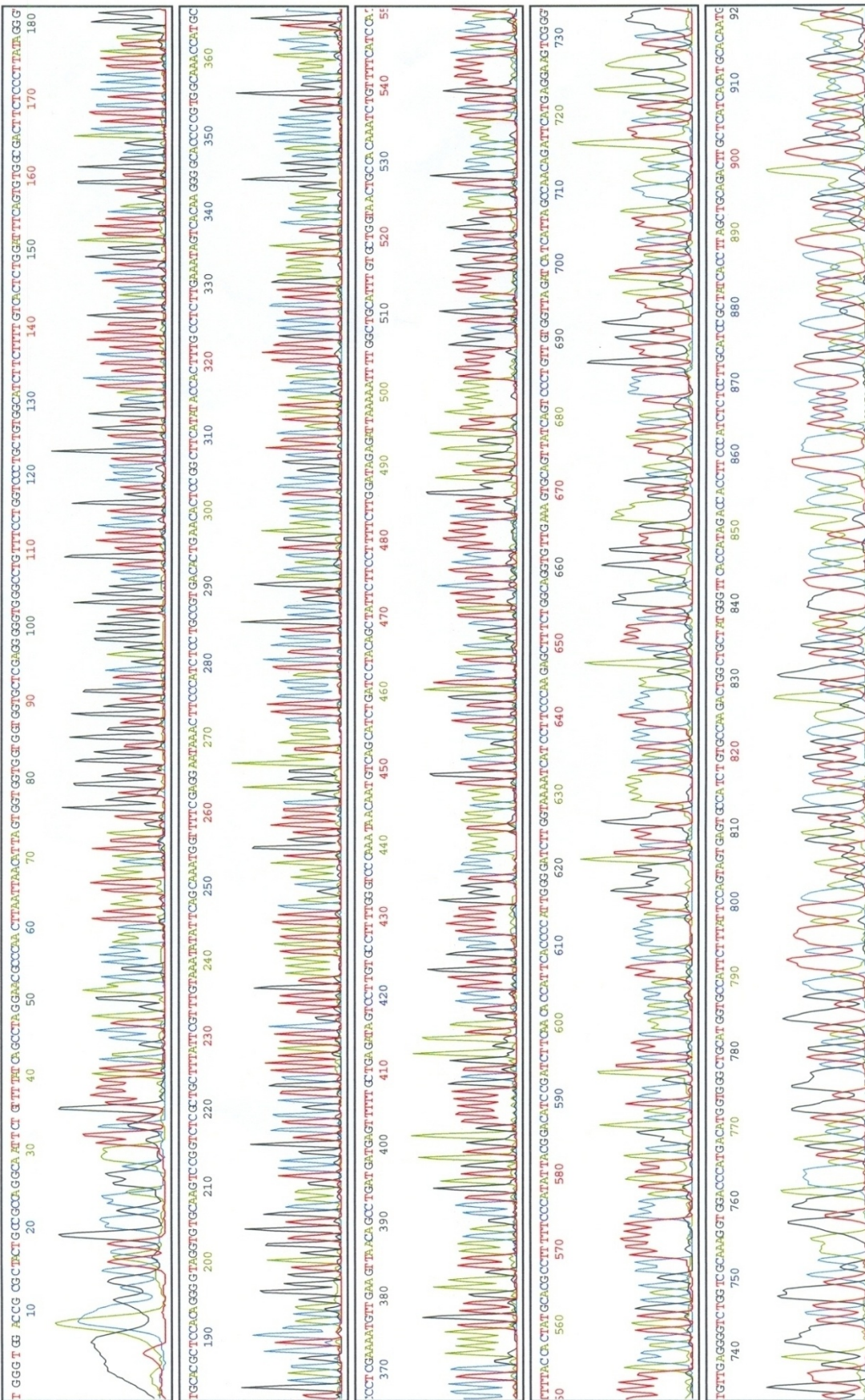


Figure 9. *hdhp-pET-43.1a* (+) construct DNA sequencing. The DNA sequence of *hdhp-pET-43.1a* (+) construct was analyzed according to Sanger Method by MISSION BIOTECH Company.



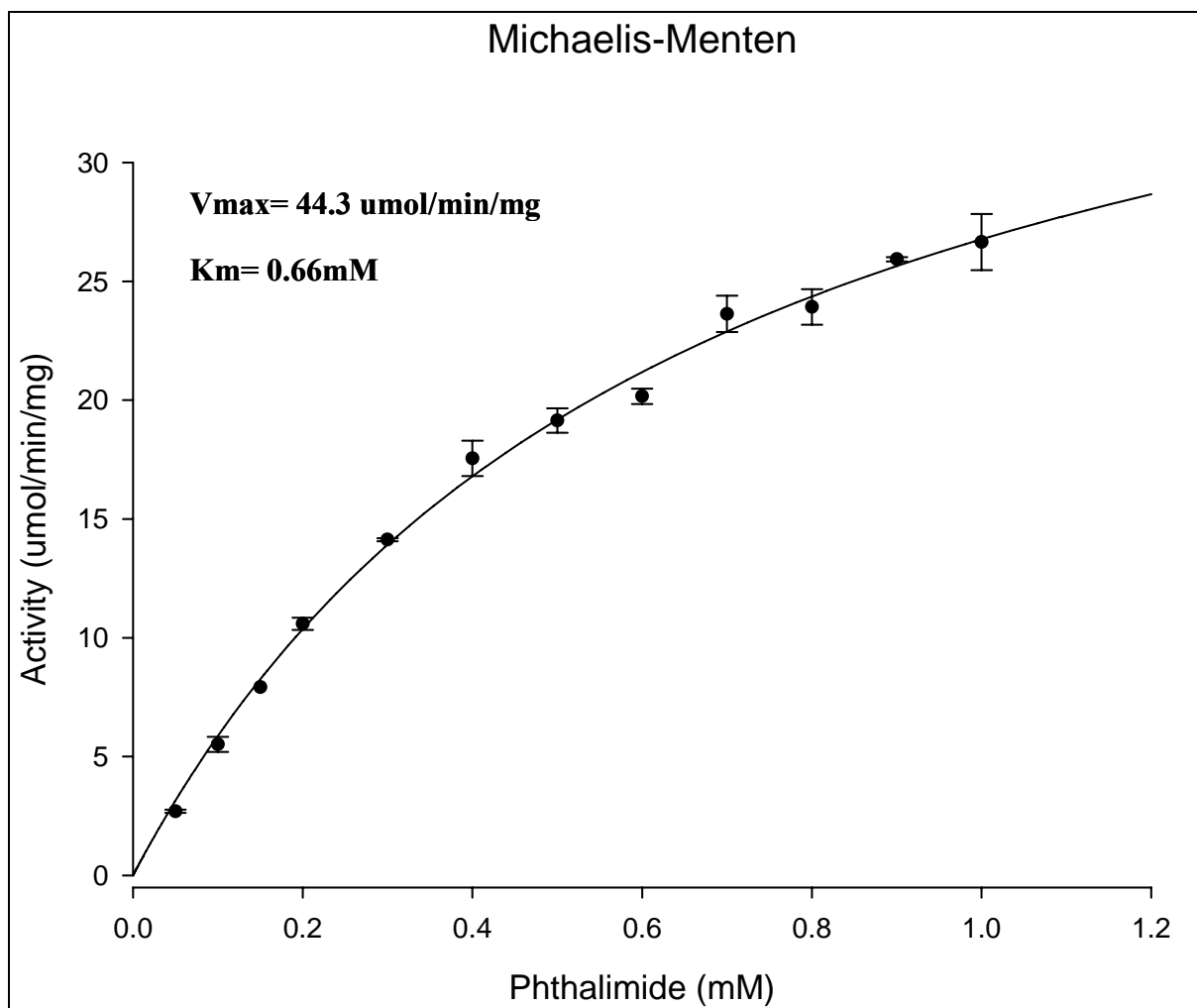


Figure 10. Enzyme Kinetics of *h*DHP with Phthalimide. Measurement of kinetic constant of each substrate was performed by varying the concentration of one substrate, while keeping the other substrate at a fixed and near saturating concentration. The apparent K_m and V_{max} were determined using nonlinear regression by Sigmaplot 2001,V7.0 and Enzyme Kinetics Module, V1.1.

Appendix



Appendix 1. The structure organization and chromosome localization of *hdhp* gene.

(http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000147647)

```

atggcggcggccctcgcggctcctgatccgcgggggtcgcgtggfcaacgatgactctcgg
M A A P S R L L I R G G R V V N D D F S
gaggtggcgcagctgctggaggacggcgtagtgcgggcaactcgggcacgacctgctg
E V A D V L V E D G V V R A L G H D L L
cctcccggggcctcctcgcgggctcgcggctcctcgcagccgcggcaagctcgtctg
P P G G A P A G L R V L D A A G K L V L
cccggaggcatcgacacacacacgcacatgcagttccccctcatgggctcgcggctccatc
P G G I D T H T H M Q F P F M G S R S I
gacgacttccaccagggcaccaaggctgctctcaggaggcaccaccatgattattgat
D D F H Q G T K A A L S G G T T M I I D
ttcggcatctctcagaaaggtagctcctcattgagccctcgagacctggcgaagctgg
F A I P Q K G G S L I E A F E T W R S W
gctgatcccaaagtttgcctgcactacagccttcatgtggcagtgacgtggaggatgac
A D P K V C C D Y S L H V A V T W W S D
caggtfaaagaagaatgaaaatccttggcgaagataaagggttaactcttcaagatg
Q V K E E M K I L V Q D K G V N S F K M
ttatggcctataaagatctgtacatggtagacagacctggagctgtacgaagcctctct
F M A Y K D L Y M V T D L E L Y E A F S
cggctcaaggaaatggagcaatgcccaggctccatgcggaaaatggagacttaattgca
R C K E I G A I A Q V H A E N G D L I A
gagggagc aaagaagatgttggctctgggga taacaggccctgagggccacgacctgtgc
E G A K K M L A L G I T G P E G H E L C
cgcccagaggcagtgaggcagaggccacgctgagagccatcaccatagccagcgtgtg
R P E A V E A E A T L R A I T I A S A V
aacgtctctctacatgtgcatgtgatgagcaagctcgcagctaaagtgatagcggat
N C P L Y I V H V M S K S A A K V I A D
gcaaggagagatgggaagggtgctctatggtagaacctatgcagccagctctggcacagat
A R R D G K V V Y G E P I A A S L G T D
ggcactcactactggaaataagaatggcaccatgcagccaccatgcatgggtccacct
G T H Y W N K E W H H A A H H V M G P P
ttggaccagaccctcaacaccgacttctcctgaatctgttggcctaattgatgatcta
L R P D P S T P D F L M N L L A N D D L
accacaacagggactgataactgcacttcaacacctgccagaaagctcttgggaaggat
T T T G T D N C T F N T C Q K A L G K D
gat tttaccagatccccaatgggtgaaatgggttgaagatcggatgctccgtaatatgg
D F T K I P N G V N G V E D R M S V I W
gaaaaaggcgtgcatagtgtaaaatggatgaaaacagatttggcagttaccagcac
E K G V H S G K M D E N R F V A V T S T
aatgcagc caaaat tttaatctctatccaagaaaaggaaagaatagctgtaggatcagat
N A A K I F N L Y P R K G R I A V G S D
gctgacattgttatttgggacc caaaaggca caaggactatctcagcaaaaactcatcat
A D I V I W D P K G T R T I S A K T H H
caggtgttaactcaacat ttcgagggcattgttggccacgggggtgccccttgtgact
Q A V N F N I F E G M V C H G V P L V T
atttcaagaggc aaagtggat atgnaagccggagtgtcagtgctcaggcaggagatggg
I S R G K V V Y E A G V F S V T A G D G
aagtttatctctgaaaaccttgc tgaat atatttacaacgaataa agcagcggagac
K F I P R K P F A E Y I Y K R I K Q R D
cggacttgcacacctaccctgtggagcgtgcacctataaggagaagtcgccacctg
R T C T P T P V E R A P Y K G E V A T L
aaatccagagtgaanaagaagatgcacagcaggaccagaaacaggcccacccctga
K S R V T K E D A T A G T R K Q A H P -

```

Appendix 2. Nucleotide sequence of cDNA and deduced amino acid sequence of *hdhp*

gene. Analyze the nucleotide sequence of cDNA and deduced amino acid sequence of *hdhp* gene by Translate tool on the ExPASy sever(<http://ca.expasy.org/tools/dna.html>).

Species	Sequence identity(%)								
	<i>R. norvegicus</i>	<i>M. musculus</i>	<i>T. nigroviridis</i>	<i>D. discoideum</i>	<i>S. kuyveri</i>	<i>B. pickettii</i>	<i>A. radiobacter</i>	<i>hCRMP1</i>	<i>hCRMP2</i>
<i>H. sapien</i>	90	88	73	58	28	36	36	58	57
<i>R. norvegicus</i>		94	72	56	28	37	37	56	55
<i>M. musculus</i>			72	56	28	37	36	55	55
<i>T. nigroviridis</i>				58	28	36	35	55	54
<i>D. discoideum</i>					29	35	34	46	47
<i>S. kuyveri</i>						37	36	26	24
<i>B. pickettii</i>							92	35	31
<i>A. radiobacter</i>								35	31
<i>hCRMP1</i>									73

Appendix 3. The identity of the amino acid sequences of imidase from other species and *Homo Sapien*. Analyze the the amino acid sequences of imidase from other species and *Homo Sapien* by ClustalW2(<http://www.ebi.ac.uk/Tools/clustalw2/index.html>)

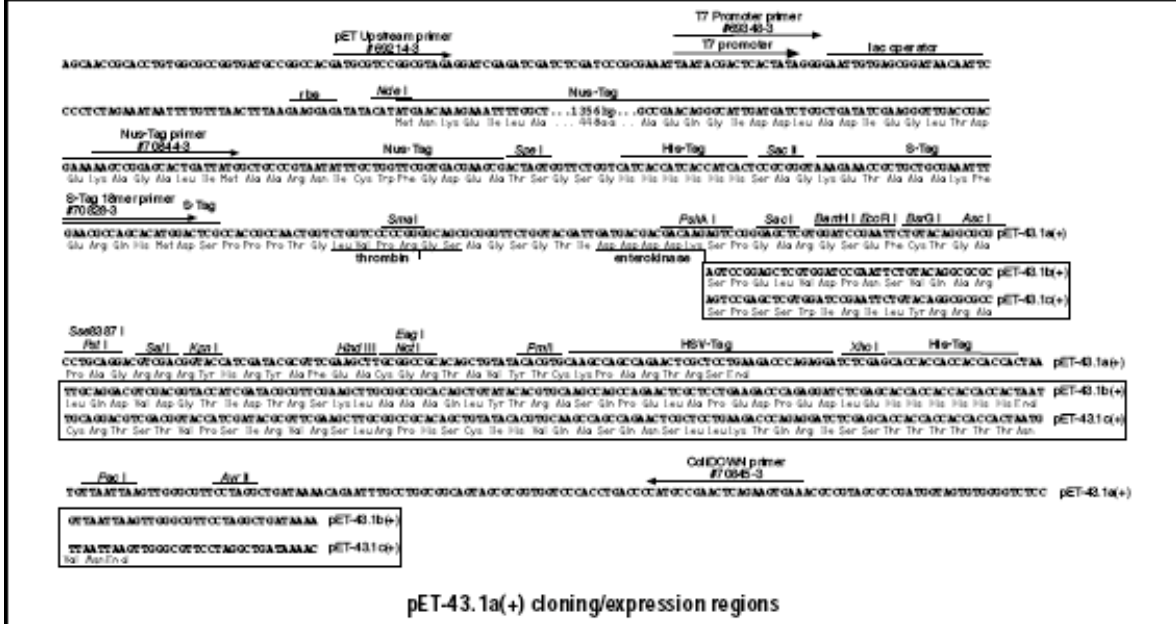
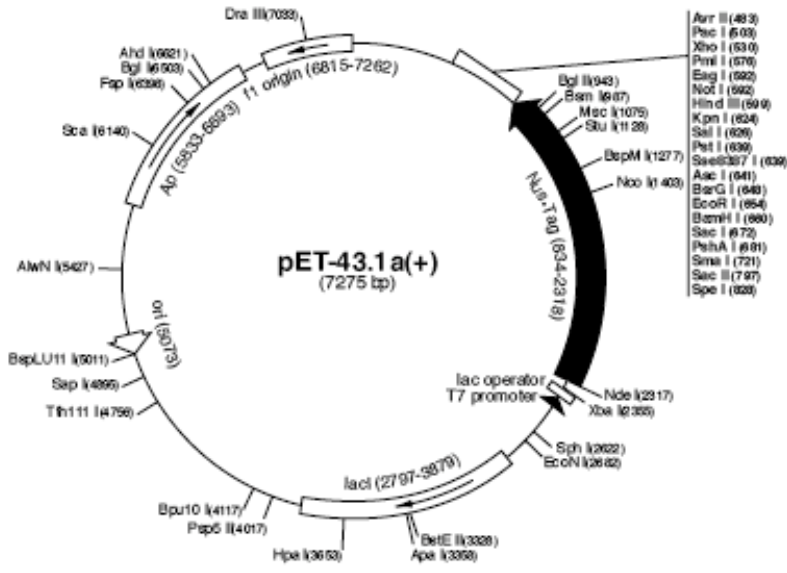
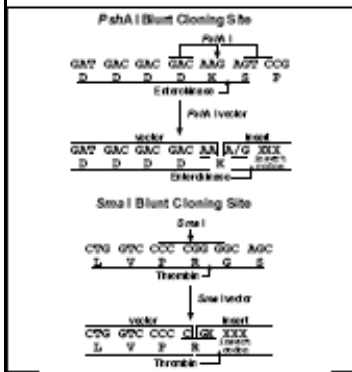
pET-43.1a-c(+) Vector

TB288 09/00

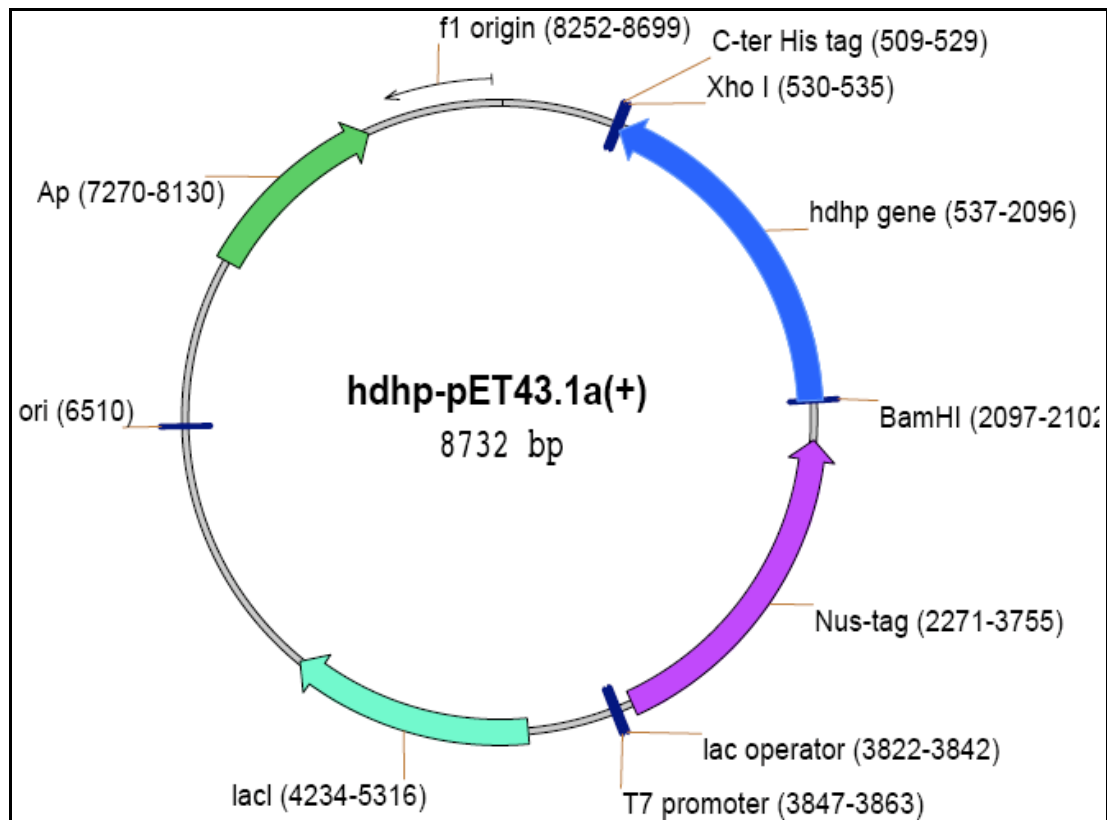
	Cat. No.
pET-43.1a(+)-DNA	70939-3
pET-43.1b(+)-DNA	70940-3
pET-43.1c(+)-DNA	70941-3

pET-43.1a(+)-sequence landmarks	
T7 promoter	2390-2406
T7 transcription start	2390
Nus*Tag™ coding sequence	834-2318
His*Tag* coding sequence	801-818
S*Tag* coding sequence	747-791
Multiple cloning sites (<i>Sma</i> I- <i>Xho</i> I)	721-686
HSV*Tag* coding sequence	537-572
His*Tag* coding sequence	513-530
T7 terminator	27-73
<i>lac</i> I coding sequence	2797-3879
pBR322 origin	5073
<i>bla</i> (Ap) coding sequence	5833-6663
<i>fl</i> origin	6815-7262

The pET-43.1 series of vectors are designed for cloning and high-level expression of peptide sequences fused with the 491 aa Nus*Tag™ protein. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The *fl* origin is oriented so that infection with helper phage will produce virions containing single stranded DNA that corresponds to the coding strand. Therefore, single stranded sequencing should be performed using the ColIDOWN primer (cat. no. 70845-3). Vector encoded sequence can be completely removed when cloning into the *Pst*II or *Sma*I sites (as shown below) by cleaving the Nus*Tag fusion protein with enterokinase or thrombin, respectively.



Appendix 4. The map of pET-43.1a(+) vector (Novagen).



Appendix 5. The map of hdhp-pET-43.1a (+) construct. Clone the PCR products of cDNA of *hdhp* gene of *Homo sapien* on pCMV-sports6 vecto into pGEM-T Vector (Promega) and add restriction sites of BamH I and Xho I to the termini of amplified DNA with the pairing primers *hdhp*-F and *hdhp*-R . Subclone the DNA fragment containing the *hdhp* gene on pGEM-T vector into the modified *pET-43.1a(+)* vector by restriction enzyme, BamH I & Xho I, and T4 DNA Ligase.

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ProtParam

User-provided sequence:

```

      10      20      30      40      50      60
GSAGSGTIDD DDLSPGARGS MAAPSRLLR GGRVVNDDFS EVADVLVEDG VVRALGHDL
      70      80      90     100     110     120
PPGGAPAGLR VLDAAGKLVL PGGIDTHTHM QFPFMGSRSI DDFHQGTKAA LSGGTTMIID
      130     140     150     160     170     180
FAIPQKGGSL IEAFETWRSW ADPKVCCDYS LHVAVTWWSL QVKEEMKILV QDKGVNSFKM
      190     200     210     220     230     240
FMAYKDLYMV TDLELYEAFS RCKEIGAIAQ VHAENGLIA EGAKKMLALG ITGPEGHEL
      250     260     270     280     290     300
RPEAVEAEAT LRAITIASAV NCPLYIVHVM SKSAAKVIAD ARRDGKVVY G EPIAASLGT
      310     320     330     340     350     360
GTHYWNKEWH HAAHHVMGPP LRPDPSTPDF LMNLLANDDL TTTGTDNCTF NTCQKALGK
      370     380     390     400     410     420
DFTKIPNGVN GVEDRMSVIW EKVHSGKMD ENRFVAVTST NAAKIFNLYP RKGRIAVGSD
      430     440     450     460     470     480
ADIVIWDPKG TRTISAKTHH QAVNFNIFEG MVCHGVPLVT ISRGKVVEA GVFSVVTAGDG
      490     500     510     520     530     540
KFIPRPFAE YIYKRIKQRD RTCTPTPVER APYKGEVATL KSRVTKEDAT AGTRKQAHPL
GHHHHHH

```

[References](#) and [documentation](#) are available.

new Please note the [modified algorithm for extinction coefficient](#).

Number of amino acids: 547

Molecular weight: 59439.6

Theoretical pI: 6.58

Amino acid composition:

```

Ala (A) 55 10.1%
Arg (R) 27 4.9%
Asn (N) 16 2.9%
Asp (D) 39 7.1%
Cys (C) 9 1.6%
Gln (Q) 10 1.8%
Glu (E) 27 4.9%

```

Gly (G)	51	9.3%
His (H)	24	4.4%
Ile (I)	30	5.5%
Leu (L)	35	6.4%
Lys (K)	34	6.2%
Met (M)	15	2.7%
Phe (F)	19	3.5%
Pro (P)	28	5.1%
Ser (S)	28	5.1%
Thr (T)	36	6.6%
Trp (W)	8	1.5%
Tyr (Y)	12	2.2%
Val (V)	44	8.0%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 66

Total number of positively charged residues (Arg + Lys): 61

Atomic composition:

Carbon	C	2629
Hydrogen	H	4128
Nitrogen	N	744
Oxygen	O	782
Sulfur	S	24

Formula: C₂₆₂₉H₄₁₂₈N₇₄₄O₇₈₂S₂₄

Total number of atoms: 8307

Extinction coefficients:

Extinction coefficients are in units of M⁻¹ cm⁻¹, at 280 nm measur

Ext. coefficient	62380
Abs 0.1% (=1 g/l)	1.049, assuming ALL Cys residues appear as hal

Ext. coefficient	61880
Abs 0.1% (=1 g/l)	1.041, assuming NO Cys residues appear as half

Estimated half-life:

The N-terminal of the sequence considered is G (Gly).

The estimated half-life is: 30 hours (mammalian reticulocytes, in
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 24.09
This classifies the protein as stable.

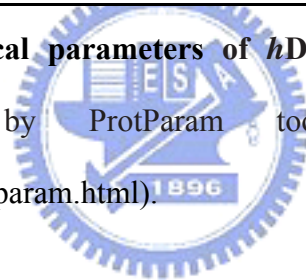
Aliphatic index: 79.73

Grand average of hydropathicity (GRAVY): -0.242

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Appendix 6. Physico-chemical parameters of *hDHP*. Analyze the Physico-chemical parameters of *hDHP* by ProtParam tool on the ExPASy server (<http://tw.expasy.org/tools/protparam.html>).



R.norvegicus	LVLPGGIDTHHEM ⁶⁷ QFFPMGS-QSVDDFHQGTKAALAGGTTMIIDFAIPQKGS ⁶⁹ SLIEAFET	116
M.musculus	LVLPGGIDTHHEM ⁶⁷ QFFPMGS-QSVDDFYQGTKAALAGGTTMIIDFAIPQKGS ⁶⁹ SLIEAFET	116
H.sapiens	LVLPGGIDTHHEM ⁶⁷ QFFPMGS-RSIDD ¹⁵⁹ FHQGTKAALSGGTTMIIDFAIPQKGS ¹⁹² SLIEAFET	116
T.nigroviridis	LVI ¹⁹² PGGIDTHHEMELAFMGT-RAVDDFHIGTKAALAGGTTMILDFVMTQKQ ²⁴⁸ SLLEAYDL	112
D.discoideum	LLLPGGIDTHHEFQLPFMGT-VSVDDFDIGTQAAVAGGTTPIIDFVIPT ²⁴⁸ RGQSLLEAYDQ	115
B.pickettii	YVFPGGIDVHHEVETV ¹⁹² SFNT-QSADTFATATVAAACGGTTTIVDFCQ ²⁴⁸ QDRGHS ³²⁶ LAEAVAK	106
A.radiobacter	YVFPGGIDVHHEVETV ¹⁹² SFNT-QSADTFATATVAAACGGTTTIVDFCQ ²⁴⁸ QDRGHS ³²⁶ LREAVAK	106
S.kluyveri	FITPGGIDAHV ¹⁹² DEPLKLLGDVVD ²⁴⁸ TMEHATRS ³²⁶ AVAGGTTTVVAFSTQDVSKK ³⁶⁵ GPSALAE	112
R.norvegicus	-----WRNWADPKVCCDYS ¹⁹² LHVAVTW ²⁴⁸ WS-----DKVKEEMKTLAQDKGVNS ³²⁶ EKKMF ³⁶⁵ MAY	164
M.musculus	-----WRNWADPKVCCDYS ¹⁹² LHVAVTW ²⁴⁸ WS-----DKVKEEMKTLARDKGVNS ³²⁶ EKKMF ³⁶⁵ MAY	164
H.sapiens	-----WRSWADPKVCCDYS ¹⁹² LHVAVTW ²⁴⁸ WS-----DQVKEEMKILVQDKGVNS ³²⁶ EKKMF ³⁶⁵ MAY	164
T.nigroviridis	-----WRKTADPKVCCDYS ¹⁹² LHVAVTW ²⁴⁸ WS-----DEVKDEMRTLAQERGVNS ³²⁶ EKKMF ³⁶⁵ MAY	160
D.discoideum	-----WKKWADEKVNCDYS ¹⁹² LHVAVITW ²⁴⁸ WS-----EQVSR ³²⁶ EMEILVKERGVNS ³⁶⁵ EKKMF ³⁶⁵ MAY	163
B.pickettii	-----WDGMAGGKSAIDYGYHIIVLDPT-----DSVIEE ¹⁹² LEVLP-DLGITSP ²⁴⁸ XV ³²⁶ FMAY	153
A.radiobacter	-----WDGMAGGKSAIDYGYHIIVLDPT-----DSVIEE ¹⁹² LEVLP-DLGITSP ²⁴⁸ XV ³²⁶ FMAY	153
S.kluyveri	SVKLDVDEYSEQ ¹⁹² TLYCDYGLHLILFQIEKPSVEARELLDVQLQAAYNDYGVSSV ³²⁶ KMF ³⁶⁵ MTY	172
R.norvegicus	KDLYMVQDQQMYAAFSQCKEIG ¹⁹² AIQVHAENGDLIAEGAKKMLALGITGPEGHEL ²⁴⁸ CRPEA	224
M.musculus	KGLYMVQDEQLYAAFSQCKEIG ¹⁹² AIQVHAENGDLIAEGAKKMLALGITGPEGHEL ²⁴⁸ CRPEA	224
H.sapiens	KDLYMVTDLLEYAFSRCKEIG ¹⁹² AIQVHAENGDLIAEGAKKMLALGITGPEGHEL ²⁴⁸ CRPEA	224
T.nigroviridis	KGLFMLR ¹⁹² DDLEYAVFSRCKEIVGAIQVHAENGDLIAEGAKKMLSLGITGPEGHEL ²⁴⁸ CRPEA	220
D.discoideum	KNSFMVTDQEMYHIFKRCKELGAIQVHAENGDMVFE ¹⁹² GQKML ²⁴⁸ EMGITGPEGHEL ³²⁶ SRPEA	223
B.pickettii	RCNMIDDVTL ¹⁹² LKTL ²⁴⁸ DKAVK ³²⁶ TGSLVMVHAENGDAADYL ³⁶⁵ RDKFV ³⁶⁵ AE ³⁶⁵ GKTAPIYHALSR ³⁶⁵ PPR	213
A.radiobacter	RCNMIDDVTL ¹⁹² LRL ²⁴⁸ DKAAKTGSLVMVHAENGDAADYL ³⁶⁵ RDKFV ³⁶⁵ ADGKTAPIYHALSR ³⁶⁵ PPR	213
S.kluyveri	PGLQIS-DYDIMSAMYAT ¹⁹² RKNGFTTMLHAENGDMV ²⁴⁸ KWMI ³²⁶ EAL ³⁶⁵ EEQGLT ³⁶⁵ DAYYHG ³⁶⁵ VS ³⁶⁵ RPSI	231
R.norvegicus	VEAEATLRAITIASAVNCP ¹⁹² LYIVHVM ²⁴⁸ SKSAAKVIADAKREGKV ³²⁶ VYGEPIAAG-----	276
M.musculus	VEAEATLRAITIASAVNCP ¹⁹² LYIVHVM ²⁴⁸ SKSAAKVVADARRAGNV ³²⁶ VYGEPIAAG-----	276
H.sapiens	VEAEATLRAITIASAVNCP ¹⁹² LYIVHVM ²⁴⁸ SKSAAKVIADARRDGKV ³²⁶ VYGEPIAAS-----	276
T.nigroviridis	VEAEATQRAITIASAVNCP ¹⁹² LYIVHVM ²⁴⁸ SKSAADVSKARKDGRV ³²⁶ VGEPIAAS-----	272
D.discoideum	LEAEATNRAIV ¹⁹² ADSVCTPVYIVHVM ²⁴⁸ QSIGAADVIC ³²⁶ KHRKEG ³⁶⁵ VRVYGEPIAAG-----	275
B.pickettii	VEAEATARALALAEIVNAPIYIVHVTCEESLEE ¹⁹² VMRAKSRG ²⁴⁸ VRALAE ³²⁶ TCTHY-----	265
A.radiobacter	VEAEATARALALAEIVNAPIYIVHVTCEESFDEL ¹⁹² MRKARG ²⁴⁸ VHALAE ³²⁶ TCTQY-----	265
S.kluyveri	VEGEATNRAITLATTMDTPILFVH ¹⁹² VSSPQAAEVIKQAQTKGLK ²⁴⁸ VYAETCPQYALLSDAIT	291
R.norvegicus	DLTTTGS ¹⁹² DNCTFN-TCQKALG-----KDDFTKI ²⁴⁸ PNGVNGVEDRMS ³²⁶ VIWEKGVH	365
M.musculus	DLTTTGS ¹⁹² DNCTFN-TCQKALG-----KDDFTKI ²⁴⁸ PNGVNGVEDRMS ³²⁶ VIWEKGVH	365
H.sapiens	DLTTTGD ¹⁹² NCTFN-TCQKALG-----KDDFTKI ²⁴⁸ PNGVNGVEDRMS ³²⁶ VIWEKGVH	365
T.nigroviridis	DLTLTGIDNCTFS-RCQKALG-----KDDFTRI ¹⁹² PNGVNGVEDRMS ²⁴⁸ VIWEKGVH	361
D.discoideum	DLDCVGD ¹⁹² NCTFC-ADQKAME-----KDDFTKI ²⁴⁸ PNGVNGVEDRMS ³²⁶ IVWENGVN	364
B.pickettii	VFETVSSD ¹⁹² HCSWLFK ²⁴⁸ GHKDRG-----RNDFRAI ³²⁶ PNGAPGVEERLMMVYQ-GVN	352
A.radiobacter	VLETVSSD ¹⁹² HCSWLFEGHKDRG-----RNDFRAI ³²⁶ PNGAPGVEERLMMVYQ-GVN	352
S.kluyveri	TPTIVGSD ¹⁹² HCSYNYEKTSTASKHRAFD ²⁴⁸ PENKNKGEFRYIPNGLPGVCTRMPLLYDYGYL	410

Appendix 7. Multiple amino acid sequences alignment of imidase from other species

and *Homo Sapien*. Analyze the Multiple amino acid sequences alignment of imidase from

other species and *hDHP* by ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

The labeled amino acids, such as His67, His 69, Lys159, His192, His 248, and Asp 326 are responsible for metal binding.