

科技部補助

大專學生研究計畫研究成果報告

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* 計畫 : 二氫嘧啶水解酵素與其抑制劑 kaempferol 的複合結晶 *
* 名稱 : 結構之解析:催化功能、結構特性與抑制機制之研究 *
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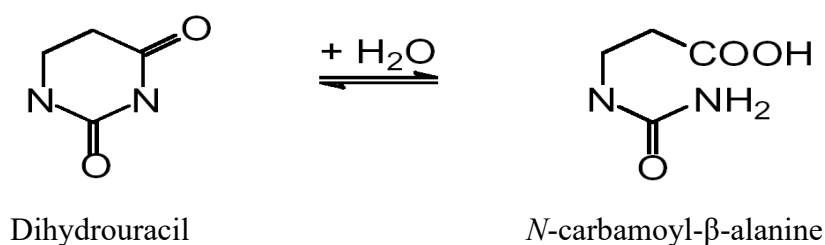
(一) 摘要

本計畫欲利用二氫嘧啶水解酵素的分子結構來研究其抑制劑 kaempferol 的結合與抑制催化反應等相關特性，目前此計畫的初步成果包含此酵素的 X-ray 結晶結構之解析以及結構特性已發表於 SCI 期刊，本人為此期刊論文的第一作者 [Tzeng, C.T. (曾敬婷), Huang, Y.H., Huang, C.Y.* (2016) Crystal structure of dihydropyrimidinase from *Pseudomonas aeruginosa* PAO1: Insights into the molecular basis of formation of a dimer. *Biochem. Biophys. Res. Commun.*, 478, 1449-1455.]。二氫嘧啶水解酵素 (dihydropyrimidinase) 是代謝 DNA 鹼基途徑中極重要的酵素，並廣泛存在於各生物間，然而細菌與真核生物來源的二氫嘧啶水解酵素的差異極大，因此若是能開發具有可以抑制細菌二氫嘧啶水解酵素活性的化合物，則可能可以阻斷細菌 DNA 鹼基代謝與生合成進而影響細菌的生長，就如同與已廣泛使用的磺胺劑抗生素相當。此計畫執行過程經過相當多的結晶條件篩選，終於找到數個不同的條件可將來自於綠膿桿菌的二氫嘧啶水解酵素結晶並利用位於新竹科學園區的國家同步輻射中心的 13C1 光束線得到其繞射數據，接著使用分子置換法解出了此酵素的 X-ray 繞射晶體結構 (解析度 2.1 Å; PDB ID: 5E5C)。雖然此酵素的三級結構與其他家族酵素一樣皆擁有一典型的 $(\beta/\alpha)_8$ -barrel structure 與一延伸的 β -sandwich domain，然而令人驚訝的是，此酵素為雙套體(dimer)而非四套體(tetramer)，因而讓我們另有興趣研究其四級結構之不同處與成因為何。我們利用胺基酸序列-結構對應-演化保留分析發現，原本高度保留的雙套體-雙套體介面 (dimer-dimer interface) 在綠膿桿菌的二氫嘧啶水解酵素中都不保留，然而在其 C 端卻多出一段額外延伸的區域，可能用於穩定其雙套體結構。我們根據結晶結構分析與膠體過濾法(gel filtration analysis)分析後的結果提出了”大象與章魚模型”(elephant versus octopus)，用此簡單易懂的卡通模型來說明為何其他的家族酵素皆為四套體而本酵素卻是雙套體。另外，在此計畫結案前，此酵素與抑制劑 kaempferol 的複合結晶結構仍在解析階

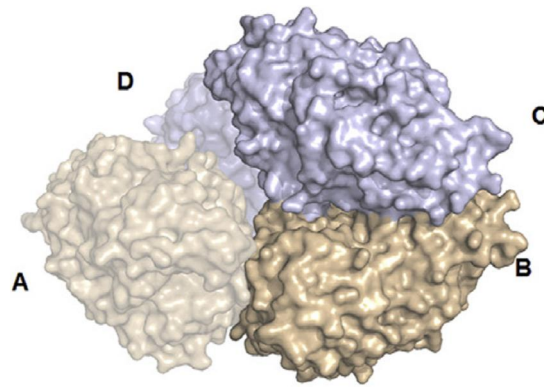
段，我們亦利用分子對接法(molecular docking)來模擬並試圖說明此抑制劑與綠膿桿菌的二氫嘧啶水解酵素的結合模型。

(二) 緒論

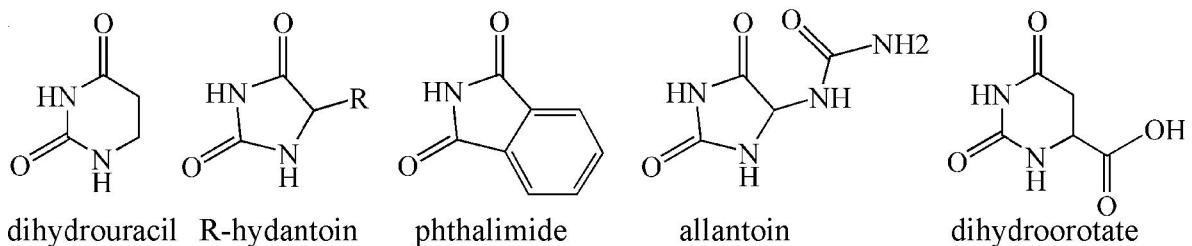
二氫嘧啶水解酵素(dihydropyrimidinase)廣泛存在於細菌、真菌、植物與動物中，催化二氫嘧啶開環並產生溶解度高的產物，為 DNA 鹼基合成中極關鍵的反應之一。圖一說明二氫嘧啶水解酵素可催化 dihydrouracil 與 dihydrothymine 變成 *N*-carbamoyl- β -alanine 與 *N*-carbamyl- β -amino-isobutyrate，這些產物在 DNA 鹼基代謝途徑與各種生理反應中均扮演極重要的角色(1-3)。二氫嘧啶水解酵素家族成員，包括真核與原核生物來源，目前均已知為四套體(tetramer)，其中每個次單元(subunit)均擁有一典型的 $(\beta/\alpha)_8$ -barrel structure 與一延伸的 β -sandwich domain，並且均含有兩個金屬離子(圖二)。由於二氫嘧啶水解酵素所催化的水解反應是將環狀含氮化合物例如在雜環上的醯亞胺等官能基做專一的切割，因此被歸類在 cyclic amidohydrolase family 的一員中，其中在此家族裡的酵素尚包括 allantoinase、dihydroorotase、hydantoinase 與 imidase (1,4,5)；歸類在 cyclic amidohydrolase 家族的酵素雖然催化相似的化合物，然而其受質專一性皆不同(圖三)。



圖一：二氫嘧啶水解酵素在生理上可催化 dihydrouracil 與 dihydrothymine 變成 *N*-carbamoyl- β -alanine 與 *N*-carbamyl- β -amino-isobutyrate；這些產物在 DNA 鹼基代謝途徑與各種生理反應中均扮演極重要的角色。



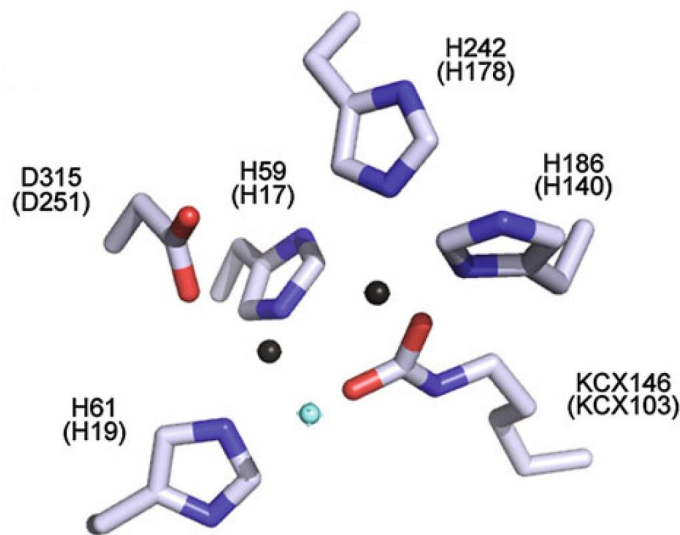
圖二:二氫嘧啶水解酵素為一兩兩對稱的四套體結構，每一獨立的單套體含有一個 $(\beta/\alpha)_8$ -barrel structure 與一延伸的 β -sandwich domain，並且在其活性中心含有兩個金屬離子。另外，擁有 $(\beta/\alpha)_8$ -barrel structure 構型的酵素通常相當耐熱，熱穩定性亦高，適合於工業應用(6-8)。此圖來自於文獻(9)。



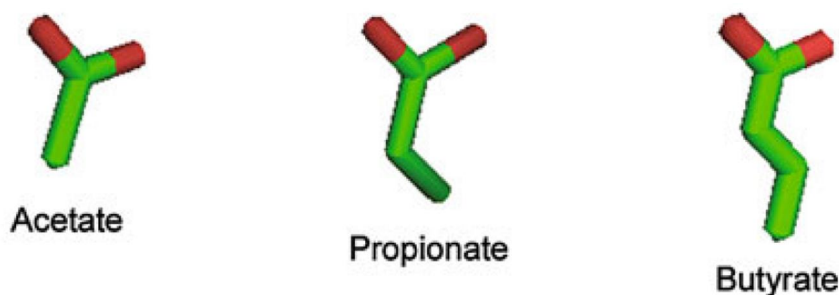
圖三: cyclic amidohydrolyase family 中不同酵素可接受的受質結構。二氫嘧啶水解酵素被歸類在 cyclic amidohydrolyase family 的一員中，其中在此家族裡的酵素尚包括 allantoinase、dihydroorotase、hydantoinase 與 imidase；此家族的酵素群雖然催化相似的化合物，然而其受質專一性皆不同，例如 allantoinase 就僅能催化 allantoin。此圖來自於文獻(1)。

二氫嘧啶水解酵素的活性中心含有 4 個 His、1 個 Asp、1 個 Lys 與 2 個金屬離子；總的來說，一般若未經波長掃描或原子光譜鑑定分析前皆假設為此兩金屬為鋅離子(4)。能夠螯合金屬的氨基酸一般來說是 Asp、Glu、Cys 與 His residues；Lys 可以當成金屬螯合殘基是十分罕見的(4,10,11)。尤其令人驚訝的是，經過詳細的結晶結構分析與突變研究發現，與一般的具雙金屬活性中心的酵素不同的是，在二氫嘧啶水解酵素中，此雙金屬活性中心與一顆活化的水分子，是以一個含有轉譯後修飾的羧化賴氨酸

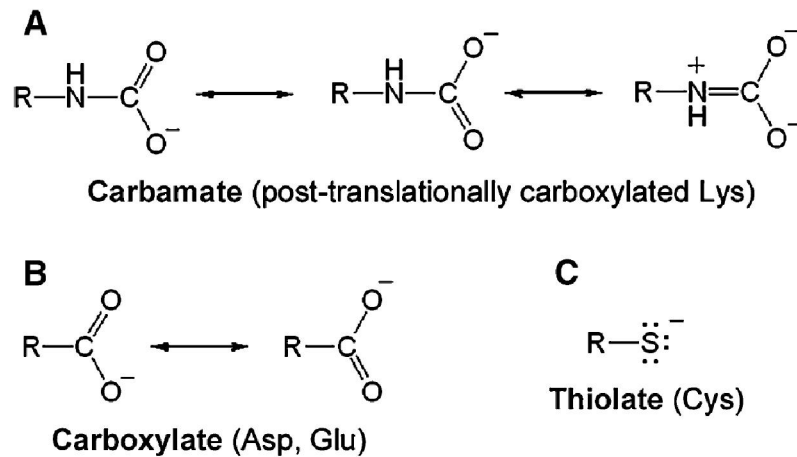
(posttranslational carboxylated lysine)所連結而成的，此在原核生物來源的金屬酵素十分罕見(4,5,10)。圖四說明了此雙金屬中心是如何的被 4 個 His、1 個 Asp 與 1 個經 CO₂ (carboxylated)後修飾的 Lys 所組成，並且此後修飾的 Lys 若突變後(以及其他的金屬螯合殘基)將使此酵素完全失去活性。然而此喪失的活性亦可經由加入短鏈的酸(short-chain acids)所產生的化學修復(chemical rescue)回復部分酵素活性(圖五)，因此此罕見的原核生物後修飾 Lys 程序，對於其酵素功能為必須。圖六說明為了催化效率，為何生物演化的過程中必須將 Lys 後修飾而不是使用現成的天然氨基酸 Asp 或 Cys 來螯合此雙金屬(10)。



圖四: cyclic amidohydrolase family 中的雙金屬催化中心。通常在 cyclic amidohydrolase family 中，雙金屬是由 4 個 His、1 個 Asp 與 1 個經 CO₂ (carboxylated)後修飾的 Lys 所螯合。淡青色為水分子，用來在水解反應中作為親核試劑。此圖來自於文獻(10)。



圖五：二氫嘧啶水解酵素若後修飾的 Lys 突變，可使用短鏈的酸所產生的化學修復 (chemical rescue) 回復部分酵素活性，暗示此 CO₂ (carboxylated) 修飾為必須。此圖來自於文獻(10)。



圖六：二氫嘧啶水解酵素中電子轉移與金屬螯合情形。為了催化效率，生物演化的過程中必須將 Lys 後修飾而不是使用現成的天然氨基酸 Asp 或 Cys 來螯合此雙金屬此圖來自於文獻(10)。

近年由於過度使用抗生素，導致細菌演化出多種抵抗抗生素的機制，這些具有多重抗藥性的細菌造成了許多公共衛生的問題，包括嚴重的群落感染(12-16)。最早於印度被報導的超級細菌，即 NDM-1 腸道菌感染症，帶有 NDM-1 基因的克雷白氏菌、綠膿桿菌與大腸桿菌目前正逐步流行至全球各地。MRSA (Methicillin-Resistant *S. aureus*) 及 VRE (Vancomycin-Resistant *Enterococcus*) 等具有抗藥性的細菌在院內感染中占的比例也逐年上升。這些細菌所引發的感染症第一線治療用藥均是使用 β -lactam 類抗生素治療，然而這些細菌已經經由突變或是交換基因等方式發展出對抗這些傳統抗生素的能力，尤其是 β -lactam 及 aminoglycoside 類抗生素，具有多重抗藥性的細菌不斷被發現與報導，尤其像是綠膿桿菌已被發現至少超過 120 種以上的 β -lactamases (15,16)，因此在臨床治療中不得不選用具有較多副作用的後線抗生素或甚至面臨無藥可醫的情況。因此若能開發出新

的攻擊標靶，也許對此棘手的問題將可能有所幫助。

本計畫所研究的二氫嘧啶水解酵素，為細菌鹼基代謝中必須之酵素，且其結構與功能與真核生物之水解酵素相比有顯著的不同，因此我們相信據此開發出的藥物可能對人體較無副作用。二氫嘧啶水解酵素雖廣泛存在於細菌、真菌、植物與動物中，催化二氫嘧啶開環並產生溶解度高的產物，為 DNA 鹼基合成中關鍵的反應之一。然而各物種的二氫嘧啶水解酵素經長時間的演化，其結構與功能迥異；細菌來源的二氫嘧啶水解酵素不像人類來源的酵素喜歡催化六環類的嘧啶，反而較喜歡催化五環 hydantoin 相關類似物，其特異性相差至少百倍(4)，因此給了我們一個如同與磺胺劑抗生素藥物研發相當的理由，來據之以研究。近年來，具有多重抗藥性的細菌在公共衛生與群落感染造成了許多問題，新的、不同以往的分子標靶，不是針對細胞壁也不是核糖體攻擊的小分子抗生素應當需要被開發出來，例如帶有 NDM-1 基因的超級細菌在最後一線的臨床用藥上僅剩 2 個抗生素有效 (13,14)，也因此本來簡單輕鬆服用抗生素即可治療的感染症或在醫院開刀預防投藥時有時將可能甚至面臨無藥可醫的情況。

最近開始有此酵素抑制劑的相關研究被報導，部分類黃酮物質發現竟有相當好的抑制能力，例如利用 dihydromyricetin 加入酵素反應時其 IC_{50} 為 48 μM (1)。類黃酮在行光合作用的生物中廣泛的存在，在許多研究中指出類黃酮具有抗發炎、抗腫瘤、抗自由基及抗微生物等功能；在開花植物中，類黃酮具有吸引傳粉者的功能並在葉片中具有抗真菌病原體等功能 (17)。主要類黃酮結構的核心具有兩個芳香環，並與一個 pyrane 連接，一般可分為以下六大類：flavonols (黃酮醇)、flavones、flavanones、flavanols、anthocyanidins、與 isoflavones。由於類黃酮多年來累積的研究均顯示對人體有益而無害，因此我們相信在研發新型抗生素時，以類黃酮為基礎的衍生物將較不具副作用。在此計畫，我們解出了綠膿桿菌的二氫嘧啶水解酵素的結晶結構(此部分結果已於去年發表)，並且我們找到類黃酮 kaempferol 可抑制此酵素；同時間我們亦結晶出綠膿桿菌二氫嘧啶水解酵

素與此類黃酮 kaempferol 的複合結晶結構。在計畫結案前雖仍在解析階段，我們亦利用分子對接法(molecular docking)來模擬並試圖說明此抑制劑與綠膿桿菌的二氫嘧啶水解酵素的結合模型，期望這些研究所帶出的研究結果，將可能讓吾人更加了解二氫嘧啶水解酵素其更詳細的反應機制，以及成為不同於傳統攻擊細胞壁合成等的新的抗生素研發的分子標靶。希望在未來，在臨床應用上能得到數個對於原核水解酵素專一性高的前導抑制化合物，以便對抗越趨嚴峻的抗藥性細菌現況。

(三) 材料與方法

3.1 實驗材料

- (1) 聚合酶連鎖反應中所使用的 PCR buffer 以及 *Taq* polymerase 購買自 MDBio (Frederick, MD, USA)。
- (2) PCR 產物純化所使用的 kit 購買自 VIOGENE (Taipei, Taiwan)。
- (3) 質體抽取所使用的 kit 購買自 MDBio (Frederick, MD, USA)。
- (4) Gel extraction 所使用的 kit 購買自 GE Healthcare (Piscataway, NJ, USA)。
- (5) 製備 Clone 的限制酶購買自 NEB (Ipswich, MA, USA)。
- (6) 勝任細胞 (competent cell) 為 *ECOSTM 21*、*ECOSTM 101* 購買自 YEASTERN (Taipei, Taiwan)。
- (7) 接合反應所使用的 T4 DNA ligase 和 buffer 購買自 Promega (Madison, WI, USA)。
- (8) 細菌生長所使用的 LB Broth 購買自 BD (Franklin Lakes, NJ, USA)。
- (9) 抗生素 Ampicillin 及 Kanamycin 購買自 MDBio (Frederick, MD, USA)。
- (10) 蛋白質表現所使用的 isopropyl β -D-1-thiogalactopyranoside (IPTG) 購買自 MDBio (Frederick, MD, USA)。
- (11) 純化用的膠體管柱為 HisTrapTM HP 及 SuperdexTM 200 HR 10/30 購買自 GE Healthcare (Piscataway, NJ, USA)。
- (12) SDS-PAGE 所使用的 SDS、TEMED、Tris-HCl (pH 8.8)、Tris-HCl (pH 6.8)、30% Acrylamide/Bis 及 APS 購買自 BIO-RAD (Hercules, CA, USA)。
- (13) Bacterial agar 購買自 AMRESCO (Solon, OH, USA)。

(14) DNA 電泳使用的 Agarose I™ 購買自 AMRESCO (Solon, OH, USA)。

1% 瓊脂膠製作配方：12.5 g 的 agarose 加入 70 ml 的 0.5X TAE 中，沸騰至透明呈清狀，冷卻至 55 °C 後倒入鑄膠槽中。

(15) SDS PAGE 製作配方：

Stacking gel (4 %)：

1. ddH ₂ O	1.50 ml
2. 30 % Acrylamide/Bis	0.33 ml
3. 0.5 M Tris-HCl (pH 6.8)	0.63 ml
4. 10 % SDS	25 μl
5. 10 % APS	25 μl
6. TEMED	1 μl

Resolving gel (12 %)：

1. ddH ₂ O	1.65 ml
2. 30 % Acrylamide/Bis	2.00 ml
3. 1.5 M Tris-HCl (pH 8.8)	1.25 ml
4. 10 % SDS	50 μl
5. 10 % APS	50 μl
6. TEMED	1.5 μl

Resolving gel (15 %)：

1. ddH ₂ O	1.05 ml
2. 30 % Acrylamide/Bis	2.50 ml
3. 1.5 M Tris-HCl (pH 8.8)	1.25 ml
4. 10 % SDS	50 μl
5. 10 % APS	50 μl
6. TEMED	1.5 μl

10X running buffer (1 L) 製作配方 (使用時稀釋為 1X)：

1. Tris	30.2 g	(final concentration = 250 mM)
2. Glycine	144 g	(final concentration = 1.92 M)
3. SDS	20 g	(final concentration = 1 %)

Destain buffer (1 L)製作配方：

1. Methanol	200 ml
2. Acetic acid	100 ml
3. ddH ₂ O	700 ml

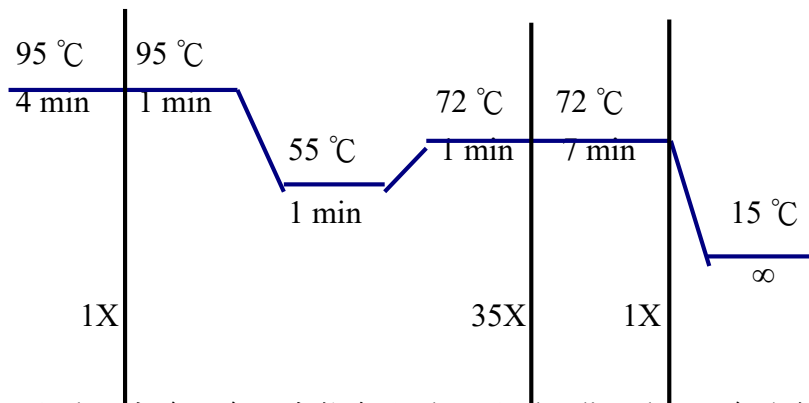
1X running buffer pH 8.3 (1 L)製作配方：

1. Tris	3 g	(final concentration = 250 mM)
2. Glycine	14 g	(final concentration = 1.92 M)
3. ddH ₂ O	1000 ml	

3.2 聚合酶連鎖反應

加入下列之 PCR 實驗反應物至200 µl 微量離心管中並混和均勻，使用的儀器是 BIO-RAD Thermocycler，反應條件如下所示。

Ragent	Quality
Template DNA	0.5 µl
10 mM dNTP mix	1 µl
Primer-forward	1 µl
Primer-revers	1 µl
<i>Taq</i> DNA Polymerase	0.7 µl
10X <i>Taq</i> buffer	5 µl
Sterile deionized water	40.8 µl
Final	50 µl



取 4 μl 反應後混合液產物與 1 μl 的 6X loading dye 混合均勻後，注入到 1% 瓊脂膠 (0.5X TAE buffer and 1% agarose) 內進行電泳 (electrophoresis)，於 0.5X TAE buffer 中以電流 110 V 電泳分離 20 分鐘，經 EtBr 染色 10~20 分鐘後用紫外光照膠確認反應後產物是否正確。

3.3 PCR 產物的純化

- (1) 將 PCR 產物 (總體積 50 μl) 加入 500 μl 的 PX buffer 混合均勻。
- (2) 將混合好的反應物加入 Clean up column 中，13000 rpm 離心，1 分鐘。
- (3) 倒掉下層液，加入 500 μl 的 WF Buffer，以 13000 rpm 離心，1 分鐘。
- (4) 倒掉下層液，加入 500 μl 的 WS Buffer，以 13000 rpm 離心，1 分鐘。
- (5) 再以 13000 rpm 離心，2 分鐘 (將 WS buffer 內的 ethanol 去除乾淨)。
- (6) 換新的 tube 後加入 50 μl 的 ddH₂O，放置室溫 10 分鐘，以 13000 rpm 離心 2 分鐘，保存至 -20 °C。

3.4 質體抽取及質體製備

質體抽取步驟為：

- (1) 將含有 pET21b 質體之單一菌落挑起，加入含有 4 μl 100 mg/ml ampicillin 的 4 ml LB 培養液中，置於 37 °C 培養箱，搖晃培養約 6 小時，OD₆₀₀=1.0。
- (2) 之後在 Tube 中分次加入 1000 μl 菌液，離心 12000 rpm，1 分鐘，去除上清液，重覆 4 次。
- (3) 加入 250 μl 的 Solution I 將離心下來的菌塊溶散，冰上反應 2 分鐘。
- (4) 加入 250 μl 的 Solution II，用手緩慢搖晃混合均勻，室溫反應 1 分鐘。
- (5) 加入 250 μl 的 Solution III，上下緩慢混合均勻，室溫反應 1 分鐘。
- (6) 離心 11000 rpm，10 分鐘，然後將上清液取出 (勿吸取到細胞碎片的白色沉澱

物)。

- (7) 將 750 μl 上清液吸至 Collect tube 中，靜置室溫 2 分鐘，離 6400 rpm，1 分鐘。
- (8) 除去 column 之下層液，加入 500 μl Wash Solution 至 column 中，離心 7200 rpm，1 分鐘，重複兩次。
- (9) 再以 8800 rcf 離心 2 分鐘 (將 Wash Solution 中的 ethanol 去除乾淨)。
- (10) 最後加入 50 μl 的 elution buffer 放置 37 $^{\circ}\text{C}$ 反應 5 分鐘，以 8800 rcf 離心 2 分鐘，完成質體抽取。
- (11) 利用瓊脂膠進行電泳，確認質體大小是否正確，即完成質體抽取 (質體未使用時保存至-20 $^{\circ}\text{C}$)。

質體製備步驟為：

- (1) 在質體中加入 10 μl 10X 4 號 enzyme cutting buffer 及 36 μl ddH₂O，再加入限制酶 *NdeI* 及 *XhoI* 各 2 μl 進行剪切，混合均勻，置於 37 $^{\circ}\text{C}$ 培養箱反應 8 小時。
- (2) 將剪切完成的質體依 gel extraction 步驟操作，除去剪切下來的多餘小片段，以免妨礙接合作用。gel extraction 實驗步驟如下：
 1. 將質體和 6X loading dye 混合均勻後，注入到 1% 瓊脂膠內進行電泳，於 0.5X TAE buffer，電流 110 mV 跑膠 20 分鐘，經 EtBr 染色 10~20 分鐘後，用紫外光照膠確認內容產物大小。
 2. 將正確大小之質體片段膠體切下，置於 1.5 ml eppendorf 中秤重，以每 10 mg 加入 10 μl 的比例在管中加入 capture buffer type 3，置於 60 $^{\circ}\text{C}$ 乾浴槽 10 分鐘直到瓊脂膠完全溶解。
 3. 將 eppendorf 內之液體以 600 μl /次 加入 GFX MicroSpinTM column 和 Collect Tube 中，置於室溫反應 1 分鐘，離心 14000 rpm，1 分鐘。
 4. 除去 column 之下層液，加入 Wash buffer type 1，離心 16000 rcf，1 分鐘。
 5. 除去 column 之下層液，離心 14000 rpm，1 分鐘 (將 Wash buffer 中的 ethanol 去除乾淨)。
 6. 最後加入 50 μl 的 Elution buffer type 4 靜置室溫 1 分鐘，離心 14000 rpm，1 分鐘，保存至-20 $^{\circ}\text{C}$ ，完成 gel extraction。
- (3) 完成 pET21b 之質體製備，保存至-20 $^{\circ}\text{C}$ 。

3.5 表現質體 pET21b-PaDHT 之製備

- (1) 將 PCR 純化後之產物以限制酶 *NdeI* 及 *XhoI* 進行剪切，反應條件為限制酶 *NdeI*

及 *XhoI* 各 2 μl 、10X 4 號 enzyme cutting buffer 10 μl 、48 μl 的 PCR 產物及 36 μl ddH₂O，混合均勻後置於 37 °C 培養箱中反應 8 小時。

- (2) 將剪切完成的基因片段依 PCR 產物的純化步驟操作，除去剪切下來的多餘小片段，以免妨礙接合作用。
- (3) 將純化過的基因產物及製備好的質體混合，以 7:1 的比例進行接合作用。反應條件為 0.5 μl 的 T4 DNA ligase、0.5 μl 的 10X ligase buffer 及等比例的基因產物與質體混合物，總體積 5 μl ，在室溫反應 4 小時 (或室溫反應 1 小時之後移置 4 °C 冰箱中反應 8 小時)。

3.6 質體轉型作用

- (1) 取 50 μl 之經過特殊處理的大腸桿菌勝任細胞 DH5 α (*ECOS*TM 101) 和上述已經處理好的 clone 反應物 5 μl 混合均勻 (以 10:1 的比例)。
- (2) 之後置於 42 °C 反應 45 秒進行熱衝擊 (heat shock)，使大腸桿菌細胞外鞘的油脂變性而刺激質體 DNA 進入細菌細胞內。
- (3) 隨即置於冰上 1 分鐘，而後將菌液均勻塗抹在 LB 培養盤，放置於 37 °C 培養箱，直到長出菌落。
- (4) 之後進行質體抽取的動作，以質體 4 μl 限制酶、ddH₂O 36 μl 、*NdeI* 和 *XhoI* 各 0.5 μl 及 10X 4 號 enzyme cutting buffer 1 μl 將產物作雙切，置於 37 °C 培養箱，反應時間 2 小時。
- (5) 取 10 μl 內容產物與 2 μl 的 6X loading dye 混合均勻後注入到 1% 瓊脂膠內進行電泳，於 0.5X TAE buffer 中以電流 110 V 電泳分離 20 分鐘，經 EtBr 染色 10~20 分鐘後，用紫外光照膠確認內容產物是否正確。若 clone 成功則可看見質體與 *PaDHT* 的兩種不同大小片段。

3.7 蛋白質表現測試與蛋白質表現菌體儲存

- (1) 將定序結果正確的質體轉型至 (*ECOS*TM 21) 中。
- (2) 用挑菌棒取出 plate 中的單一菌落，種入含 4 μl 100 mg/ml ampicillin 的 4 ml LB 培養液中，置於 37 °C 培養箱，搖晃培養至 OD₆₀₀=1.0。
- (3) 將 O.D 1.0 的菌液平分成兩管，一管做蛋白表現測試，一管做菌液儲存，各加入 2 ml LB 培養液，搖晃培養至 OD₆₀₀=1.6。
- (4) 從蛋白表現測試管中取 200 μl 菌液至 eppendorf 中，原菌液加入 1 M isopropyl

β -D-1-thiogalactopyranoside (IPTG) 4 μ l，再培養 4 小時後取 150 μ l 表現後菌液至 eppendorf 中。

- (5) 用 12000 rpm 離心，1 分鐘，用 20 μ l ddH₂O 回溶菌塊後跑 SDS-PAGE 進行確認，步驟如下：
 1. 加入 4X loading dye 10 μ l 混合均勻，以 98 °C 加熱 1 小時。
 2. 將 marker 與 sample 注入 SDS-PAGE 之後，以 70 V 跑膠約 2 小時。
 3. 跑膠完成後，將膠片置於 stain solution (coomassie blue) 中染色 1 小時。
 4. 染色完後以 destain solution，退染約 1 小時。
 5. 最後以玻璃紙封膠，風乾後確認結果。
 6. 相較於誘導前，誘導後將出現一明顯藍色色帶位於 20 kDa 左右。
- (6) 取菌液儲存管，在 tube 中分次加入 1000 μ l 菌液，離心 12000 rpm，1 分鐘，去除上清液，重覆 4 次。
- (7) 加入 400 μ l LB 培養液回溶菌塊，再加入 50% glycerol 100 μ l 後，保存至 -80°C，完成菌液儲存。

3.8 細菌大量培養與蛋白質表現

- (1) 取出儲存菌液 15 μ l，加入含 4 μ l 100 mg/ml ampicillin 的 4 ml LB 培養液中，置於 37 °C 培養箱，搖晃培養至菌液濃度達到 OD₆₀₀=1.0。
- (2) 將培養至 OD₆₀₀=1.0 的菌液加入含 200 μ l 100 mg/ml ampicillin 的 500 ml LB 培養液中，此次共培養 6 瓶共 3 L 細菌培養液，置於 37 °C 培養箱，搖晃培養至菌液濃度達到 OD₆₀₀=1.6 (約 4~6 小時)。
- (3) 於濃度約為 OD₆₀₀=1.6 時加入 1 M IPTG 125 μ l 誘導表現蛋白生成，繼續培養 8 小時。

3.9 蛋白質純化

- (1) 將培養好的菌液分置於 250 ml 離心瓶，以高速冷凍離心機 (Beckman) 12000 rpm 於 4 °C 低溫離心 15 分鐘。
- (2) 除去上清液，每瓶以 20 ml 的 binding buffer (5 mM imidazole, 500 mM NaCl, pH 7.4) 將底部菌塊重新回溶，將回溶的菌液以 50 ml 離心管平分成 3 管，即每管 40 ml。
- (3) 以超音波破碎機 (sonicator) 將溶於 binding buffer 內的菌體打碎。

- (4) 之後以 14000 rpm 於 4 °C 低溫離心 20 分鐘，收集上清液。
- (5) 將純化用 column His Trap™ HP 依序通入 25 ml 的 ddH₂O、30 ml 的 50 mM NiSO₄、30 ml 的 ddH₂O 及 30 ml 的 5 mM imidazole, 500 mM NaCl, pH 7.4) buffer。
- (6) 接著將含有蛋白質之上清液通入 column 中。
- (7) 通完上清液後，依序使用含有 500 mM NaCl pH 7.4 的不同濃度 imidazole (5 mM、60 mM、100 mM、200 mM、300 mM、500 mM) 通入 column。並收集洗出之蛋白質各 35 ml。
- (8) 最後使用 100 mM EDTA pH 8.00 與 2 M NaCl 清洗 column，並用 20 % EtOH 保存 column。之後以 SDS-PAGE 確認蛋白質狀況，至此蛋白質純化完成。

3.10 Beckman 高速離心機操作

- (1) 核對並確認使用離心機適合的 rotor 型號及離心體積：

rotor 型號	最高轉速 (rpm)	離心體積 (ml)
JA-14	14000	250
JA-17	17000	50
JA-20	20000	15

- (2) 自冰箱取出 rotor 並確認 rotor 上的 O-ring 有無脫落。
- (3) 確認 rotor 與離心機是否有多餘水分殘留並拭乾。
- (4) 離心之樣本秤重平衡至小數點第二位，並將樣品注入於離心管 (需達八分滿以上，以免高速離心後離心管變形卡住)。
- (5) 確認平衡與對稱，rotor 放入前需確認橫桿對準儀器軸心之 Y 型凹槽。
將 rotor 上蓋與軸心一同鎖緊，勿用力推壓上蓋以免儀器軸心變形，並關上離心機蓋子，開始離心。
- (6) 儀器設定如下：

Rotor 鍵-輸入 Rotor 號碼 → 按 Enter → SPEED 鍵-設定轉速 → 按 Enter
 → TEMP 鍵-設定溫度 → 按 Enter → TIME 鍵-設定離心時間 → 按 Enter →
 Show set 可從螢幕檢閱設定值 → 按 Enter → 按 Start 啟動儀器 (按 Start 後需
 立即施力按壓上蓋兩側邊緣，直到儀器確實啟動為止)
 若儀器發出警示聲，則按 CE 鍵，再按 Show set → 按 Enter → 按 Start 啟動
 儀器，並迅速按壓上蓋兩側邊緣，當聽到馬達聲響起，再按 Show set → 按

Enter → 按 Start 啟動儀器。儀器啟動後若發出異常聲響或轉速升不上去，則立即按 Stop 鈕停止，並確認 rotor 是否鎖緊、樣本是否平衡、位置是否對稱。

- (7) 離心完成後，按 DOOR 鍵打開儀器，取出樣品，將 rotor 放回冰箱。
- (8) 離開前將儀器門打開，以保持離心槽乾燥，關閉電源。

3.11 KUBOTA 高速冷凍離心機操作

- (1) 使用離心機型號為 3700 型，使用的 rotor 為：

rotor 型號	最高轉速 (rpm)	離心體積 (ml)
AK-5004CA	12000	50
- (2) 確認 rotor 與離心機是否有多餘水分殘留並拭乾。
- (3) 離心之樣本秤重平衡至小數點第二位，並將樣品注入於離心管需為八分滿（不可至全滿），以免高速離心後離心管變形卡住或溢出。
- (4) 確認平衡與對稱，rotor 放後需確認卡榫是否有嵌入 rotor 之凹槽，並且為水平後，將 rotor 上蓋與軸心一同鎖緊，並關上離心機蓋子，開始離心。
- (5) 儀器設定如：TEMP LIMIT 旋鈕-設定溫度 → SPEED 旋鈕-設定轉速 → TEMP 旋鈕-設定溫度按 → Start 啟動儀器
- (6) 離心完成後，按 OPEN 鍵打開儀器，取出樣本。
- (7) 使用完畢後將儀器門打開，並保持離心槽乾燥，關閉電源。

3.12 蛋白質透析

實驗使用之透析膜為 Thermo Snake Skin[®] Pleated Dialysis Tubing。

- (1) 剪下適當長度後，將透析膜浸濕於 ddH₂O，大約 2 分鐘。
- (2) 將蛋白質與要置換之 buffer 以 1：1 比例慢慢混合後，觀察蛋白質狀態，以確定蛋白質是否適用於置換之 buffer，避免蛋白質聚集沉澱。
- (3) 將透析膜浸濕於要置換的 buffer (此實驗使用之 buffer 為 20 mM Tris-HCl, 300 mM NaCl, pH 8.0 及 20 mM Tris-HCl, 20 mM NaCl, pH 8.0)，2 分鐘。
- (4) 將浸濕的透析膜拉平，先以長型夾子夾住下端密封，放入欲置換 buffer 之蛋白質，將上端也以長型夾子夾住密封，並預留約 1 公分之置換空間。
- (5) 根據其置換蛋白質之體積不同，使用不同的置換 buffer 體積，如 20 ml 之蛋白質則需置於 1000 ml 之 buffer，50 ml 之蛋白質則置於 3000 ml 之 buffer。
- (6) 將蛋白質懸掛於欲置換之 buffer 中，並以磁石攪動 buffer，轉速約 500 rpm，

置於 4 °C 環境下置換 4 小時，即完成蛋白質透析。

3.13 蛋白質濃縮

實驗使用之濃縮管為 Millipore Amicon ultra-10K。

- (1) 取出濃縮管後，以酒精和 ddH₂O 小心沖洗 (以免破壞過濾膜)。
- (2) 加入 10 ml ddH₂O 至濃縮管中，將濃縮管放置在 50 ml 離心管內，3000 rpm 於 4 °C 低溫離心 10 分鐘。
- (3) 清洗和檢測完畢後，加入 10 ml 透析完的蛋白質溶液，3000 rpm 於 4 °C 低溫離心 25 分鐘，重覆此步驟至蛋白質濃度達到實驗條件為止 (需注意蛋白質狀態是否有聚集沉澱)。
- (4) 使用完畢後，以 ddH₂O 和酒精小心沖洗，保存至 20 % 酒精中。

3.14 膠體過濾色層分析法

實驗使用之 Gel filtration column 為 Superdex™ 200，使用的儀器為 AKTA prime plus-FPLC。操作步驟如下：

- (1) 先開 AKTA-FPLC，再開電腦。
- (2) 將管線中的 buffer 換成 ddH₂O，模式設定為：
- (3) Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 3 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Load → Start Run，清洗 10~20 分鐘，將管線中所有的液體清洗乾淨。
- (4) 用 ddH₂O 和針筒清洗 Sample Loop，重覆數次。
- (5) 將管線中的 ddH₂O 換成 Gel filtration buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8.0)，模式設定為：
Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 3 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Load → Start Run，清洗 20 分鐘，直到管線中所有的液體換成 Gel filtration buffer。
- (6) 用 Gel filtration buffer 和針筒清洗 Sample Loop，重覆數次。

- (7) 將 Gel filtration column 接上 AKTA prime plus 模式設定為:
Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 0.5 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Load → Start Run , 清洗 10~20 分鐘。
- (8) 用針筒取 500 μ l 的 SSB 蛋白質注入 Sample Loop , 模式設定為:
Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 0.5 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Injec → Start Run , 當總收集量到達 100~120 ml (超過 Gel filtration column 總體積)時, 停止 FPLC , 並分析所得到的數據。
- (9) 清洗 Gel filtration column , 模式設定為:
Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 0.5 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Load → Start Run , 清洗 10~20 分鐘。
- (10) 卸下 Gel filtration column , 將 AKTA prime plus 管線中的 buffer 換成 ddH₂O , 模式設定為:
Manual Run → Set Method Base : ml → Set Concentration %B : 0% → Set Gradient: Off → Set Flow Rate : 3 ml/min → Set Fraction Base : ml → Set Fraction Size : 0 ml → Set Pressure Limit : 1.0 MPa → Set Buffer Valave : Pos 1 → Set Injec Valve Pos : Load → Start Run , 清洗 10~20 分鐘 , 將管線中所有的液體清洗乾淨。
- (11) 重覆步驟 9 , 將 AKTA prime plus 管線中的 buffer 換成 20% EtOH , 即完成實驗。

3.15 蛋白質的結晶

- (1) 在二氫嘧啶水解酵素晶體生成前需先將 buffer 的條件換成 40 mM Tris, 200 mM NaCl, pH 8 , 並將蛋白質濃縮至 14.5 mg/ml 。
- (2) 二氫嘧啶水解酵素初步的晶體篩選使用的是懸滴氣相擴散法 (hanging-drop vapor diffusion method)(圖三) , 懸滴氣相擴散法就是將滴含有沉澱劑的純蛋白

質溶液懸吊在在含有大量沉澱劑的密閉容器中，一段時間後，因容器內沉澱劑的溶液濃度高於蛋白質溶液濃度，在密閉容器中為了達到平衡，蛋白質溶液內的水分子會氣化移置容器內的溶液中，使得蛋白質溶液中的沉澱劑濃度提高，蛋白質濃度也逐漸增加到可析出結晶的狀態。

- (3) 我們在點晶用膠帶上滴入二氫嘧啶水解酵素及 Crystal screen kit buffer (為沉澱劑) 各 1 μ l 成為蛋白質混合液，在 24 孔盤中加入 Crystal screen kit buffer 後 (每個 well 皆為不同 buffer 編號，勿放錯)，將蛋白質混合液懸吊在 24 孔盤上，密封。
- (4) 利用顯微鏡觀察其變化狀況，利用數千種已商業化的篩選劑套組篩選出可能的沉澱劑條件。
- (5) 篩選出在 10% PEG8000, 100mM HEPES sodium salt, 200mM Calcium Acetate 與 28% PEG 6000, 100mM HEPES pH 7.5, 200mM Lithium acetate 的條件下有初步的晶體生成。

3.16 X 光繞射收集

蛋白質晶體在高輝度 X 光照射下的承受能力不若小分子晶體。因此，需要先把這些蛋白質晶體凍在液態氮下，然後在同步輻射 X 光照射過程中不斷以 100K 的氮氣噴吹，以減少 X 光的傷害。再收集一張張連續的蛋白質晶體繞射點圖譜，最後分析這些繞射點圖譜，決定出在蛋白質晶體中規則排列的蛋白質結構。

3.17 蛋白質結構預測

以二氫嘧啶水解酵素為例，操作步驟如下：

- (1) 首先進入 SWISS-MODEL 網站 <http://swissmodel.expasy.org/>。
- (2) 在左列的 Modeling 部分選擇 Automated mode。
- (3) 填入 e-mail、project title、欲預測之蛋白質序列。
- (4) 在下方 Advanced option 可選擇欲比較之模板。
- (5) 以 PDB code: 3DC8 之 A chain 當作模板進行二氫嘧啶水解酵素之蛋白質結構預測。
- (6) 按下 submit，等待預測結果。

3.18 蛋白質序列比對

以二氫嘧啶水解酵素為例，操作步驟如下：

- (1) 首先進入 SWISS-MODEL 網站 <http://swissmodel.expasy.org/>。
- (2) 下載 ClustalX2 和 DNAMAN 兩種程式。
ClustalX2：<http://www.windows7download.com/win7-clustalx/atehudch.html>。
DNAMAN：<http://www.lynnon.com/>。
- (3) 在 NCBI 上找出 *PaDHT*、*PfDHT*、*KxDHT*、*NnDHT*、*TmDHT* 及五種序列。
- (4) 將序列以 FASTA 格式複製到記事本檔案中。
- (5) 打開 ClustalX2 程式，在上方介面操作 File → Append Sequences，將含有 FASTA 序列格式の記事本檔案載入。
- (6) 在上方介面操作 Alignment → Do Complete Alignment，進行序列比對。
- (7) 打開 DNAMAN 程式，在上方介面操作 File → Open Special → Multiple Alignment，將剛剛進行序列比對所產生的 aln 檔打開。
- (8) 在序列比對左上方視窗的 Option 裡，可以進行顏色及字元的設定。
- (9) 在序列比對左上方視窗的 Output 裡，可以將檔案輸出成 EMF 圖檔。

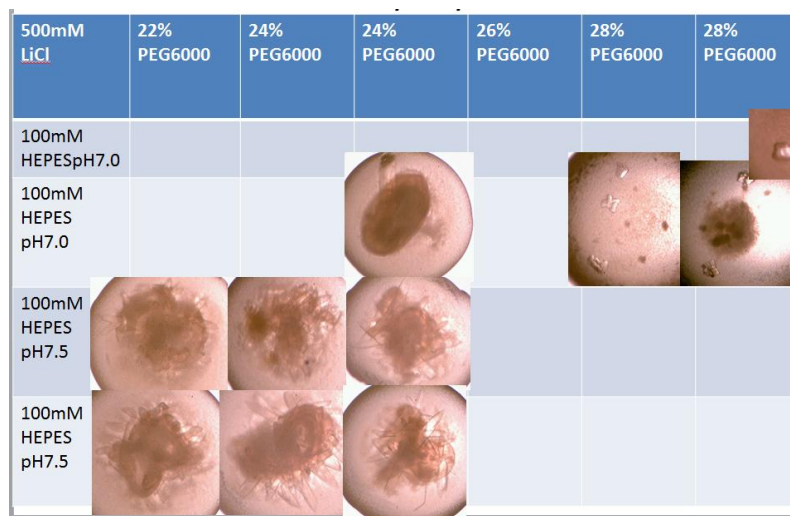
3.19 PyMol 使用顯示蛋白質結構圖像

顯示蛋白質結構操作步驟如下 (以 *PaDHT* 為例)：

- (1) 首先在 PyMol 打開 "*PaDHT* model. pdb" (為 *PaDHT* 經過 SWISS-MODEL 預測後的 pdb 檔名)。
- (2) 在右方的物件部分選擇 H (Hide) → everything，將線圖及水分子隱藏。
- (3) 在右方的物件部分選擇 S (Show) → cartoon，顯示出緞帶卡通圖。
- (4) 使用指令 "bg_color white"，將背景設為白色。
- (5) 選擇上方欄中的 Display 選項中的 sequence 可顯示胺基酸序列。
- (6) 對準圖像任意處點住滑鼠左鍵拖曳可任意旋轉動圖像。
- (7) 對準圖像任意處點住滑鼠右鍵拖曳可放大或縮小圖像。
- (8) 對準圖像任意處點住滑鼠中鍵拖曳可移動圖像。
- (9) Ctrl+Shift+滑鼠中鍵可設定圖像的旋轉中心。
- (10) 在右方的物件部分選擇 C (Color)，自訂顏色。
- (11) 使用指令 "ray"，將檔案結構美化。
- (12) 使用指令 "png *PaDHT* modeling"，將結構檔案輸出為 png 圖片檔，檔名為 *PaDHT* modeling。

(四) 實驗結果與討論

1. 為了容易看懂以下研究結果，此部分報告方式是以圖表來直接書寫與歸納。
2. 綠膿桿菌的二氫嘧啶水解酵素的結晶。圖七：綠膿桿菌的二氫嘧啶水解酵素利用晶體篩選試劑的 classic 4 B5 修飾最適條件。在含有 500 mM LiCl 中，不同的酸鹼值與 PEG 6000 可得到大小與數量不一的晶體；然經 x-ray 繞射分析其解析度不佳，約僅 8 Å。



3. 綠膿桿菌的二氫嘧啶水解酵素的結晶。圖八：綠膿桿菌的二氫嘧啶水解酵素利用晶體篩選試劑的 classic 5 B1 修飾最適條件。我們利用不同的晶體成長條件篩出 PEG 8000 是好的蛋白質結晶主成份。我們接著嘗試細分各濃度與酸鹼值，發現在 pH 7.0-7.5 可得長方形柱狀晶體，而 pH 8.0 會有針狀蛋白質晶體產生。最重要的是控制 PEG 8000 的濃度須在 16%-20%；相對來說我們結論以 18% 的 PEG 8000 為最佳主沉澱劑。然經 x-ray 繞射分析其解析度不佳，約僅 7 Å。

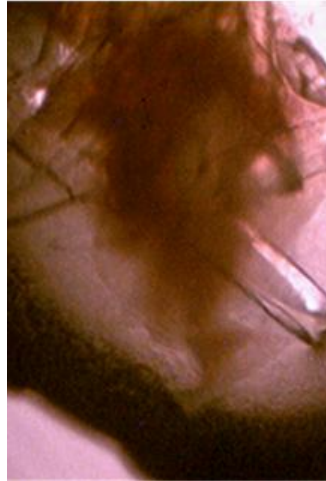
200mM Calcium Acetate	16% PEG8000	16% PEG8000	18% PEG8000	18% PEG8000	20% PEG8000	20% PEG8000
100mM HEPES PH7						
100mM HEPES PH7.5						
100mM HEPES PH7.5						
100mM HEPES PH8						

4. 綠膿桿菌的二氫嘧啶水解酵素的結晶。圖九：綠膿桿菌的二氫嘧啶水解酵素利用晶體篩選試劑的 classic 4 B5 修飾最適條件。在含有 500 mM LiCl 中，較鹼的酸鹼值(pH 8.5 and pH 9.0) 與 24%-28% PEG 6000 可得到大小與數量不一的晶體；然經 x-ray 繞射分析其解析度不佳，約僅 4Å。

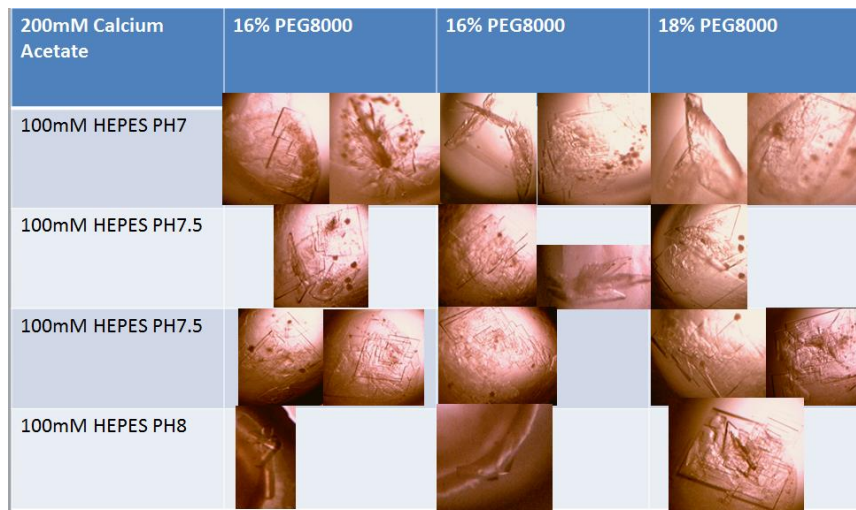
500mM Lithium chloride	24% PEG6000	26% PEG6000	28% PEG6000	28% PEG6000	30% PEG6000	30% PEG6000
100mM Tris HCL PH7.5						
100mM Tris HCL PH8						
100mM Tris HCL PH8.5						
100mM Tris HCL PH9						

5. 綠膿桿菌的二氫嘧啶水解酵素的結晶。圖十：綠膿桿菌的二氫嘧啶水解酵素修飾最適結晶條件。我們成功在 28% PEG 6000、100 mM HEPES pH 7.5 與 200 mM lithium acetate 長出長方晶體；經 x-ray 繞射分析其解

析度佳，約 2.1 Å。

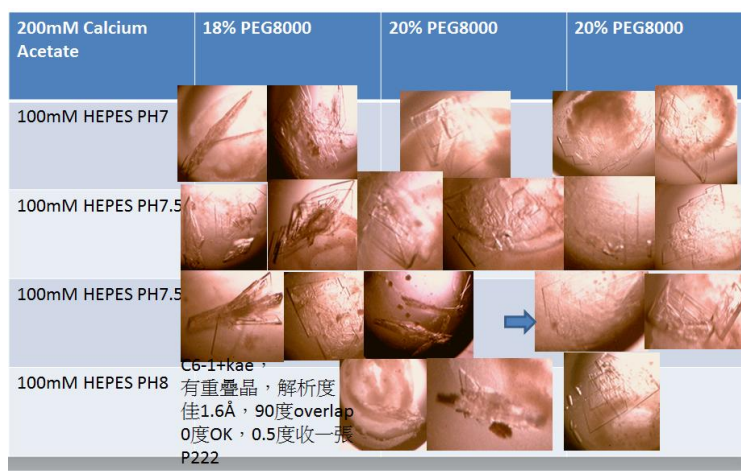


6. 綠膿桿菌的二氫嘧啶水解酵素與抑制劑 kaempferol 的複合結晶。圖十一：綠膿桿菌的二氫嘧啶水解酵素利用晶體篩選試劑的 classic 5 B1 修飾最適條件。在含有 200 mM CaAcetate 中，酸鹼值在 pH 7-8 與 16%-18% PEG 8000 可得到大小與數量不一的複合晶體；經 x-ray 繞射分析其解析度高，約 2.6 Å。

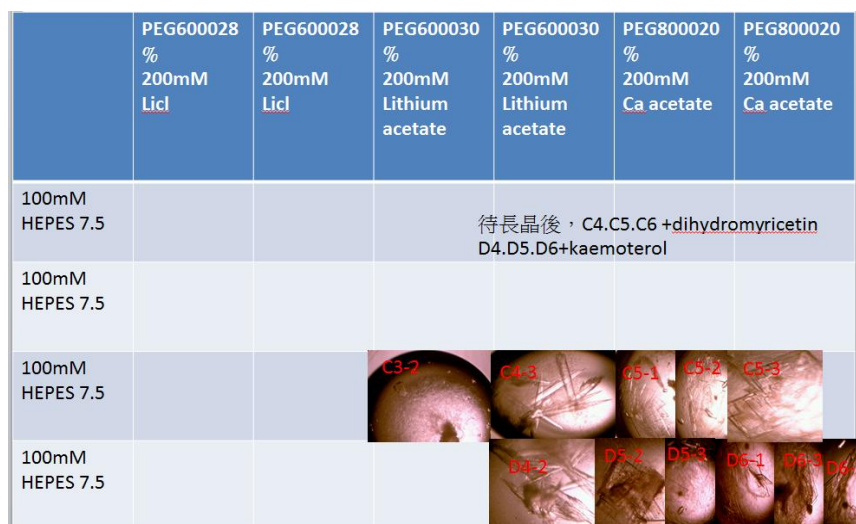


7. 綠膿桿菌的二氫嘧啶水解酵素與抑制劑 kaempferol 的複合結晶。圖十二：綠膿桿菌的二氫嘧啶水解酵素利用晶體篩選試劑的 classic 5 B1 修飾最適條件。在含有 200 mM CaAcetate 中，酸鹼值在 pH 7-8 與 18%-20% PEG 8000 可得到大小與數量不一的複合晶體；經 x-ray 繞射

分析其解析度非常高，約 1.6 Å。其 space group 也變為 P222。

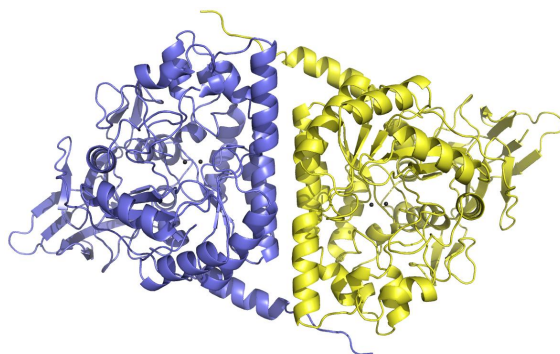


8. 綠膿桿菌的二氫嘧啶水解酵素與抑制劑 kaempferol 以及 dihydromyricetin 的複合結晶。圖十三：綠膿桿菌的二氫嘧啶水解酵素修飾最適晶體條件。在含有 200 mM Ca Acetate 或 Li Acetate 中，30% PEG 6000 與 20% PEG 8000 可得到各大小與數量不一的複合晶體。



9. 綠膿桿菌的二氫嘧啶水解酵素其 X-ray 蛋白質結構的解出。圖十四：綠膿桿菌的二氫嘧啶水解酵素的結晶結構。我們將綠膿桿菌的二氫嘧啶水解酵素晶體使用同步輻射光 13C1 光束線蒐集到繞射數據，並利用同為此家族的酵素結構作為模板解出此結構，其解析度 2.1 Å。此結構為

一著名的 TIM barrel 構型，穩定的桶狀結構恰可解釋為何此酵素如此的耐熱。另外，與所有已知的此家族酵素不同的是，我們的結構是一個相互對稱的雙套體，而非四套體。這成果著實讓我們感到好奇，因而我們將部分力氣找尋原因。



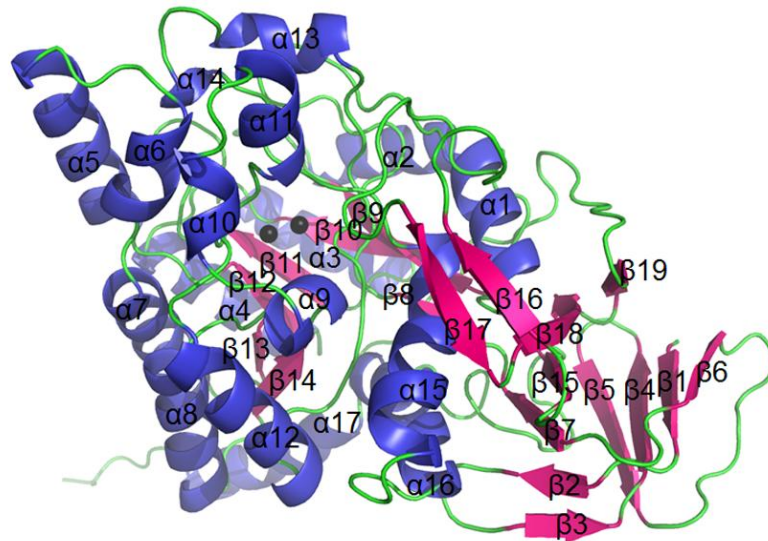
10. 蛋白質晶體 X-ray 繞射統計表(圖十五)。

Data collection and refinement statistics.

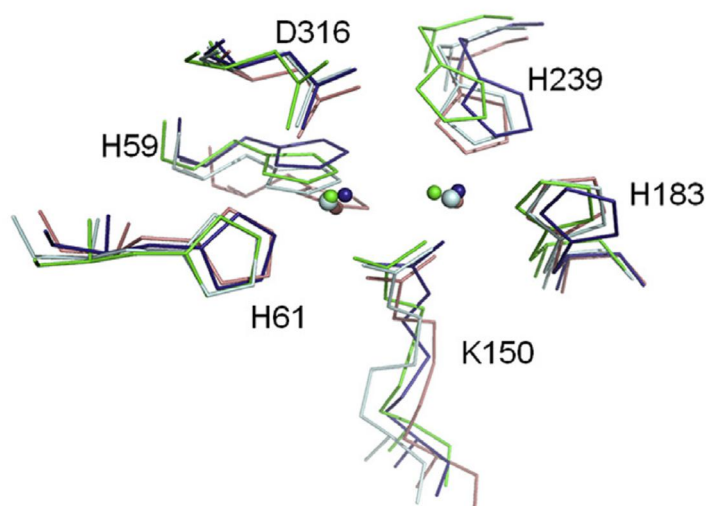
Data collection	
Crystal	<i>P. aeruginosa</i> dihydropyrimidinase
Wavelength (Å)	0.975
Resolution (Å)	97.11–2.1
Space group	$P3_121$
Cell dimension (Å)	$a = 112.13 \alpha = 90$ $b = 112.13 \beta = 90$ $c = 161.05 \gamma = 120$
Completeness (%)	99.9 (99.9) ^a
$\langle I/\sigma \rangle$	19.21 (3)
R_{sym} or R_{merge}	0.084 (0.59)
Redundancy	6.0 (5.9)
Refinement	
Resolution (Å)	30–2.1
No. reflections	68799
$R_{\text{work}}/R_{\text{free}}$	0.1696/0.2155
No. atoms	
Protein	1299
Water	338
R.m.s deviation	
Bond lengths (Å)	0.0196
Bond angles (°)	1.8795
Ramachandran plot	
In preferred regions	903 (94.75%)
In allowed regions	43 (4.51%)
Outliers	7 (0.73%)
PDB entry	5E5C

^a Values in parentheses are for the highest resolution shell.

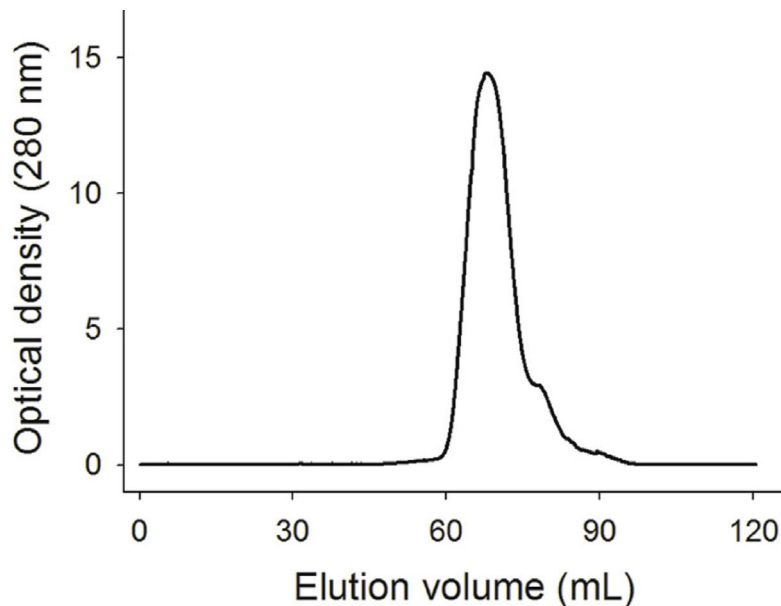
11.圖十六: 綠膿桿菌的二氫嘧啶水解酵素其次單元結構分析。此次單元含有 17 個螺旋與 19 個摺板結構，當然此結構亦含有兩個金屬與一個轉譯後修飾的 carboxylated Lys 胺基酸。



12.圖十七: cyclic amidohydrolase family 中不同家族酵素其活性中心分析。二氫嘧啶水解酵素被歸類在 cyclic amidohydrolase family 的一員中，其中在此家族裡的酵素尚包括 allantoinase、dihydroorotase、hydantoinase 與 imidase；此家族的酵素群雖然催化相似的化合物，然而其受質專一性(受質結構)皆不同。此分析發現雖然來自綠膿桿菌的二氫嘧啶水解酵素是雙套體(其他來源為四套體)，但活性區(將不同活性中心疊在一起)與其他家族酵素皆極相似。



13.圖十八:膠體過濾分析法。由於結構顯示來自綠膿桿菌的二氫嘧啶水解酵素是雙套體(其他來源為四套體),因此我們想證明在溶液中此酵素也是雙套體。利用膠體過濾分析法(分子篩)的結果經過迴歸計算其他標準品(具有標準的分子量與流出體積),我們確定此酵素確為雙套體。另外,由於是單一波峰,也可推斷此雙套體相當穩定的存在於溶液中(否則可能會有兩個以上的波峰,例如可能也會有四套體的波峰)。

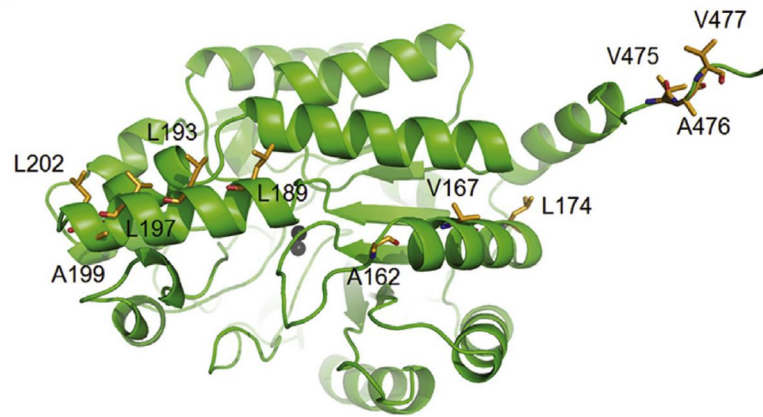


14.圖十九:綠膿桿菌的二氫嘧啶水解酵素結晶結構經審查員審查各係數與繞射資料是否正確後上傳至 PDB 供科學界與大眾公開查詢與使用。

The screenshot shows the PDB website interface. At the top, there is a search bar and navigation links. Below the search bar, there is a section for '1 Unreleased Structure'. The 'Query Parameters' section shows 'Status Search: Structure ID=SESC'. The 'Status Code Legend' section lists various status codes and their meanings. The main entry for SESC is displayed, showing the title 'Crystal structure of dihydropyrimidinase from Pseudomonas aeruginosa PAO1', the deposition date '2015-10-08', and the release date 'n/a'. The authors listed are Huang, C.C., Huang, Y.H., Hsieh, Y.C., Tzeng, C.T., Chen, C.J., and Huang, C.Y. The sequence availability is marked as 'NO'.

15.圖二十:綠膿桿菌的二氫嘧啶水解酵素單套體-單套體界面。許多的疏

水性胺基酸提供了強的疏水界面而形成雙套體，包括 Ala162 (loop), Val167 ($\alpha 4$), Leu174 ($\alpha 5$), Leu189 ($\alpha 5$), Leu193 ($\alpha 5$), Leu197 ($\alpha 5$), Ala199 ($\alpha 5$), Leu202 (loop), Leu210 ($\alpha 6$), Val475 (C-terminal tail), Ala476 (C-terminal tail), and Val477 (C-terminal tail)。

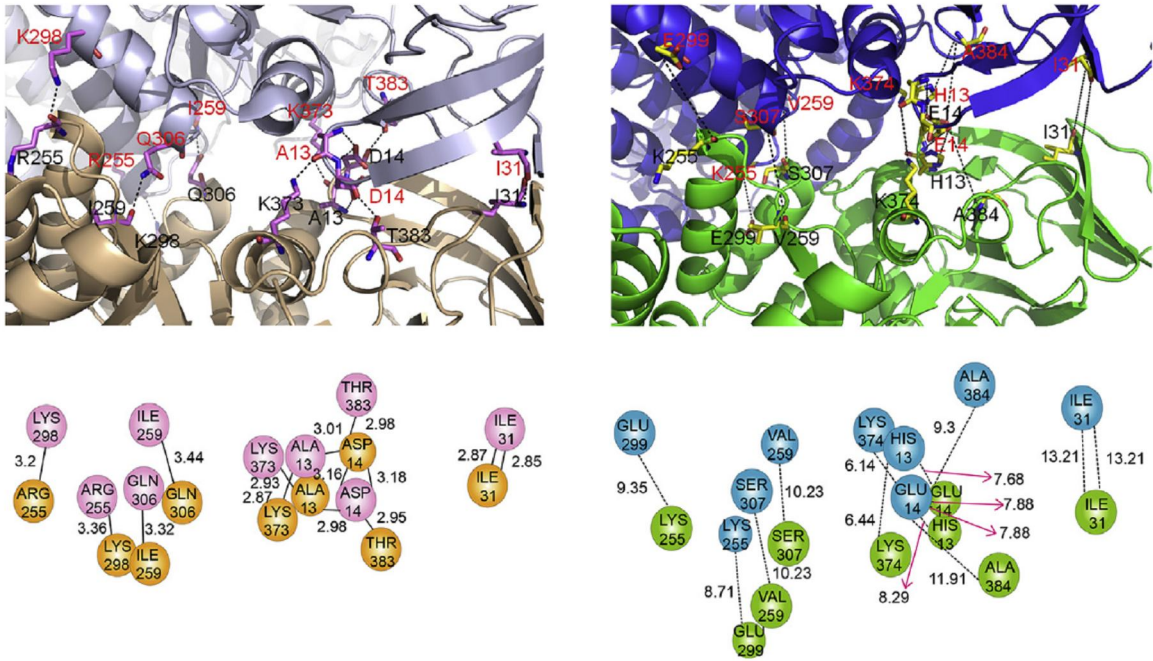


16.圖二十一:綠膿桿菌的二氫嘧啶水解酵素單套體-單套體界面其詳細的作用力與距離分析。

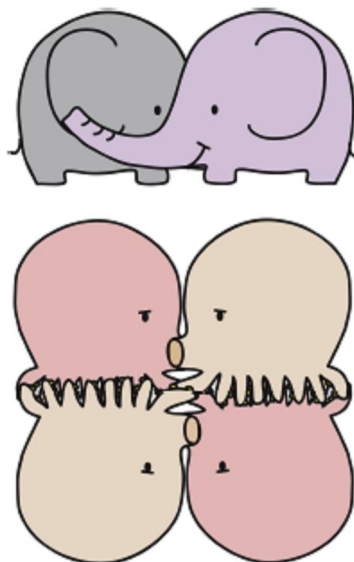
The formation of hydrogen bonds and salt bridges at the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase.

Hydrogen bonds			Salt bridges		
Subunit 1	Subunit 2	Dist. [Å]	Subunit 1	Subunit 2	Dist. [Å]
N186 [ND2]	D163 [OD2]	2.99	H192 [NE2]	E171 [OE1]	3.76
H192 [NE2]	E171 [OE2]	3.2	H192 [NE2]	E171 [OE2]	3.2
R479 [NH1]	E206 [OE1]	2.51	R479 [NH1]	E206 [OE1]	2.51
V477 [N]	E206 [OE2]	2.74	R479 [NH1]	E206 [OE2]	3.7
Q215 [NE2]	T230 [OG1]	2.86	R223 [NE]	E219 [OE2]	3.74
K255 [NZ]	E244 [OE2]	3.03	K255 [NZ]	E244 [OE2]	3.03
Y251 [OH]	D247 [OD2]	2.44	R226 [NH2]	E248 [OE2]	3.48
R226 [NH1]	E248 [OE2]	2.94	R226 [NH1]	E248 [OE2]	2.94
R479 [NH2]	H277 [O]	2.5	E171 [OE2]	L196 [NZ]	3.12
R479 [NE]	P278 [O]	3.4	E206 [OE1]	R479 [NH1]	3.53
R276 [NH1]	V475 [O]	3.05	E206 [OE1]	R479 [NH2]	2.78
R276 [NH2]	V475 [O]	2.73	E206 [OE2]	R479 [NH2]	3.98
D163 [OD2]	N186 [ND2]	2.95	E219 [OE2]	R479 [NE]	3.92
E206 [OE1]	R479 [NH2]	2.78	E244 [OE2]	K255 [NZ]	3.19
E206 [OE2]	V477 [N]	3.24	E248 [OE2]	R226 [NH1]	2.92
E218 [O]	R226 [NH1]	3.49	E248 [OE2]	R226 [NH2]	3.57
T230 [OG1]	Q215 [NE2]	2.93			
E244 [OE2]	K255 [NZ]	3.19			
D247 [OD2]	Y251 [OH]	2.55			
E248 [OE2]	R226 [NH1]	2.92			
Y275 [O]	R479 [NH1]	3.44			
H277 [O]	R479 [NH1]	2.7			
V475 [O]	R276 [NH1]	2.88			
V475 [O]	R276 [NH2]	2.82			

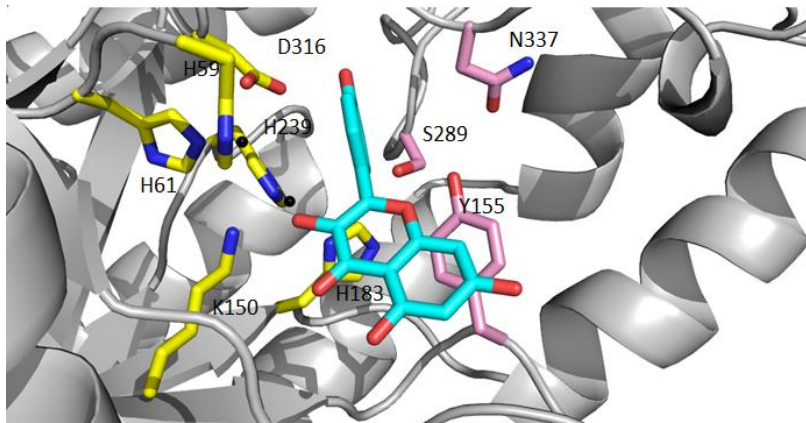
17.圖二十二：綠膿桿菌的二氫嘧啶水解酵素與其他二氫嘧啶水解酵素的雙套體-雙套體界面分析。為何綠膿桿菌的二氫嘧啶水解酵素是雙套體？我們發現在其他物種的二氫嘧啶水解酵素中(左圖)的雙套體-雙套體界面並不保留於綠膿桿菌的二氫嘧啶水解酵素中(右圖)。我們同時利用結晶堆疊查看若有四套體，綠膿桿菌的二氫嘧啶水解酵素中的這些胺基酸其距離為何。右圖顯示這些胺基酸距離都過遠(>4Å 以上)，因此無法形成四套體。



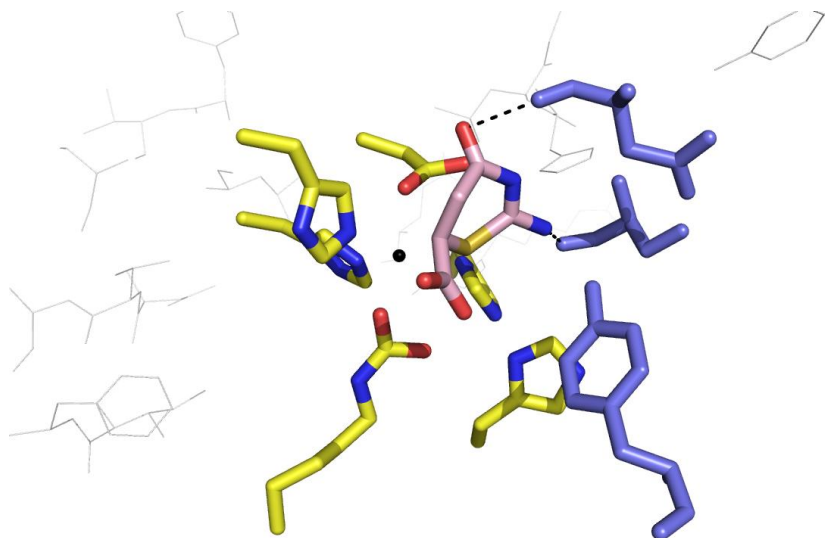
18.圖二十三：綠膿桿菌的二氫嘧啶水解酵素與其他二氫嘧啶水解酵素的結構卡通模型示意圖。由於綠膿桿菌的二氫嘧啶水解酵素是雙套體，且其 C 端遠比其他物種的二氫嘧啶水解酵素來的長，因此我們將之比擬為兩隻大象的組合。不若其他物種的二氫嘧啶水解酵素其雙套體-雙套體界面有強的交互作用(如同章魚的腳相互纏繞)，綠膿桿菌的二氫嘧啶水解酵素幾乎沒有來自雙套體-雙套體的穩定力量，因此僅為雙套體而非四套體。



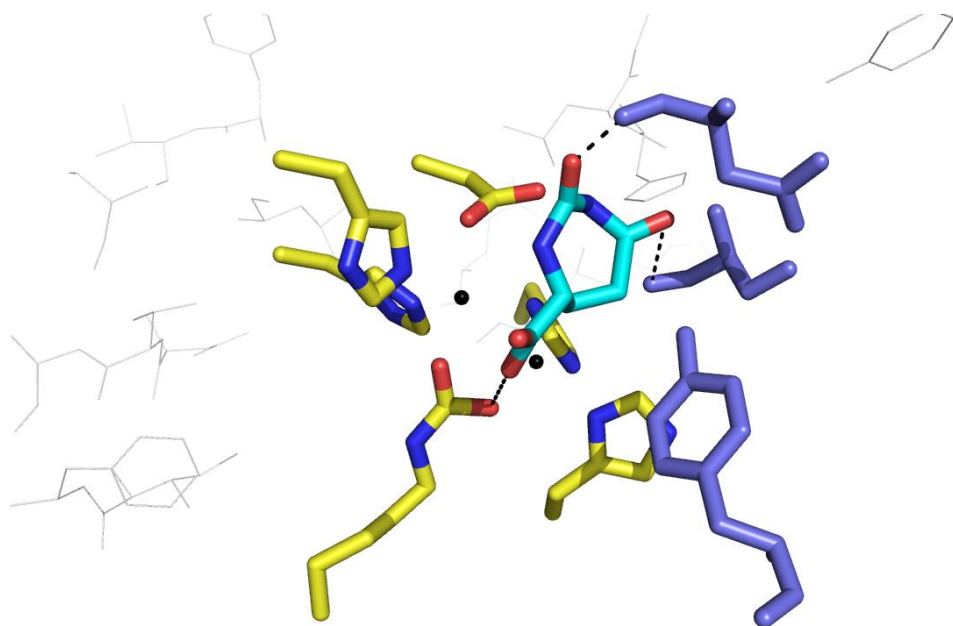
19.圖二十四：綠膿桿菌的二氫嘧啶水解酵素與抑制劑 kaempferol 對接 (docking)分析。我們發現 kaempferol 可能抑制此酵素的原因來自於對活性區的結合(可能為競爭型抑制劑)。由於 kaempferol 的結構並不類似於綠膿桿菌的二氫嘧啶水解酵素所使用的受質，因此到底 kaempferol 是如何達到可辨識此酵素的活性區，對於後續藥物設計亦將是相當重要的下一階段的問題。



20.圖二十五：綠膿桿菌的二氫嘧啶水解酵素與抑制劑 2-imino-4-oxo-[1,3]thiazinane-6-carboxylic acid 對接分析。在分析結晶結構的同時，我們亦發現 2-imino-4-oxo-[1,3]thiazinane-6-carboxylic acid 能抑制綠膿桿菌的二氫嘧啶水解酵素。我們並利用此資訊來優化，希望能根據結構與功能的特性來找到更強的抑制劑。(黃色胺基酸為金屬結合殘基，紫藍色為受質結合位)。



21.圖二十六：綠膿桿菌的二氫嘧啶水解酵素與抑制劑 2,7-Dioxo-[1,3]diazepane-4-carboxylic acid 對接分析。在分析結晶結構的同時，我們亦發現 2,7-Dioxo-[1,3]diazepane-4-carboxylic acid 能抑制綠膿桿菌的二氫嘧啶水解酵素。我們並利用此資訊來優化，希望能根據結構與功能的特性來找到更強的抑制劑。(黃色胺基酸為金屬結合殘基，紫藍色為受質結合位)



(五) 結論與檢討

我們在此計畫篩選了許多結晶條件並解出綠膿桿菌的二氫嘧啶水解酵素的結晶結構，首次發現綠膿桿菌的二氫嘧啶水解酵素是雙套體，不同於其他物種來源是四套體，並且詳細的利用結構中各種物理作用力與距離大小來說明為何綠膿桿菌的二氫嘧啶水解酵素無法形成四套體。我們亦發現了數種化合物可抑制綠膿桿菌的二氫嘧啶水解酵素，並且得到其重要抑制劑 kaempferol 的複合結晶，解析度達 1.6 Å，並且其 space group 也變為 P222(原為 P3₁21)。因為此計畫的執行也同時產生出後續更多需要被繼續研究的問題，例如雙套體與四套體二氫嘧啶水解酵素的特點有何不同、不像受質的物質如何能結合並抑制此酵素活性、結合模式、藥物優化與後續

不傷人體但可抑制細菌重要生理功能的抗生素開發。此計畫部分結果已發表(9)，論文全文附於附錄供參考。

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(七)附錄：論文發表



Crystal structure of dihydropyrimidinase from *Pseudomonas aeruginosa* PAO1: Insights into the molecular basis of formation of a dimer



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ABSTRACT

Dihydropyrimidinase, a tetrameric metalloenzyme, is a member of the cyclic amidohydrolase family, which also includes allantoinase, dihydroorotase, hydantoinase, and imidase. In this paper, we report the crystal structure of dihydropyrimidinase from *Pseudomonas aeruginosa* PAO1 at 2.1 Å resolution. The structure of *P. aeruginosa* dihydropyrimidinase reveals a classic $(\beta/\alpha)_8$ -barrel structure core embedding the catalytic dimetal center and a β -sandwich domain, which is commonly found in the architecture of dihydropyrimidinases. In contrast to all dihydropyrimidinases, *P. aeruginosa* dihydropyrimidinase forms a dimer, rather than a tetramer, both in the crystalline state and in the solution. Basing on sequence analysis and structural comparison of the C-terminal region and the dimer–dimer interface between *P. aeruginosa* dihydropyrimidinase and *Thermus* sp. dihydropyrimidinase, we propose a working model to explain why this enzyme cannot be a tetramer.

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1. Introduction

Dihydropyrimidinase catalyzes the reversible cyclization of dihydrouracil or dihydrothymine to *N*-carbamoyl- β -alanine or *N*-carbamyl- β -aminoisobutyrate, respectively, in the second step of the pyrimidine degradation pathway [1,2]. Dihydropyrimidinase can also detoxify xenobiotics with an imide functional group, ranging from linear imides to heterocyclic imides [3–5]. Homologous enzymes from microorganisms are known as hydantoinase because of their role as biocatalyst in the synthesis of D- and L-amino acids; these amino acids are used for industrial production of precursors for semisynthesis of antibiotics, active peptide, hormones, and pesticides [6,7]. Although dihydropyrimidinase and hydantoinase generally possess a similar active site, their overall sequence identity and substrate specificity differ [8]. For example, dihydropyrimidinases from the yeast *Saccharomyces kluyveri* and the slime mold *Dictyostelium discoideum* do not hydrolyze hydantoin [9]. In addition, several bacterial hydantoinases are named and identified as dihydropyrimidinase because of their catalytic activity

toward natural substrates, namely, dihydrouracil and dihydrothymine. These bacterial enzymes include *Pseudomonas aeruginosa* and *Thermus* sp. dihydropyrimidinases [10,11].

Dihydropyrimidinases, hydantoinases, imidases, allantoinases, and dihydroorotases belong to the cyclic amidohydrolase family because of their functional and structural similarities [12]. Members of this enzyme family catalyze the ring-opening hydrolysis of the cyclic amide bond of each substrate in either five- or six-membered rings in the metabolism of purines, pyrimidines, and many xenobiotics. Despite their similar functions, these enzymes present relatively low amino acid sequence identity. Most of the active sites of dihydropyrimidinases, hydantoinases, allantoinases, and dihydroorotases contain four histidines, one aspartate, and one post-carboxylated lysine residue, which are required for metal binding and catalytic activity [4,10,13–15]. The presence of a post-carboxylated lysine in hydantoinase is also required in self-assembly of binuclear metal center [16] and increases the nucleophilicity of the hydroxide for catalysis [17]. Structural studies of dihydropyrimidinases have been conducted to elucidate their catalytic properties and mechanism of post-lysine carbamylation [11,18–20].

All known dihydropyrimidinases are tetramers to date. The global architecture of dihydropyrimidinase monomer consists of two domains, namely, a large domain with a classic $(\beta/\alpha)_8$ -barrel

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structure core embedding the catalytic dimetal center and a small β -sandwich domain [11,18–20]. The C-terminal tails of each subunit extend toward another monomer in a swapping-like manner, which may be involved in protein tetramerization [19,20]. However, the C-terminal region of dihydropyrimidinases varies greatly in terms of length and similarity from different organisms [21]. Thus, the structure of a dimeric dihydropyrimidinase must be evaluated to determine the oligomerization mechanism.

Previous studies indicated that hydantoinase from *Pseudomonas putida* YZ-26 functions as a dimer [22,23]. To confirm their results and determine how this enzyme can form a dimer, instead of tetramer, the structure is needed to obtain more information in molecular level. Currently, crystal structure of the dimeric dihydropyrimidinase has not been reported yet. In the present study, we identified that dihydropyrimidinase from *P. aeruginosa* PAO1 is active as a dimer. We also determined the crystal structure of *P. aeruginosa* PAO1 dihydropyrimidinase at 2.1 Å resolution. Structural comparison with other dihydropyrimidinases indicated that several residues crucial for tetramerization are not found in *P. aeruginosa* dihydropyrimidinase.

2. Materials and methods

2.1. Cloning, protein expression and purification

Construction of the *P. aeruginosa* dihydropyrimidinase expression plasmid has been reported [10]. Recombinant *P. aeruginosa* dihydropyrimidinase was expressed and purified using the protocol described previously [10]. The protein purified from the soluble supernatant by Ni²⁺-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was eluted with Buffer A (20 mM Tris-HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9) and dialyzed against a dialysis buffer (20 mM HEPES and 100 mM NaCl, pH 7.0; Buffer B). Protein purity remained >97% as determined by SDS-PAGE (Mini-PROTEAN Tetra System; Bio-Rad, CA, USA).

2.2. Gel-filtration chromatography

Gel-filtration chromatography was carried out by the AKTA-FPLC system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). In brief, purified protein (2 mg/mL) in Buffer B was applied to a Superdex 200 HR 10/30 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with the same buffer. The column was operated at a flow rate of 0.5 mL/min, and the proteins were detected at 280 nm. The column was calibrated with proteins of known molecular weight: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa). The K_{av} values for the standard proteins and *P. aeruginosa* dihydropyrimidinase were calculated from the equation: $K_{av} = (V_e - V_o)/(V_c - V_o)$, where V_o is the column void volume, V_e is the elution volume, and V_c is the geometric column volume.

2.3. Crystallography

Before crystallization, *P. aeruginosa* dihydropyrimidinase was concentrated to 20 mg/mL in Buffer B. Crystals were grown at room temperature by hanging drop vapor diffusion in 28% PEG 6000, 100 mM HEPES, 200 mM lithium acetate, pH 7.5. Data collection and refinement statistics for the crystal of *P. aeruginosa* dihydropyrimidinase are shown in Table 1. Data were collected using an ADSC Quantum-315r CCD area detector at SPXF beamline BL13C1 at NSRRC (Taiwan, ROC). All data integration and scaling were carried out using HKL-2000 [24]. There were two *P. aeruginosa* dihydropyrimidinase monomers per asymmetric unit. The crystal

Table 1
Data collection and refinement statistics.

Data collection	
Crystal	<i>P. aeruginosa</i> dihydropyrimidinase
Wavelength (Å)	0.975
Resolution (Å)	97.11–2.1
Space group	<i>P</i> 3 ₁ 21
Cell dimension (Å)	<i>a</i> = 112.13 α = 90 <i>b</i> = 112.13 β = 90 <i>c</i> = 161.05 γ = 120
Completeness (%)	99.9 (99.9) ^a
<1/ σ >	19.21 (3)
R _{sym} or R _{merge}	0.084 (0.59)
Redundancy	6.0 (5.9)
Refinement	
Resolution (Å)	30–2.1
No. reflections	68799
R _{work} /R _{free}	0.1696/0.2155
No. atoms	
Protein	1299
Water	338
R.m.s deviation	
Bond lengths (Å)	0.0196
Bond angles (°)	1.8795
Ramachandran plot	
In preferred regions	903 (94.75%)
In allowed regions	43 (4.51%)
Outliers	7 (0.73%)
PDB entry	5E5C

^a Values in parentheses are for the highest resolution shell.

structure of *P. aeruginosa* dihydropyrimidinase was solved at 2.1 Å resolution with the molecular replacement software AMoRe [25] using *D. discoideum* dihydropyrimidinase [20] (with 51.7% identity to *P. aeruginosa* dihydropyrimidinase) as model (PDB entry: 2FTW). After molecular replacement, model building was carried out using XtalView [26]. CNS was used for molecular dynamic refinement [27]. The final structure was refined to an *R*-factor of 0.1696 and an *R*_{free} of 0.2155. Atomic coordinates and related structure factors have been deposited in the PDB with accession code 5E5C.

3. Results and discussion

3.1. Structure of the *P. aeruginosa* dihydropyrimidinase monomer

We crystallized *P. aeruginosa* dihydropyrimidinase and determined its structure at a resolution of 2.1 Å (Table 1). The cell unit contains two monomers of *P. aeruginosa* dihydropyrimidinase (Fig. 1A). The majority of the electron density for *P. aeruginosa* dihydropyrimidinase exhibited good quality, and no discontinuity was observed. Briefly, the overall structure of each *P. aeruginosa* dihydropyrimidinase unit consists of 17 α -helices, 19 β -sheets, and two zinc ions (Fig. 1B). The architecture of the *P. aeruginosa* dihydropyrimidinase monomer consists of two domains, namely, a large domain with a classic (β/α)₈-barrel structure core embedding the catalytic dimetal center and a small β -sandwich domain. The overall structure and architecture of the active site of *P. aeruginosa* dihydropyrimidinase are similar to those of other dihydropyrimidinases (Fig. 1C) and other members of the amidohydrolase family of enzymes, such as hydantoinases, dihydroorotases, and allantoinases (Fig. 1D). The active sites of these enzymes contain four histidines, one aspartate, and one post-carboxylated lysine residue, which are required for metal binding and catalytic activity [9,10,14,16,28–30].

3.2. *P. aeruginosa* dihydropyrimidinase as a dimer in solution

All known dihydropyrimidinases are tetramers. Given that the

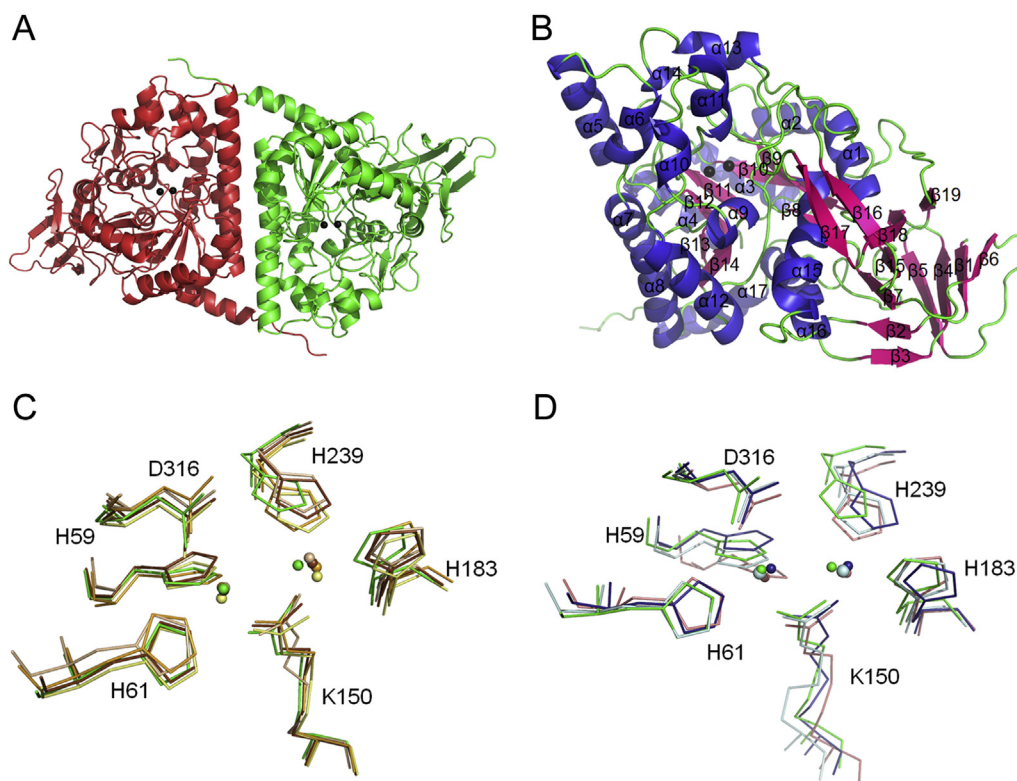


Fig. 1. Crystal structure of *P. aeruginosa* dihydropyrimidinase. (A) Ribbon diagram of a *P. aeruginosa* dihydropyrimidinase dimer. Each *P. aeruginosa* dihydropyrimidinase monomer is color-coded. Two zinc ions in the active site are presented as black spheres. (B) Ribbon diagram of a *P. aeruginosa* dihydropyrimidinase monomer with the secondary structures labeled. (C) Superposition of the active site of dihydropyrimidinases. Their active sites contain four histidines, one aspartate, and one post-carboxylated lysine residue, which are required for metal binding and catalytic activity. Dihydropyrimidinases from *P. aeruginosa* (PDB entry: 5E5C; green), *S. kluyveri* (PDB entry: 2FTY; orange), *Thermus* sp. (PDB entry: 1GKQ; wheat), *Sinorhizobium meliloti* (PDB entry: 3DC8; brown), and *Tetraodon nigroviridis* (PDB entry: 4H01; paleyellow) are shown. The architecture of these active sites is similar. (D) Superposition of the active site of members of the amidohydrolase family. Their active sites contain four histidines, one aspartate, and one post-carboxylated lysine residue, which are required for metal binding and catalytic activity. *P. aeruginosa* dihydropyrimidinase (PDB entry: 5E5C; green), *Escherichia coli* allantoinase (PDB entry: 3E74; palecyan), *Burkholderia pickettii* hydantoinase (PDB entry: 1NFG; deepblue), and *E. coli* dihydroorotase (PDB entry: 1J79; salmon) are shown. The architecture of these active sites is similar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure implies that *P. aeruginosa* dihydropyrimidinase forms a dimer in the crystalline (Fig. 1A), we performed biochemical verification to confirm the oligomerization state. To confirm that *Pseudomonas* hydantoinases/dihydropyrimidinases function as a dimer, we conducted gel filtration chromatography. Results revealed a single peak (Fig. 2A). Assuming that the shape and partial specific volume of *P. aeruginosa* dihydropyrimidinase are similar to those of standard proteins, the native molecular mass of *P. aeruginosa* dihydropyrimidinase was estimated to be 117 kDa, which was calculated from the standard linear regression equation: $K_{av} = -0.2765 (\log Mw) + 1.7210$ (Fig. 2B). The native molecular mass of *P. aeruginosa* dihydropyrimidinase is approximately two times higher than the molecular mass of a monomer (53 kDa). Thus, we conclude that *P. aeruginosa* dihydropyrimidinase in solution is a stable dimer, similar to bacterial hydantoinase from *P. putida* YZ-26 [22,23].

3.3. Monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase

Crystal structure of *P. aeruginosa* dihydropyrimidinase shows that the two subunits are tightly associated (Fig. 1A). *P. aeruginosa* dihydropyrimidinase monomers are interconnected through many hydrophobic interactions. As shown in Fig. 2C, the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase is stabilized by the hydrophobic core and the C-terminal tail region, namely, Ala162 (loop), Val167 ($\alpha 4$), Leu174 ($\alpha 5$), Leu189 ($\alpha 5$),

Leu193 ($\alpha 5$), Leu197 ($\alpha 5$), Ala199 ($\alpha 5$), Leu202 (loop), Leu210 ($\alpha 6$), Val475 (C-terminal tail), Ala476 (C-terminal tail), and Val477 (C-terminal tail). However, analysis of the amino acid sequences of 497 sequenced dihydropyrimidinase homologs using ConSurf [31–33] indicates that these residues at the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase are variable (Supplementary Fig. 1). Only Leu189 ($\alpha 5$) and Val477 (C-terminal tail) are conserved.

We also analyzed the formation of hydrogen bonds and salt bridges at the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase by using PISA (Protein Interfaces, Surfaces and Assemblies) [34], which is an automatic analytical tool for macromolecular assemblies in the crystalline state. Table 2 summarizes the hydrogen bonds and salt bridges at the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase. Based on ConSurf analysis (Supplementary Fig. 1), only the hydrogen bonds of D163–N186, E206–R479, E206–V477, and Y275–R479 as well as the salt bridges of E206–R479, E219–R479, and R223–E219 are highly conserved. Previous works indicated that the C-terminal Arg479 in bacterial hydantoinase plays an important role in dimerization [22,23]. In the present study, the structure provides the evidence that the subunit association of *P. aeruginosa* dihydropyrimidinase is mediated by the C-terminal Arg479 through hydrogen bonds and salt bridges. We also found that only six residues (G425, G434, G448, G450, R455, and R479) are highly conserved in the C-terminal region 421–479 of the 497 sequenced dihydropyrimidinase homologs (Supplementary Fig. 1).

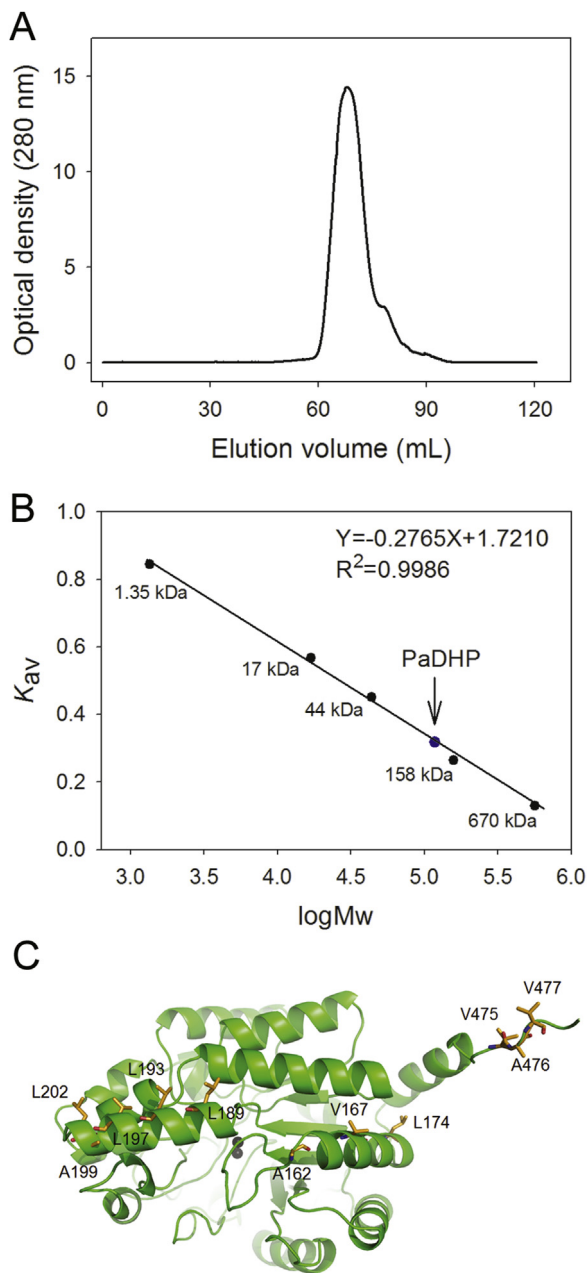


Fig. 2. The gel filtration chromatographic analysis and the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase. (A) Gel-filtration chromatography was carried out by the AKTA-FPLC system. The corresponding peak shows the eluting *P. aeruginosa* dihydropyrimidinase. (B) The column was calibrated with proteins of known molecular weight: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa). The K_{av} values for the standard proteins and *P. aeruginosa* dihydropyrimidinase were calculated from the equation: $K_{av} = (V_e - V_0)/(V_c - V_0)$, where V_0 is the column void volume, V_e is the elution volume, and V_c is the geometric column volume. A standard linear regression curve was generated by plotting the log of the molecular mass of the calibration proteins against their K_{av} values. (C) The monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase is stabilized by the hydrophobic core and the C-terminal tail region, namely, Ala162 (loop), Val167 ($\alpha 4$), Leu174 ($\alpha 5$), Leu189 ($\alpha 5$), Leu193 ($\alpha 5$), Leu197 ($\alpha 5$), Ala199 ($\alpha 5$), Leu202 (loop), Leu210 ($\alpha 6$), Val475 (C-terminal tail), Ala476 (C-terminal tail), and Val477 (C-terminal tail).

Table 2

The formation of hydrogen bonds and salt bridges at the monomer–monomer interface of *P. aeruginosa* dihydropyrimidinase.

Hydrogen bonds			Salt bridges		
Subunit 1	Subunit 2	Dist. [Å]	Subunit 1	Subunit 2	Dist. [Å]
N186 [ND2]	D163 [OD2]	2.99	H192 [NE2]	E171 [OE1]	3.76
H192 [NE2]	E171 [OE2]	3.2	H192 [NE2]	E171 [OE2]	3.2
R479 [NH1]	E206 [OE1]	2.51	R479 [NH1]	E206 [OE1]	2.51
V477 [N]	E206 [OE2]	2.74	R479 [NH1]	E206 [OE2]	3.7
Q215 [NE2]	T230 [OG1]	2.86	R223 [NE]	E219 [OE2]	3.74
K255 [NZ]	E244 [OE2]	3.03	K255 [NZ]	E244 [OE2]	3.03
Y251 [OH]	D247 [OD2]	2.44	R226 [NH2]	E248 [OE2]	3.48
R226 [NH1]	E248 [OE2]	2.94	R226 [NH1]	E248 [OE2]	2.94
R479 [NH2]	H277 [O]	2.5	E171 [OE2]	L196 [NZ]	3.12
R479 [NE]	P278 [O]	3.4	E206 [OE1]	R479 [NH1]	3.53
R276 [NH1]	V475 [O]	3.05	E206 [OE1]	R479 [NH2]	2.78
R276 [NH2]	V475 [O]	2.73	E206 [OE2]	R479 [NH2]	3.98
D163 [OD2]	N186 [ND2]	2.95	E219 [OE2]	R479 [NE]	3.92
E206 [OE1]	R479 [NH2]	2.78	E244 [OE2]	K255 [NZ]	3.19
E206 [OE2]	V477 [N]	3.24	E248 [OE2]	R226 [NH1]	2.92
E218 [O]	R226 [NH1]	3.49	E248 [OE2]	R226 [NH2]	3.57
T230 [OG1]	Q215 [NE2]	2.93			
E244 [OE2]	K255 [NZ]	3.19			
D247 [OD2]	Y251 [OH]	2.55			
E248 [OE2]	R226 [NH1]	2.92			
Y275 [O]	R479 [NH1]	3.44			
H277 [O]	R479 [NH1]	2.7			
V475 [O]	R276 [NH1]	2.88			
V475 [O]	R276 [NH2]	2.82			

in the crystalline state and in the solution. However, all known dihydropyrimidinases are tetramers. To assess why *P. aeruginosa* dihydropyrimidinase cannot form a stable tetramer, we compared the structure of this enzyme with that of *Thermus* sp. dihydropyrimidinase (PDB entry: 1GKQ). These enzymes differ in terms of the dimer–dimer interface (Fig. 3) and the length of the C-terminal tail. Although their amino acid sequences are highly conserved (Fig. 3A), the important residues located at the dimer–dimer interface for tetramer formation are quite different (Table 3). For the tetramer formation of *Thermus* sp. dihydropyrimidinase, many hydrogen bonds with close distance were found; these bonds (<3 Å) include K373–A13, T383–D14, I31–I31, and D14–A13 (Fig. 3B). However, these interactions, except for I31–I31, were not identified in the crystallographically related dimer–dimer interface of *P. aeruginosa* dihydropyrimidinase (Fig. 3C). The distance of I31–I31 in *P. aeruginosa* dihydropyrimidinase is 13.21 Å, which suggests that this bond has no role in the interaction. Considering that these residues are not conserved (Fig. 3A) and the two dimers of *P. aeruginosa* dihydropyrimidinase are associated via slight contact (Fig. 3C), *P. aeruginosa* dihydropyrimidinase could not form a tetramer (see Fig. 4).

Comparison by superimposition indicated that the length of C-terminal tails significantly differs between *P. aeruginosa* and of *Thermus* sp. dihydropyrimidinases (Fig. 3D). The residue Arg479 crucial for dimerization of *P. putida* hydantoinase [22,23] is not found in *Thermus* sp. dihydropyrimidinase (Fig. 3A), and mutation of this Arg residue of *S. meliloti* dihydropyrimidinase does not cause the dissociation of the tetramer [19]. Thus, the length of the C-terminal region of *P. aeruginosa* dihydropyrimidinase may be not an important factor to form a tetramer, given that *Thermus* sp. dihydropyrimidinase can efficiently form a tetramer.

3.4. Comparison of the tetrameric and dimeric structures using *Thermus* sp. dihydropyrimidinase and crystallographically related dimer–dimer interface of *P. aeruginosa* dihydropyrimidinase

We identified *P. aeruginosa* dihydropyrimidinase as a dimer both

3.5. Structural insights into dimer formation of dihydropyrimidinase

In this study, we identified *P. aeruginosa* dihydropyrimidinase as a dimer both in the crystalline state and in solution (Fig. 2). The

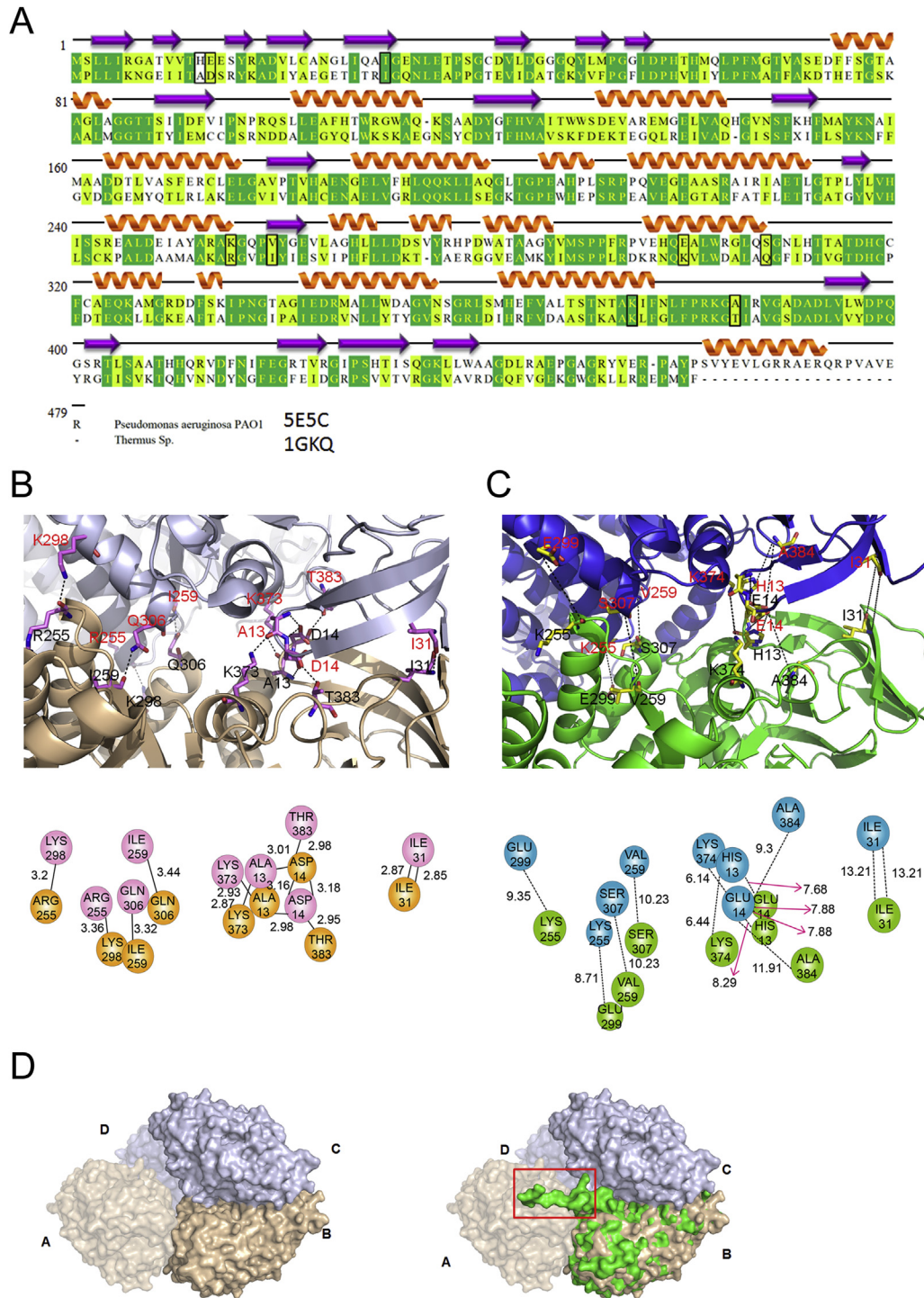


Fig. 3. Comparison of the tetrameric and dimeric structures using *Thermus sp.* dihydropyrimidinase and crystallographically related dimer–dimer interface of *P. aeruginosa* dihydropyrimidinase. (A) Structure-based sequence alignment of dihydropyrimidinases from *P. aeruginosa* and *Thermus sp.* The labeled secondary structural elements derived from this work are shown above the alignment. The amino acids that are involved in dimer–dimer interface of *Thermus sp.* dihydropyrimidinase are boxed. (B) Structural analysis of the dimer–dimer interface of *Thermus sp.* dihydropyrimidinase. The distance (Å) of the residues is shown. (C) The residues, crucial for forming hydrogen bonds at the dimer–dimer interface of *Thermus sp.* dihydropyrimidinase, were not identified in the crystallographically related dimer–dimer interface of *P. aeruginosa* dihydropyrimidinase. (D) Comparison by superimposition of dihydropyrimidinases from *Thermus sp.* and *P. aeruginosa*. The length of C-terminal tails significantly differs between *P. aeruginosa* (green) and *Thermus sp.* dihydropyrimidinases (dimer of dimer). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure of the tetrameric *Thermus sp.* dihydropyrimidinase and dimeric *P. aeruginosa* dihydropyrimidinase was compared (Fig. 3). Many important residues for *Thermus sp.* dihydropyrimidinase tetramer formation are different from those for *P. aeruginosa*

dihydropyrimidinase. This condition might be the reason why *P. aeruginosa* dihydropyrimidinase could not form a tetramer similar to *Thermus sp.* dihydropyrimidinase because these critical residues are not found in the former. Basing on the results as well as

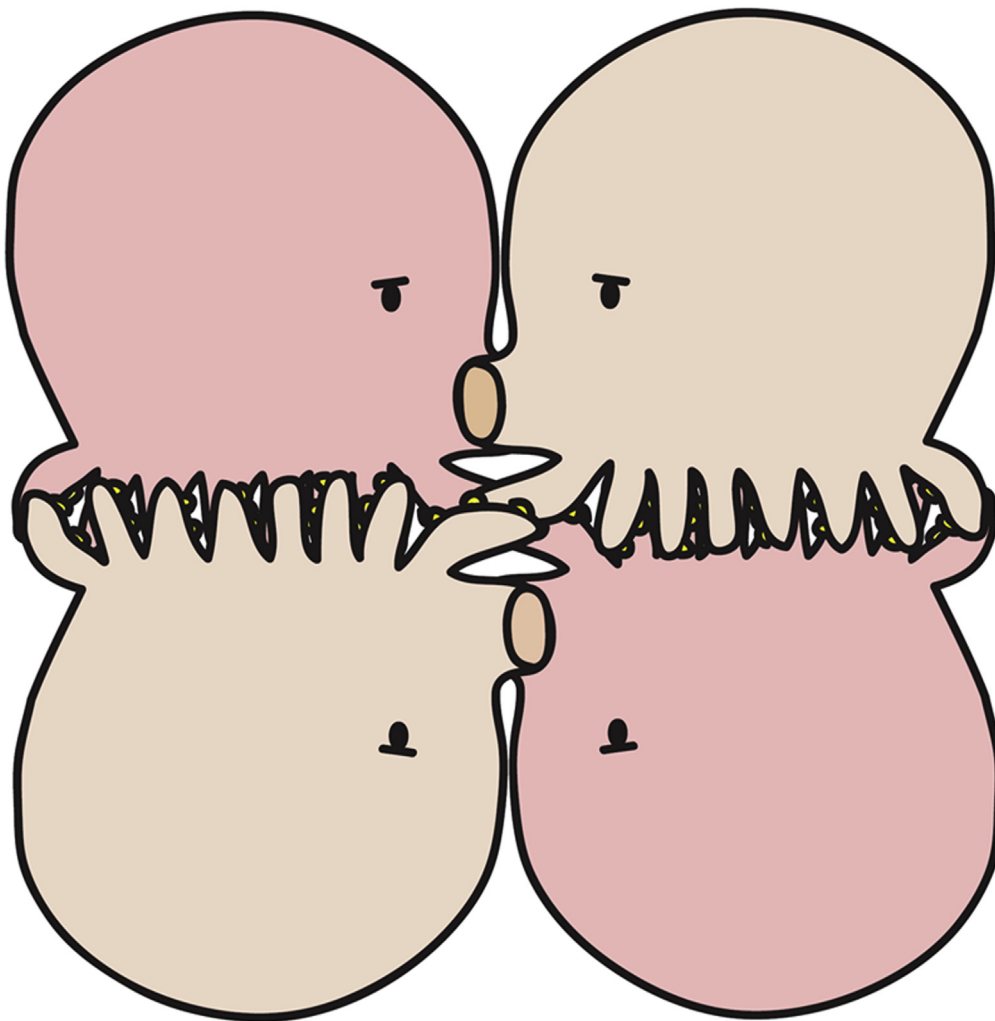
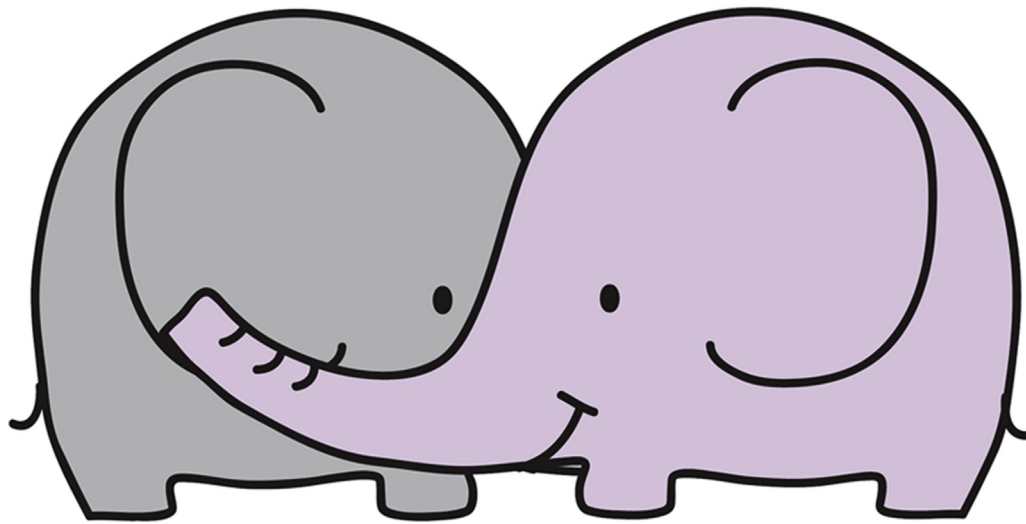


Fig. 4. A working model to explain why this enzyme cannot be a tetramer. In this study, we identified *P. aeruginosa* dihydroxyrimidinase as a dimer both in the crystalline state and in solution. The structure of the tetrameric *Thermus* sp. dihydroxyrimidinase and dimeric *P. aeruginosa* dihydroxyrimidinase was compared, and many important residues for *Thermus* sp. dihydroxyrimidinase tetramer formation are different from those for *P. aeruginosa* dihydroxyrimidinase. This condition might be the reason why *P. aeruginosa* dihydroxyrimidinase could not form a tetramer. Basing on the results as well as sequence and crystal structural comparisons, we propose a cartoon model (elephant versus octopus) to show why *P. aeruginosa* dihydroxyrimidinase (elephant) could not form a tetramer similar to other dihydroxyrimidinases (octopus). Given only a minimal contact (lack of many important and conserved residues similar to octopus legs), this working model may explain why *P. aeruginosa* dihydroxyrimidinase is a dimer, not a tetramer. Although *P. aeruginosa* dihydroxyrimidinase has a longer C-terminal region (shown as elephant nose) than that of *Thermus* sp. dihydroxyrimidinase, this region was proven to play a crucial role for dihydroxyrimidinase dimerization by structural information and mutational analyses.

Table 3

The formation of hydrogen bonds at the dimer–dimer interface of *Thermus* sp. dihydropyrimidinase and the corresponding residues at the crystallographically related dimer–dimer interface of *P. aeruginosa* dihydropyrimidinase.

<i>Thermus</i> sp. dihydropyrimidinase (PDB entry 1GKQ)			<i>P. aeruginosa</i> dihydropyrimidinase (PDB entry 5E5C)		
Subunit 1	Subunit 2	Dist. [Å]	Subunit 1	Subunit 2	Dist. [Å]
Hydrogen bonds					
K373 [NZ]	A13 [O]	2.93	K374 [NZ]	H13 [O]	6.14
T383 [OG1]	D14 [O]	2.98	A384 [N]	E14 [O]	9.3
I31 [N]	I31 [O]	2.87	I31 [N]	I31 [O]	13.21
A13 [O]	K373 [NZ]	2.87	H13 [O]	K374 [NZ]	6.44
D14 [O]	T383 [OG1]	2.95	E14 [O]	A384 [N]	11.91
D14 [OD2]	A13 [N]	2.98	E14 [OE2]	H13 [N]	8.29
I31 [O]	I31 [N]	2.85	I31 [O]	I31 [N]	13.21

sequence and crystal structural comparisons, we propose a cartoon model (elephant versus octopus) to show why *P. aeruginosa* dihydropyrimidinase (elephant) could not form a tetramer similar to other dihydropyrimidinases (octopus). Given only a minimal contact (lack of many important and conserved residues similar to octopus legs), this working model may explain why *P. aeruginosa* dihydropyrimidinase is a dimer, not a tetramer. Although *P. aeruginosa* dihydropyrimidinase has a longer C-terminal region (shown as elephant nose) than that of *Thermus* sp. dihydropyrimidinase, this region was proven to play a crucial role for dihydropyrimidinase dimerization by structural information and mutational analyses.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2016.08.144>.

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