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去氧核醣核酸水解

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摘　要

去氧核醣核酸水解 (deoxyribonuclease DNase)為 一 種 核 酸 (endonuclease) 可水解基質去氧核糖核酸(DNA)中之磷酸雙酯鍵(phosphodiester bond)。非限制性的 DNase 根據目前研究可大致區分為三大類, 包含：DNase I、DNase II 與近年來研究細胞凋亡(apoptosis)之熱潮中甫被發現的 caspase-activated DNase (CAD)。本文中除對上述三者之基本性質及應用略作介紹外, 另將著眼於首先被發現且目前被研究最為透徹的牛胰臟甲型去氧核醣核酸水解 (bovine pancreatic DNase I, bpDNase), 分別就其基本結構特性、可能作用機轉、與鈣離子之關係、其雙硫鍵之生物功能及其 N-與 C-端片段參與 bpDNase 活性蛋白摺疊之重要性等方面近年來研究之重要成果作詳細之介紹；最後提出本實驗室進行 DNases 相關結構與功能研究之未來展望。

關鍵詞：去氧核醣核酸水解 、牛胰臟甲型去氧核醣核酸水解 、鈣離子、雙硫鍵、蛋白質摺疊

Deoxyribonuclease

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Abstract

Deoxyribonuclease (DNase) is an endonuclease capable of hydrolyzing phosphodiester bonds in double-stranded DNA. Non-restrictive DNases can be divided into three major categories based on the current studies, including DNase I, DNase II and caspase-activated DNase (CAD), which arose from the apoptosis researches recently. In this review, we will introduce not only the basic properties and applications of these three groups of DNases, but also will focus on the firstly-discovered and most thoroughly-studied DNase, bovine pancreatic DNase I (bpDNase). The structural characteristics, possible catalytic mechanism, calcium effects, biological functions of the disulfides, and the involvement of the N- and C-terminal fragments in the active protein folding of bpDNase will be described, and future perspectives of our investigations for the structure and functions of DNase will be discussed.

Key words: deoxyribonuclease (DNase), bovine pancreatic DNase (bpDNase), calcium, disulfide, protein folding

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前　言

一、去氧核醣核酸水解　基本性質與分類

去氧核醣(deoxyribonuclease)為一種核酸內切(endonuclease)，可水解基質去氧核糖核酸(DNA)中之磷酸雙酯鍵(phosphodiester bond)，以下簡稱 DNase。自然界中絕大部份的生命體都含有 DNase，其中以牛胰臟中之 DNase 最具代表性(Moore, 1981)，其主要之功能是用來消化食物中之 DNA。而在動植物細胞內之(lysosome)亦含有 DNase，此類酵素則是用以分解細胞內外之 DNA。另外在 DNA 合成、修補以及遺傳基因轉換時也需要 DNase 之參與(Mosbaugh and Linn, 1983)；而在分子生物學研究中經常使用之限制(restriction enzyme)亦為 DNase 之一種。非限制性的 DNase 根據目前研究可大致區分為三類，即 DNase I (EC.3.1.21.1)、DNase II (EC.3.1.22.1)與近年來研究細胞凋亡(apoptosis)之熱潮中甫被發現的 caspase-activated DNase (CAD) (Enari *et al.*, 1998)。DNase I 及 DNase II 主要的差別在於：DNase I 需要二價金屬離子之參與來活化其酵素活性，且其最適生理作用條件為中性或微鹼性；而 DNase II 則反而會被二價金屬離子抑制其反應，最適作用條件則為酸性環境。在酵素水解產物方面，DNase I 為 2-4 base 長帶有 5’端磷酸根(phosphate)的寡核(oligonucleotide)，DNase II 則為 2-4 base 長帶有 3’端磷酸根的寡核酸；以下就 DNases 之特性作進一步的介紹。

二、甲型去氧核醣核酸水解　(DNase I)

DNase I 主要分布於動物的分泌性腺體中，如胰臟、唾腺即為哺乳動物體內 DNase I 含量最多的器官，其主要功能為分解消化道中食物的 DNA。然而，在哺乳動物體內許多其他不同的器官、組織及體液中也有 DNase I 的存在，但含量較少；例如人類之血球(Kishi *et al.*, 1990)、胃部(Tsutsumi *et al.*, 2001)、腦下垂體(Yasuda *et al.*, 2002)、兔之尿液(Yasuda *et al.*, 1997)、唾液(Johnson and Kalu, 1988)、腎臟(Nadano *et al.*, 1991)、老鼠之肝臟(Malicka-Blaszkiewicz, 1990)、胃部(Tsutsumi *et al.*, 2001)、腎臟(Basnakian *et al.*, 2002)、雞之眼球(Counis *et al.*, 1991)、鳥類之卵母細胞(Stepinska and Olszanska, 2003)等。另外，也有許多研究著眼於不同物種來源之 DNase I，分別將之萃取、純化並研究其結構與功能；這方面的報告包括有豬、牛、羊之胰臟(Abe and Liao, 1983)、肌肉細胞(Los *et al.*, 2000)、狗(Kaneko *et al.*, 2003)、兔子(Yasuda *et al.*, 1997)、雞之胰臟(Hu *et al.*, 2003)、兩生類(蛙、蟾蜍、蠑螈) (Takeshita *et al.*, 2001)、爬蟲類(蛇)(Stoynov *et al.*, 1997; Takeshita *et al.*, 2003)、魚之肝胰臟(Hsiao *et al.*, 1997; Mogi *et al.*, 2003)、蝦之肝胰臟(Chou and Liao, 1990; Wang *et al.*, 2000)、昆蟲(Schernthaner *et al.*, 2002)、發芽之大麥(Liao, 1977)、蘆筍(Wang and Ng, 2001)、真菌(Chen *et al.*, 1993; Ho and Liao, 1999)、細菌(Focareta and Manning, 1991; Hasegawa *et al.*, 2002)和病毒(Hsiang *et al.*, 1998; Liu *et al.*, 1998)等。另有首次以營養學角度探討 DNase I 在大鼠腮腺與小腸中被飢餓與再餵食調控其表現的重要發現(Lu *et al.*, 2003)。

DNase I 在醫藥之應用上也逐漸受到重視(Liao, 1997)。人類之 DNase I 基因已成功被選殖(Shak *et al.*, 1990)，並進而製造大量重組人類 DNase I (rhDNase I)以治療纖維性囊腫(cystic fibrosis)患者之肺中積痰(Cantin, 1998; Furuya *et al.*, 2001; Diot *et al.*, 2003; Geller *et al.*, 2003; Jones and Wallis, 2003; Le Bourgeois, 2003)，和全身紅斑性狼瘡(systemic lupus erythematosus, SLE)(Pan *et al.*, 1998)。美國大藥廠 Genetech 之研究群進一步利用定點突變法改變 rhDNase I 對 actin 抑制之抗性(Pan and Lazarus, 1998)並增強其與基質 DNA 之結合能力(Pan *et al.*, 1998)，以得到更有效力的 rhDNase I。近年來 rhDNase I 更進一步廣泛應用在多種呼吸道疾病之治療，如：早產兒之出生性肺膨脹不全症(atelectasis) (Kupeli *et al.*, 2003)、支氣管炎(bronchitis) (Manna, 2003)、胸蓄膿症(empyema thoracis) (Simpson *et al.*, 2003)等。最近也在臍帶血解凍過程中有效地被用來防止凝集現象的產生(Beck *et al.*, 2003; Eichler *et al.*, 2002)。另外小白鼠 DNase I 之基因也被

選殖出來(Takeshita *et al.*, 1997)；而用化學方法合成之牛胰臟 DNase I之基因亦可在 *E. coli* 中表現(Worrall and Connolly, 1990)，並作定點突變研究其與 DNA 結合之部位(Doherty *et al.*, 1991)、生產高效率(Pan and Lazarus, 1997)及具選擇性之 DNase I (Warren *et al.*, 1997)；最近牛胰臟 DNase I 之 cDNA 也被選殖完成並定出其序列(Chen *et al.*, 1998)。某些疾病，尤其是癌症被認為與血液中之 DNase I 活性有關(Economidou-Karaoglou, 1990)；近來許多研究也明確指出在全身紅斑性狼瘡患者體內發生了 DNase I 基因突變或缺失的現象(Simmonds *et al.*, 2002; Chakraborty *et al.*, 2003; Lachmann, 2003)。高劑量之 methotrexate 治療(Reitz and Gutjahr, 1991)及腎功能衰退(Adamiec *et al.*, 1991)等之患者，亦有 DNase I 升高的現象。另外白喉毒素也被証實具有 DNase I之活性(Wisnieski *et al.*, 1998)。利用亂點突變法也找到了細胞致死根源之 DNase I 活性是在 Colicin E9 的 C-端(Garinot-Schneider *et al.*, 1996)。DNase I 經點滴輸入老鼠體內被證實可以防止肺癌細胞之擴散(Tokita *et al.*, 1995)。DNase I 之活性測定也可作為許多癌症的指標(Dewez *et al.*, 1993; Stolzenberg *et al.*, 1996)，對癌細胞有特殊結合能力之蛋白質如與 DNase I 作共價結合後，可產生針對癌細胞之殺滅能力(Linardou *et al.*, 1994)。牛之 DNase I 具多樣性(Chang *et al.*, 1994)，人類之各種體液所含有之 DNase I 亦然，因此可以利用高靈敏度之電泳偵測法，得到不同的個人型式(Yasuda *et al.*, 1995)；此種 DNase I 多樣性已可應用在人類之犯罪證據搜查(Yasuda *et al.*, 1996)。而在測定古生物 DNA 時，DNase I 則可用來防止其他 DNA 之污染(Eshleman and Smith. 2001)。

三、乙型去氧核醣核酸水解 (DNase II)

DNase II 則 主 要 存 在(lysosome)中，其與溶 體 外 膜 之 結(Chang and Liao, 1990)；另亦有少量分布於細胞核內。由於在巨噬細胞(macrophage)中也有它的存在，因此 DNase II 被認為與分解外來 DNA (細菌、病毒)有關。DNase II 已被發現廣泛分布於人類、豬、大鼠與小鼠體內各器官與體液中(Yasuda *et al.*, 1992)，另在昆蟲性器官中之 DNase II 則可受荷爾蒙之調節(Dutta-Gupta and Sridevi, 1991)。本實驗室已於 1998 年由王政清博士完成豬脾臟 DNase II 之 cDNA 選殖及蛋白質結構決定(Wang *et al.*, 1998)，同時人類及其他物種 DNase II 之 cDNA 亦被發表出來(Baker *et al.*, 1998; Shiokawa and Tanuma, 1998; Yasuda *et al.*, 1998)。另一方面，老鼠體內亦存在有一種類似 DNase II 的酸性 DNase (Tanuma and Shiokawa, 1993)。另在線蟲(Wu *et al.*, 2000)、果蠅(Evans *et al.*, 2002)及其他寄生蟲(Maclea *et al.*, 2003)體內也相繼發現類似 DNase II 的酸性 DNase。研究顯示，DNase II 在老鼠胎兒肝臟的造血作用中扮演重要角色(Kawane *et al.*, 2001)。另外美日研究群相繼在老鼠肝臟及人類唾腺發現新型的 DNase II，其生理功能原本尚不清楚，但最近一項重要的研究顯示在老鼠水晶體中富含此酵素，一旦缺失將造成白內障之主要成因(Nishimoto *et al.*, 2003)；起初科學家們將此酵素命名為 DLAD (DNase II-like acid DNase) (Shiokawa and Tanuma, 1999)，之後統一正名為 DNaseIIβ (Krieser *et al.*, 2001)，同時將原型改名為 DNase IIα。最近人類 DNase IIα 用以形成活性結構的重要決定因子如訊息序列、活性區胺基酸、醣基化作用及雙硫鍵形成等都已被研究清楚(MacLea *et al.*, 2003)。人類 DNase IIα在 HL60 細胞分化過程中的轉錄作用也被發現由 Sp1 與 Sp3 參與作正向調控(up-regulation) (Chou *et al.*, 2003)。另在基因治療發展的過程中，DNase II 也被認為是主要影響轉染(transfection)效率的障礙(Howell *et al.*, 2003)。

四、去氧核醣核酸水解 與細胞凋亡

近年來，細胞之計畫性死亡(programmed cell death)、又稱細胞凋亡(apoptosis)成為相當熱門的研究課題；陸續有研究指出，包含 CAD (Enari *et al.*, 1998)、DNase I (Liu *et al.*, 1997; Nitahara *et al.*, 1998)、DNase II (Pio *et al.*, 1998)、L-DNase II (Torriglia *et al.*, 1998)與 DNaseγ (Shiokawa and Tanuma, 1998)等在內之多種 DNases，都可能參與細胞凋亡，執行細胞核內 DNA 之分解作用。其中被研究得最為清楚，已建

立其可能作用機制的就是近年來甫被發現的 CAD，平時存在細胞質中與其抑制物(Inhibitor of CAD，簡稱 ICAD) 維持結合的狀態且不具活性，直到 caspase-3 活化後，casepase-3 可切除 ICAD 並釋放出活化態的 CAD (Wolf *et al.*, 1999)。由於 CAD 帶有 nuclear localization sequence (NLS)，被活化後的 CAD 可進入細胞核執行 DNA fragmentation 的工作(Lechardeur *et al.*, 2000)。CAD 之最適作用 pH 為 7.5，產物為帶有 5 ' 端磷酸根的 [illegible]AD與ICAD 目前均已在 lymphoma cells 之細胞質中被找到(Sakahira *et al.*, 1998)。先前研究中所發現之 DNA fragmentation factor (DFF)是由 DFF45 與 DFF40 組成，其中 DFF40 就是CAD，而 DFF45 則是 ICAD (Halenbeck *et al.*, 1998; Liu *et al.*, 1998)。CAD 之基因已被成功選殖(Mukae *et al.*, 1998)，其中負責調節及催化活性之部位也被決定出來(Inohara *et al.*, 1999)。心室敗壞以及脊髓受傷後所產生之 apoptosis 被發現均與 CAD/ICAD 有關(Jiang *et al.*, 1999; Springer *et al.*, 1999)。而在人類淋巴細胞及單細胞之分泌胞器中之 DNase 在自體免疫病人中有上昇的現象(Pio *et al.*, 1998)。CAD 重要之胺基酸已被決定出來(Meiss *et al.*, 2001; Korn *et al.*, 2002)，對離子之需求亦已有進一步研究(Widlak and Garrard, 2001)；CAD 與 ICAD 的立體結構已相繼被決定(Ding *et al.*, 2003; Li *et al.*, 2003; Scholz *et al.*, 2003)；而兩者間交互調控表現的機制也被發表出來(Nagase *et al.*, 2003)。

其次是 DNase II，多項研究顯示趨向計劃性死亡的細胞(apoptotic cells)在被巨噬細胞吞噬後，則由 DNase II 執行 DNA 降解的工作(Krieser *et al.*, 2002; Kawane *et al.*, 2003; Nagata *et al.*, 2003)。而在 DNase γ 方面也有許多重要的成果，它被發現參與人類神經細胞(Saito *et al.*, 2003)與 T 淋巴球(Higami *et al.*, 2003)的 apoptosis，而當 DNase γ 的兩段功能性核定位訊息序列(nuclear localization signals, NLS)被決定出來後(Shiokawa *et al.*, 2003)，其與 apoptosis 的關聯性就更加明確了！另外尚有一種 L-DNase II，其前驅物為 leucocyte elastase inhibitor (LEI)，經轉譯後修飾作用才形成活化態的 L-DNase II (Torriglia *et al.*, 1998)；研究指出細胞內產生酸化現象時可能產生此轉換作用，並進一步導致 apoptosis (Altairac *et al.*, 2003)。

而在 DNase I 與細胞凋亡的相關研究方面，Tschopp 與 Mannherz 研究群早在 1992 年即已在小鼠觀察到 DNase I 可能與 apoptosis 有關(Peitzch *et al.*, 1992)，之後 10 年間也陸續發現 DNase I 可能參與多種大鼠組織及細胞(Polzar *et al.*, 1994; Rauch *et al.*, 1997)、人類 T 細胞淋巴癌(von Kobyletzki *et al.*, 2000)及皮膚角質細胞(Mass *et al.*, 2003)等之細胞凋亡過程。Puccetti 研究群亦研究指出 DNase I 可能與人類細胞株 JA3 (Jurkat T cell clone)、K562 (erythroleukemia)、M14 (melanoma)與 CEM (T cell lymphoma) (Oliveri *et al.*, 2001)等之細胞凋亡有關；最近甚至提出 DNase I 可能作為一轉錄因子(transcription factor)，用以調節人類細胞中 Fas 之表現(Oliveri *et al.*, 2004)。另外 Counis 與 Arruti 等人之研究結果顯示 DNase I 可能參與雞(Chaudun *et al.*, 1994)及牛(De Maria and Arruti, 2001; De Maria and Arruti, 2004)水晶體纖維細胞之細胞凋亡過程。

由以上得知，近年來 DNase 的研究正方興未艾，而本實驗室是少數能做到對 DNase 純化與蛋白結構探討的實驗室之一；我們嘗試藉由研究 DNase 之結構與功能上的聯結，以解答許多生理及病理上的問題，並希望能進一步應用在臨床治療及生物科技產業上。

牛胰臟去氧核醣核酸水解 之構造及作用機轉

一、基本結構之特性

牛胰臟去氧核[illegible]ovine pancreatic DNase I，以下簡稱 bpDNase)是最早被發現、也是目前被研究得最為透徹的(Moore, 1981)。BpDNase 為一醣蛋白(glycoprotein)，分子量為 31 kDa (Lindberg, 1967)。其一級構造已在 1973 年由

Liao 等人決定出來(Liao *et al.*, 1973)。1970 年，Salnikow 等人利用 phosphocellulose 管柱純化 Worthington DP grade bpDNase，可分離出 DNase A、B、C、D 四種 isoforms (Salnikow *et al.*, 1970)。DNase A 為四者中含量最多的，DNase B 與之不同處僅在於其醣鏈中含有 sialic acid，為一 sialoglycoprotein；而 DNase C 與 DNase A 不同之處在於其胺基酸序列 His^{121} 被 Pro 所取代，DNase D 則與 DNase C 之胺基酸序列相同，差異僅在其醣鏈如同 DNase B 一般具有 sialic acid。後來尚有一些次 isoforms 被發表出來，如：DNase E、F、X 等。其蛋白質三維晶體構造已由 Suck 與 Oefner 等人利用 X 光繞射法分析得到，解析度達 2.0 (Oefner and Suck, 1986; Suck and Oefner, 1986)；進而由 bpDNase 與基質 DNA-octamer 之結合複體解出其共結晶構造 (Lahm and Suck, 1991; Weston *et al.*, 1992)，進一步觀察酵素與 DNA 之結合情形。BpDNase 是 由 單 一 所構成之蛋白質分子，由 260 個胺基酸所組成；其醣基化的位置為 Asn^{18}。BpDNase 具有兩對雙硫鍵，即 Cys173-Cys209 與 Cys101-Cys104；其中 Cys173-Cys209 與維持整個酵素構形較有關，如被還原會使酵素失去活性，反之若 Cys101-Cys104 被還原，則不影響其活性(Price *et al.*, 1969)。

二、與鈣離子之關係

二價金屬離子對於 bpDNase 之構形與活性有重大影響，其活化酵素的能力依序為 $Mn^{2+}=Mg^{2+}+Ca^{2+}>Mg^{2+}>Ca^{2+}$ (Wiberg, 1958)；當只有 Ca^{2+} 存在下，酵素水解基質 DNA 之效率很差，但當僅有 Mn^{2+} 或 Mg^{2+} 存在下，添加微量 Ca^{2+} 即能對 bpDNase 活性產生顯著的加乘效果。前人研究指出 Ca^{2+} 主要功能不在於催化，而在於能改變酵素構形，降低 Km 值使酵素能更容易與基質 DNA 結合，而達到使活性上升的目的(Price, 1975)。當只有 Mg^{2+} 存在時，bpDNase 僅能對 DNA 作單股切割(single nick)，而在有 Mn^{2+} 或 $Mg^{2+}+Ca^{2+}$ 存在下則可以水解雙股 DNA (double cut) (Junowicz and Spencer, 1973; Campbell and Jackson, 1980)。除了影響酵素催化活性，Ca^{2+} 對 bpDNase 之穩定性也非常重要；當 Ca^{2+} 與 bpDNase 結合時，可使其維持緊緻(compact)的構形(Lizarraga, 1978)，進而保護 bpDNase 免於被蛋白 所分解(Price *et al.*, 1969; Poulos and Price, 1972; Hugli, 1973)；使 Cys173-Cys209 間之雙硫鍵不被 β-MSH 還原，且可使還原態 bpDNase 進行雙硫鍵正確配對，再回復成具活性之酵素蛋白(Hugli, 1973; Price, 1975)；另外亦可保護 bpDNase 免於被 nitration 反應作用而抑制其活性(Hugli and Stein, 1971)。由紫外線差異光譜(Tullis and Price, 1974)、螢光圖譜(Tullis *et al.*, 1981)、圓二色偏光光譜 (Hugli and Stein, 1971; Poulos and Price, 1972)等分析結果得知，Ca^{2+} 與 bpDNase 結合後，色胺酸(tryptophan)與酪胺酸(tyrosine)殘基會往分子內移動而造成構形上的改變。此性質亦可用於將 bpDNase 沖提出陰離子交換管柱，以達到純化之目的(Liao, 1974)。

在 pH 7.5 時，bpDNase 可與兩個 Ca^{2+} 有強烈的結合($K_d = 1.4 \times 10^{5}$ M)，同時另有三個較弱的 Ca^{2+} 結合區($K_d = 2.0 \times 10^{4}$ M)；而在 pH 5.5 時，bpDNase 則只與一個 Ca^{2+} 有強烈的結合($K_d = 2.2 \times 10^{-5}$ M) (Price, 1972)。由 X 光繞射分析法得到之 bpDNase 三維晶體構造中發現有兩個構造性的 Ca^{2+} 結合位置(Oefner and Suck, 1986; 圖 1)，Site I 位於 Asp201 與 Thr207 之間的 loop 中，由 Asp201、Thr203、Thr205、Thr207 及兩個水分子與 Ca^{2+} 作用；而 Site II 則是由 Asp99、Asp107、Glu112、Phe109 及兩個水分子組合而成。Site I 可能與保護鄰近的 Cys173-Cys209 間之雙硫鍵有關，Site II 則與固定 Cys101-Cys104 所在之 Gly100~Gly105 鬆散 loop 有關(Suck and Oefner, 1986)。本實驗室也在 1997 年研究指出 bpDNase 上有兩種與金屬離子結合的位置(Liu and Liao, 1997)，其一是與催化活性有關的催化位置(catalytic site)，此位置與 Suck 所提出的催化中心相對應，當 Mn^{2+}、Co^{2+} 或 Mg^{2+} 與之結合後，便可活化 bpDNase；另一種則是與調節活性有關的調節位置(modulation site)，當 Mn^{2+}、Co^{2+} 或 Ca^{2+} 與之結合後，可促進酵素活性，但此位置尚未能明確的對應 Suck 所提之 Site I 或 Site II。

本實驗室陳靜瑩博士於 1998 年成功選殖出 bpDNase 之 cDNA 並定出其序列(Chen *et al.*, 1998)，再

以此為模版針對結構性鈣離子結合區(Site I 與 Site II)進行定位突變，利用大腸桿菌 BL21(DE3)*p*LysE 菌株表現突變株酵素重組蛋白 brDNase(D99A) 與 brDNase(D201A)，前者為 Site II 被破壞、僅保留 Site I，後者則相反(Chen *et al.*, 2002)。在 Mg^{2+}與 Ca^{2+}同時存在或是 Mn^{2+}單獨存在下，bpDNase 與 brDNase(D99A)均保有切割 DNA 雙股之能力，而 brDNase(D201A)則喪失此能力。用胰蛋 mM Ca^{2+}存在下，bpDNase 與 brDNase(D99A)均可被鈣離子保護，只有 brDNase(D201A)會被胰蛋白水 β-MSH 處理時，在 10 mM Ca^{2+}存在下，bpDNase 與兩種突變株酵素均能被鈣離子保護而不被 β-MSH 還原而喪失活性，直到離子濃度降至 0.1 mM 時，三種酵素蛋白均會喪失活性。利用螢光光譜分析與紫外光差異光譜測定分析得知，兩種突變株酵素均與 bpDNase 相似，當與 Ca^{2+}結合時，都能造成蛋白質構形之改變。綜合以上結果推論：Site I 與 Site II 這兩個結構性鈣離子結合區與 Ca^{2+}結合後造成蛋白質構形改變及保護酵素雙硫鍵不被 β-MSH 還原的作用機制無關，但與保護 bpDNase 不被胰蛋白調節酵素活性有關。

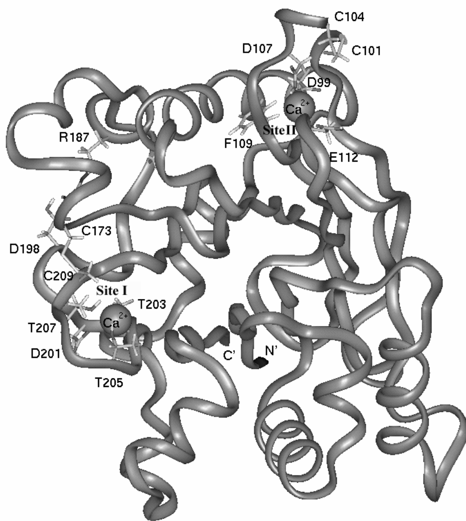

圖 1、BpDNase 之 X 光繞射晶體構造中呈現的兩個鈣離子結合區

Fig 1. The X-ray structure of bpDNase showing two Ca^{2+}-binding sites.

在 Site I 中，由 Asp201、Thr203、Thr205 及 Thr207 之氧原子與鈣離子作用，而在 Site II 中則由 Asp99、Asp107、Phe109 及 Glu112 之氧原子與鈣離子作用。兩對雙硫鍵 Cys173-Cys209 與 Cys101-Cys104 則分別位於 Site I 與 Site II 之附近。

In Site I, the calcium atom is coordinated by the oxygens of Asp201, Thr203, Thr205 and Thr207. In Site II, the calcium atom is coordinated by the oxygens of Asp99, Asp107, Phe109 and Glu112. The two conserved disulfides Cys173-Cys209 and Cys101 and Cys104 are localized in the vicinity of Site I and Site II, respectively.

三、催化機制

Price 在 1969 年提出，bpDNase 在 pH 7.2 有 Cu^{2+}的存在下，可被 idoacetate (IAA)烷基化而完全失去水解 DNA 的活性，此專一性的化學修飾在酵素 His134 上，此結果顯示，His134 極有可能位於活性區(Price *et al.*, 1969)。近年來科學家們利用化學修飾(Liao and McKenzie, 1979; Sartin *et al.*, 1980; Liao *et al.*, 1982; Liao *et al.*, 1991)與定位突變法(Doherty *et al.*, 1995; Jones *et al.*, 1996; Warren *et al.*, 1997; Evans *et al.*, 1999)也陸續決定出許多重要的胺基酸殘基。1992 年 Weston 等人依據 bpDNase 與基質 DNA-octamer 之結合複體所得共結晶(co-crystal)構造觀察酵素與 DNA 之結合情形，提出可能之催化機制，如圖 2 所示(Weston et al., 1992)。His252 扮演質子的接受者(proton acceptor)、即催化鹼(general base)，可以接受水分子的質子，之後水分子轉變成 OH，會攻擊 DNA 之磷酸雙酯鍵。另一方面，His134 則扮演質子的提供者(proton donor)、即催化酸(general acid)，將質子傳給 DNA 之 3’ 端，使磷酸雙酯鍵斷裂；而 Asp212、Glu78 可以分別穩定 His252 及 His134。Glu39 與 Asp168 則負責固定二價金屬離子，穩定整個水解過程。

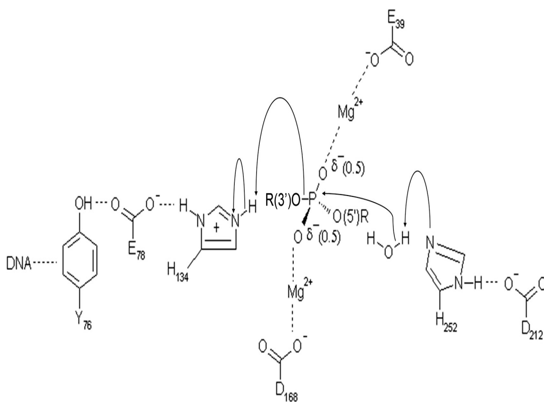

圖 2、BpDNase 之可能催化機制示意圖

Fig 2. A possible catalytic mechnism for bpDNase.

H134 與 H252 在此機制中分別扮演催化酸與催化鹼之角色。兩個分別與 E39 和 D168 作用之鎂離子則可中和被切割之磷酸根上未鍵結之氧原子，以穩定整個催化反應。H134 可經由 E78 與 Y76 這個重要的 DNA 結合殘基產生交互作用，因此 E78 對於 bpDNase 對基質 DNA 之結合與催化作用之間扮演重要的橋樑角色。R(5＇)O 與 R(3＇)O 則用以表示 DNA 分子剩餘的部分。

Neutralize the charges on the non-bridging oxygen atoms of the scissile phosphate and provide electrophilic catalysis. H134 is connected to Y76, a key DNA-binding residue, *via* E78 and so this glutamate forms a link between the binding of DNA and its hydrolysis. R(5＇)O and R(3＇)O represent the remainder of the DNA molecule.

四、雙硫鍵之生物功能

BpDNase 中有兩對雙硫鍵，即 Cys173-Cys209 與 Cys101-Cys104 (圖 3)。在鈣離子存在下、不含其他變性試劑時，僅有 Cys101-Cys104 會被 β-MSH 還原，此時酵素仍具活性；然而，在缺乏鈣離子時，兩對雙硫鍵均會被 β-MSH 還原，酵素則完全喪失活性。因此，Cys101-Cys104 被稱為「非必需」雙硫鍵，而 Cys173-Cys209 則被稱為「必需」雙硫鍵 (Price *et al.*, 1969)。另根據本實驗室歷年來研究發現，魚 DNase (Hsiao *et al.*, 1997) 僅具有一對「必需」雙硫鍵；雞 DNase (Hu *et al.*, 2003)則除了與 bpDNase 相同的兩對雙硫鍵之外，增加了第三對雙硫鍵(Cys192-Cys217)。而研究結果顯示來自魚、雞與牛三物種之 DNase 其酵素及物理性質並不完全相同，引起我們希望進一步探討其間差異的高度興趣。

我們以定位突變法分別製備了 bpDNase 中非必需(Cys101-Cys104)及必需雙硫鍵(Cys173-Cys209)之丙胺酸突變株酵素[brDNase(C101A)、brDNase(C173A)與 brDNase(C209A)]，以及另外一株具有新增第三對雙硫鍵之突變株酵素[brDNase(F192C/A217C)]，並分別探討其生化特性 (Chen *et al.*, 2004a)。研究結果顯示 bpDNase 之非必需雙硫鍵(C101-C104)雖與催化活性無關，但對酵素結構之穩定仍非常重要：若缺乏此對雙硫鍵，將造成突變株酵素[brDNase(C101A)]即使在鈣離子存在下仍會被胰性降低。另一方面，我們根據以結構為基礎之序列比對(sequence-based alignment)發現 bpDNase 之「非必需」雙硫鍵(-CESC-)與硫氧化還原素(thioredoxin)之活性區片段(-CGPC-)在序列、結構及立體空間上均非常相似，均為 CXXC motif (Holmgren, 1979a)；我們進一步利用「胰島素還原沉澱法」(Holmgren, 1979b)、以反應液混濁度升高速率(A_{650nm}/min)作為酵素活性定量依據分析還原態的 bpDNase，發現其非必需雙硫鍵在還原狀態可測得約 39% 之類似 thioredoxin 活性，另再額外添加鈣離子將可進一步提高活性至 50%。而在必需雙硫鍵(Cys173-Cys209)方面，破壞此雙硫鍵雖會造成其丙胺酸突變株[brDNase(C173A)與 brDNase (C209A)]酵素重組蛋白在大腸桿菌表現時有初步摺疊的問題而表現在內涵體(inclusion body)中，但在鈣離子協助下進行再摺疊(refolding)可回復約 10-18% 之 bpDNase 活性，因此其對 bpDNase 之催化活性似乎並非絕對必要。另外為模擬雞胰臟甲型去氧核醣核酸水解 (cpDNase)第三對雙硫鍵(Cys192-Cys217)而製備之突變株酵素[brDNase(F192C/A217C)]，則發現新增之雙硫鍵將為酵素提供進一步的穩定性。突變株酵素在缺乏鈣離子的條件下仍及β-MSH 之抑制作用，且其熱穩定性較野生型酵素為佳；實驗結果顯示此新增之雙硫鍵似可取代鈣離子對 bpDNase 提供之結構穩定作用。

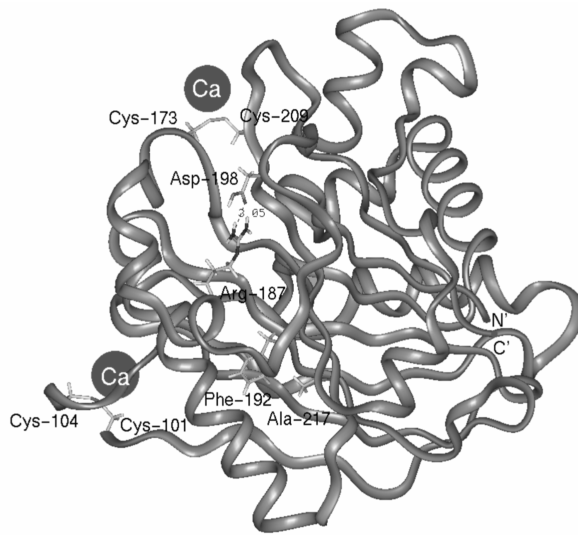

圖 3、BpDNase 之 X 光繞射晶體構造中呈現其雙硫鍵所在位置

Fig 3. The X-ray structure of bpDNase showing the locations of disulfides.

此結構圖指出 bpDNase 本身兩對雙硫鍵 Cys173-Cys209 及 Cys101-Cys104 與其鄰近之兩個鈣離子結合區在立體空間上之相對關係；同時也標示出與 cpDNase 中第三對雙硫鍵(Cys192-Cys217)位於相對應位置之 Phe192 與 Ala217。另外，Arg187 與 Asp198 兩者側鏈間之距離為 3.05 。

The structure illustrates the two intrinsic disulfides (Cys173-Cys209 and Cys101-Cys104) and the two Ca^{2+}-binding sites. The newly engineered third disulfide, Cys192-Cys217, not shown in the structure, corresponds to Phe192 and Ala217, whose side chains are as shown. The distance between the guanidinium group of Arg187 and the carboxyl group of Asp198 is 3.05 .

五、N-與 C-端片段參與活性蛋白質摺疊之重要性

BpDNase 之 X-光繞射晶體結構顯示其 N-與 C-端區域極為靠近且共同形成一反平行 β-摺板 (anti-parallel β-sheet)構造(圖 4)，而這些 N-與 C-端序列在來自不同物種之 DNase 之間具有高度保守性。為了研究此反平行 β-摺板構造對 bpDNase 摺疊形成活性蛋白構造之重要性，我們設計了一系列移除與取代突變株酵素進行分析(Chen *et al.*, 2004b)。

Leu1 與 Leu259 之一系列取代突變株酵素均具活性且比活性與 bpDNase 相當，顯示此 N-與 C-端片段並無支鏈(side-chain)交互作用，而以其主鏈(main-chain)氫鍵對此反平行 β-摺板構造之形成最為重要。僅缺少 Thr260 之突變酵素 brDNase(△260)仍保有活性，但其餘移除 C-或 N-端序列二至十個胺基酸之突變株酵素則均會表現在內涵體中且不具活性。而將這些突變蛋白先以 6 M guanidine-HCl 溶解再對含有 10 mM 鈣離子之緩衝液透析以移除變性試劑後，仍無法使其回復活性。我們合成了相對應於 N-與 C-端移除序列之胜 片段嘗試進行補償(complementation)實驗，在測試過不同胜 長度、濃度及反應時間等條件後發現以 400 倍 過 量 C10 (DHYPVEVTLT)與突變株酵素 brDNase(△251-260)在

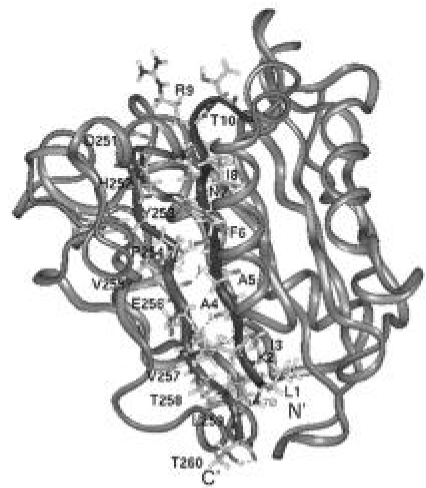

圖 4、BpDNase 之 X 光繞射晶體構造中呈現其 N-與 C-端片段之交互作用

Fig 4. The X-ray structure of bpDNase showing the interactions between the N- and C-terminal fragments.

此結構圖指出 bpDNase 之 N-與 C-端片段非常靠近並形成一反平行 β-摺版構造，N-端(L1-T10)及 C-端(D251-T260)片段各十個胺基酸之側鏈亦被清楚地標示出來。

The structure indicates that the N- and C- terminal fragments of bpDNase are in proximity forming an anti-parallel β-sheet structure. Ten residues each of the N- (L1-T10) and C-terminal (D251-T260) fragments are clearly shown with the amino acid side chains.

室溫反應八小時可使其回復 83%之 活 性 ；C10 與突變株酵素之間的非共價性結合作用其解離常數最低可達 48 μM。以圓二色偏光光譜(circular dichroism)分析發現移除突變株酵素之結構有部分未摺疊現象，且主要以α-螺旋(α-helix)為主；而在添加胜 反應後確實可協助(native) bpDNase 之β-摺板為主的結構，進而造成活性之回復。熱變性(thermal denaturation)實驗也指出 brDNase(△251-260)之 Tm 值為 55℃， 在 與 C10 反應後則可回升至 63℃，接近 bpDNase 之 65℃。我們同時發現鈣離子在此兩階段酵素摺疊(folding)與活化(activation)的過程中也扮演了重要的角色。

未來展望

本實驗室長期以來致力於核酸水解 (包含 DNase I、DNase II 與 Nuclease)之結構與功能研究，早期以傳統蛋白化學方法為主，近年來結合分子生物學技術、哺乳動物細胞培養、生物資訊學、基因體學及蛋白質體學方法，開啟新的研究領域與視野。近五年來已分別針對牛胰臟甲型去氧核醣核酸水解 、豬脾臟乙型去氧核醣核酸水解 、蝦肝胰臟核酸水解 、雞胰臟甲型去氧核醣核酸水解 、魷魚肝胰臟核酸水解 、蜆肝胰臟核酸水解 及 菌類核酸水解 等不同物種來源之核酸水解 進行一系列的研究。除了希望了解核酸水解 在演化上的意義，在 DNase I 方面，將藉由結構生物學研究解析不同物種 DNase I 之生化特性與結構之相關性；另在 DNase II 方面，則將進一步釐清其於細胞中表現後之明確定位(localization)及運輸(transport)機制、及其蛋白質之轉譯後修飾作用(post-translational modifications)與處理過程(processing)；也希望能製備並純化大量之重組蛋白，以完成 DNase II 之結晶與結構分析。

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