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挑戰聚醣胺水解酵素之自然設計(3/3)

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計畫主持人：李耀坤

計畫參與人員：鄭至玉

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中文摘要

關鍵詞: 聚醣胺水解酵素、寡醣、化學修飾、定點突變、液態層析質譜

此研究的主旨在於突破自然界賦于聚醣胺水解酵素的功能上的限制。研究所用之酵素基因來自於Bacillus circulans。由於自然界所有已發現之聚醣胺水解酵素均屬於反轉型酵素(inverting enzyme)；亦即此類酵素分解β-1,4-linkage 之聚醣胺而得還原端(reducing end)為α-form 之寡醣產物。由於此酵素類型之催化機制的特性，此酵素於轉醣反應上的應用因而受到限制。本計畫之主要工作之一是利用定點突變技術將特定胺基酸改變成半胱胺酸(cysteine)後再以化學修飾方法引進不同碳鏈之羧酸基將反轉型酵素轉變成保留型酵素(retainingenzyme)以利寡胺衍生物之製備，及其催化機制的研究。除利用基因工程技術和化學修飾法以開發單成份寡醣及其衍生物之製備外，我們亦正發展以phagedisplay 技術製備單株抗體並計畫用以分離各不同DP值之寡醣胺。

本計畫中主要工作之二還以製造變異性酵素(chimeric enzyme)之技術將內切型聚醣胺水解酵素(endo-chitosanase)轉變成外切型酵素(exo-chitosanase) 。藉由電腦模擬的酵素結構圖，找到了居於酵素表面位置的兩點氨基酸 (116&223)，並成功地以基因工程插入了兩段的蛋白片段。雖然其產物仍以幾丁二醣為主，但以幾丁六醣測試此新型酵素時，確有轉變成外切型酵素的跡象。這些新異酵素之作用機制正進一步研究中。

Abstract

Key words: Chitosanase, Chitooligosaccharides, Chemical modification,
Site-directed mutagenesis, LC/MS

For all known cases, chitosanase is an inverting enzyme, which catalyzes the hydrolysis of chitosan by an inverting manner to form α-anomeric products.The intrinsic property of the natural occurring chitosanase largely decreases itsapplication in glycosyl-transfer reaction. In this study, we have tried to convertthe *Bacillus circulan* chitosanase into a retaining enzyme by combiningsite-directed mutagenesis and chemical modifications. Although the active sitetopologies of these two classes of enzymes,inverting and retaining enzymes, aredifferent in many ways, the distance between their two essential groups arebelieved to be the major factor. The chimeric chitosanases were prepared bychemical modification of the D55C and E37C mutants with 2, 2-dithiodiacetic acid,3, 3-dithiodipropionic acid, and 4, 4-dithiobutanoic acid. LC/MS analyses revealed that all mutants were stoichiometrically labeled with all three thiol agents.The catalytic activity of D55C and E37C were largely decreased as compared with that of wild type enzyme. No significant activity can be detected on D55C/E37C double mutant. Thiolated enzyme showed a minor factor on increasing catalytic activities.

The catalytic products for all enzymes were chitobiose, chitotriose and chitotetraose. Besides of the protein engineering approach, a powerful affinity column for isolation of specificchitooligosaccharide is under developing by phage display technique. After screening through the phages library containing 10^9-10^{10} different clones of antibody. The functional phage will be massively produced and immobilized for affinity column application.

Two amino acids (116 and 223) were selected with the aid of computer simulation. Chitosanase-bridge was produced by inserting two loops onto these amino acids. From experimental results suggested that the insertion of these two loops onto amino acid transforms Chitosanase-bridge from an endo-type chitosanase to an exo-chitosanase.

一、簡介

幾丁質是由單體 N-乙醯葡萄糖胺 (N-acetylglucosamine) 以 β-(1,4) 鍵結而成之聚醣 (polysaccharide)，其功能和植物纖維一樣，具有保護及支持生物體的作用 (1 , 2) 。而幾丁質去乙醯化後就稱為幾丁聚醣，幾丁聚醣為大分子聚合物，水溶性差，導致在應用上的困擾，。N-乙醯幾丁寡醣 (N-acetyl-chitooligosaccharides) 一般是指由 2~10 個 N-乙醯葡萄糖胺以 β-1,4 醣甘鍵結所形成的低分子醣類；而幾丁寡醣 (chitooligosaccharides)則是由 2-10 個葡萄糖胺以 β-1,4 醣甘鍵結所形成的。此兩種寡醣皆可由幾丁質或幾丁聚醣製備而得。開發分子量小且水溶性佳之幾丁寡醣是目前的趨勢（3）幾丁聚醣酵素 (chitosanase EC 3.2.1.132) 為一具有催化長鏈幾丁聚醣成為短鏈幾丁聚醣或幾丁寡醣的水解酵素。根據作用型式可將幾丁聚醣酵素分類成外幾丁聚醣酵素 (exochitosanase) 及內幾丁聚醣酵素 (endochitosanase) (4,5)。幾丁聚醣酵素水解幾丁聚醣 β-醣甘鍵，一般有兩種水解途徑，一為位向保留的機制 (retention)，另一為位向反轉的機制 (inversion)，。

目前 *Baciilus circulnas* MH-K1 產生的幾丁聚醣酵素相關文獻並不多，但其立體結構已由 X-ray 晶格繞射解出，此株酵素與另一個 *Streptomyces* sp. N174 chitosanase 之結構極為相似（6）。MH-K1 chitosanase 具有 259 個胺基酸，N174 chitosanase 則有 238 個。幾丁聚醣酵素根據反應的部份乙醯化聚醣斷裂位置的不同分成三類：第一類如 N174 chitosanase，水解的位置為 GlcN-GlcN 及 GlcNAc-GlcN 鍵結；第二類如 *Bacillus sp* No. 7M chitosanase，水解的位置為 GlcN-GlcN 鍵結；第三類如 MH-K1 chitosanase，水解的位置為 GlcN-GlcN 及 GlcN-GlcNAc 鍵結。是因活化區域缺口(cleft)深度及扮演催化角色之胺基酸位置不同，而導致不同酵素辨識不同乙醯化位置的聚醣

二、方法與材料

幾丁聚醣酵素基因選殖

首先利用一般抽取質體的方式，接著根據日本教授提供的重組過質體 pNCMO2。因為文獻閱讀中，除了嘗試建構在 Bacillus megaterium expression 及 E.coli

表現系統中。在 Bacillus 中所使用的載體 pWH1520 本身的限制酶很少的關係，故可以利用 SpeI 及 KpnI 來做選殖，透過聚合酶連鎖反應（PCR）放大，SpeI : 5'—GCT CCC ATG GCT TTC ACT AGT CCT TCT CCT 及 XR-V : 5'- GAC AAT GTA ATT GTT CCC TA 來放大 chitosanase gene，經過剪接之後所得得質體為 pWH1520/cns; 在 E.coli 表現系統中，設計了兩條引子如下：cns-Nterm : 5'-GCT CCC ATG GCT TTC CAT ATG GCT TCT CCT 及 cns-Cterm 5'-GAC AAT GTA ATT GTT CCC TAC ，利用聚合酶連鎖反應（PCR）放大 chitosanase gene，且選用載體 pET22b(+)，利用 NdeI 及 NcoI 的切位，所建構得質體稱分別為 pNde/cns 及 pNco/cns。

***Bacillus megaterium* 中的表現與純化**

挑選轉形後的 B.megaterium 的單菌落，置於含有 tetracycline 的 LB 培養液中，置 37 ℃ 下，以轉速為 115 rpm 培養 12-16 小時。取 100 μL 於 500 mL LB，於 DO600 = 0.3 時，加入 0.5 % xylose 直到 DO600 = 1.5 時，收集之。將菌液於 4 ℃，以 15000 rpm 離心 20 分鐘。去除上清液，利用 pH 7 磷酸緩衝液回溶，再以超音波震盪破菌體後，離心，將細胞殘骸與上層液分開，此上層液即是胞內粗提液。

HiTrap Q 陰離子交換樹脂管柱層析

將 1.5 mL 胞內粗提取液導入預先以磷酸緩衝溶液 (20 mM ，pH 7.0)平衡之 HiTrap Desalting column 中，再將 2 mL 磷酸緩衝溶液導入 HiTrap Desalting column 中，收集除鹽後的胞內粗提取液，重複數次 直到所有的胞內粗提取液除鹽完畢。把所得去鹽胞內粗提取液，導入預先以磷酸緩衝溶液 (20 mM，pH 7.0) 平衡之 HiTrap Q 陽離子交換樹脂管柱，進行層析分離。鹽類梯度為 5 到 200 mM，流速為 2.0 mL /min，沖提體積為 60 mL。收集完畢後由試管取樣，加入 1 % chitosan，在 37 ℃觀測 400 nm 的吸光值增加率以測定其活性。

在大腸桿菌中的表現與純化

(a)胞內粗提取液的取得與硫酸銨沈澱

取單一菌落在 5 mL LB-Amp 培養液養 12~16 小時後，接種到 1000 mL

LB-Amp 培養液中，以 37 ℃水浴的培養箱轉速 115 rpm 培養 18～24 小時。收菌液在 4 ℃以 15000 rpm 離心 20 分鐘，棄去上層液，取離心下的菌體，以磷酸緩衝液 10 mL (20 mM，pH 7.0) 溶解菌體，搖晃使成均勻懸浮。以超音波震盪使菌體破裂 (45W， 60 % pulse)，在 4 ℃以 15000 rpm 離心 10 分鐘，將上層液與細胞殘骸分開，此上層液即胞內粗提取液。於冰浴下，在粗提取液中加入硫酸銨至 80 % 飽和度，靜置 20 分鐘後，在 4 ℃以 15000 rpm 離心 10 分鐘，取沈澱於 4 ℃保存。

(b)HiTrap SP 陽離子交換樹脂管柱層析

取硫酸銨飽和度為 0 到 80 % 區間的酵素，利用 HiTrap Desalting column 除鹽，收集除鹽後的胞內粗提取液，再導入預先以磷酸緩衝溶液 (20 mM, pH 7.0) 平衡之

HiTrap SP 陽離子交換樹脂管柱，進行層析分離。鹽類梯度為 5 到 200 mM，流速 2.0 mL /min，沖提體積為 60 mL。收集完畢後由試管取樣，加入 1 % chitosan，，在 37 ℃觀測 400 nm 的吸光值增加率以測定其活性。

蛋白質濃度測定及分子量的鑑定

本實驗採用的 Bicinic acid (BCA assay) 吸收值方法估計所得蛋白質的量。同時利用 SDS-PAGE 及 LC-Mass 來觀測正確的分子量。

突變株之突變選殖

在 1999 年，Saito 等人所發表的論文中 (Saito, J-i., 1999)得知其活化位置，希望能夠將其活化中心做修飾，以作為之後相關研究之探討，故挑了 E37C 及 D55C 其引子(primer)設計如下:

E37C-5’：5’—CAT CAA TAA ACC GTG CCA GGA TGA TTT G ；
E37C-3: 5’— CAA ATC ATC CTG GCA CGG TTT ATT GAT G
D55C-5’：5’—GAA GAC ATT GAA TGC GAG CGC GGG TAT AC
D55C-3’：5’—GTA TAC CCG CGC TCG CAT TCA ATG TCT TC

突變酵素與各標示物物質反應

各標示物質的配法如下: 分別配置 10 mM Iodoacetic acid、3,3-dithiodipropionic acid 、4,4-dithiobutanoic acid、2,2-dithiodiglycolic acid。取突變的酵素 400 μL 與 100 μL 標示物 (Final conc: 2 mM) 混合均勻，並置於 37 ℃下進行反應。反應結束後，將酵素溶液除鹽兩次，並以質譜儀(ESI/MS/MS)追蹤反應情形。

突變酵素與各標示物物質反應後產物分析

將各標示後的酵素除鹽跟濃縮。加入 1 % chitosan 50 μL 及 酵素 50 μL，於 37 ℃下反應數小時，將反應後的溶液離心，將有未反應完全的反應物殘留，取上層液稀釋 1000 倍。利用質譜儀(ESI/MS/MS)分析其產物的分子量。

轉 endo-chitosanase 成為 exo-chitosanase 的突變設計

我們所設計的引子如下:

CNS-116-F (5’-CA GGC GGC TCT AGA GAT CCG TGC CTG GGC GGT ACC CAT CCC GAT GGC C-3’), CNS-116-B (5’- G GCC ATC GGG ATG GGT ACC GCC CAG GCA CGG ATC TCT AGA GCC GCC TG-3’), CNS-223-F (5’-CTG AAT CAA GGC GCT ACT AGT CGT ACC TGC AAA CCG GGC TCA GAT ACG CTT C-3’) and CNS-223-B (5’-G AAG CGT ATC TGA GCC CGG TTT GCA GGT ACG ACT AGT AGC GCC TTG ATT CAG-3’).

三、結果與討論

幾丁聚醣酵素基因的建構與表現

由日本千葉大學安藤昭一 (Akikazu Ando) 教授所提供的幾丁聚醣酵素基因已

建構在 pNCMO2 載體上，命名為 chito/pNCMO2 。利用 *Bacillus* 菌屬表現重組蛋白質為本實驗室新的嘗試，所使用的 *B. megaterium* 表現系統為德國 MoBiTec 公司所發展的，已經有許多蛋白質成功的利用此系統表現。我們歷經約一年半的學習與嘗試，擁有初步的結果，已可將 MH-K1 幾丁聚醣酵素於 *Bacillus megaterium* 中表現出擁有活性的重組蛋白質。為了能夠獲得高表現量的酵素，故採用 T7 RNA polymerase system，宿主菌種為 *E.coli* BL21 (DE3)，載體為 pET22b(+)。因此，建構了兩個質體，分別是保留 pET22b(+) 上 pelB leader 及去除 pelB leader。

在除去 pelB leader 的部份，命名為 (n) chito/pET22b(+)。未除去 pelB leader 的部份，則直接將 chito/pNCMO2 與 pET22b(+)分別利用 NcoI 及 XhoI DNA 限制酶處理後再回收。隨機挑選轉形菌株抽取其質體，利用限制酶切下的片段做確認並進行 DNA 定序，獲得之正確質體，命名為 chito/pET22b(+)，DNA 序列於圖一所示。

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                         Rn- GCT TCT CCT GAC GAC AAT TTC TCC CCA GAA ACC CTG
                              A   S   P   D   D   N   F   S   P   E   T   L

CAA TTT CTT CGC AAT AAT ACG GGG CTC GAT GGC GAG CAG TGG AAC AAC ATC ATG
Q   F   L   R   N   N   T   G   L   D   G   E   Q   W   N   N   I   M

AAG CTC ATC AAT AAA CCG GAG CAG GAT GAT TTG AAC TGG ATC AAA TAC TAC GGG
K   L   I   N   K   P   E   Q   D   D   L   N   W   I   K   Y   Y   G

TAT TGT GAA GAC ATT GAA GAT GAG CGC GGG TAT ACG ATC GGT CTT TTC GGT GCT
Y   C   E   D   I   E   D   E   R   G   Y   T   I   G   L   F   G   A

ACT ACA GGC GGC TCC AGA GAT ACC CAT CCC GAT GGC CCG GAC CTC TTC AAA GCC
T   T   G   G   S   R   D   T   H   P   D   G   P   D   L   F   K   A

TAT GAC GCC GCC AAA GGA GCC AGC AAC CCG TCG GCT GAT GGC GCA TTG AAG CGC
Y   D   A   A   K   G   A   S   N   P   S   A   D   G   A   L   K   R

CTT GGC ATT AAC GGA AAA ATG AAA GGC TCG ATT CTG GAA ATT AAG GAT AGC GAA
L   G   I   N   G   K   M   K   G   S   I   L   E   I   K   D   S   E

AAG GTG TTC TGC GGC AAG ATT AAA AAG CTT CAA AAC GAT GCG GCT TGG AGA AAA
K   V   F   C   G   K   I   K   K   L   Q   N   D   A   A   W   R   K

GCG ATG TGG GAA ACA TTC TAT AAC GTG TAT ATC CGG TAC AGC GTC GAA CAA GCG
A   M   W   E   T   F   Y   N   V   Y   I   R   Y   S   V   E   Q   A

CGC CAG CGC GTT TTT ACC AGC GCG GTG ACG ATC GGA TCG TTT GTC GAT ACG GCG
R   Q   R   G   F   T   S   A   V   T   I   G   S   F   V   D   T   A

CTG AAT CAA GGC GCT ACC GGC GGC TCA GAT ACG CTT CAA GGC TTG CTA GCC CGT
L   N   Q   G   A   T   G   G   S   D   T   L   Q   G   L   L   A   R

TCT GGC AGC AGC TCG AAC GAG AAA ACC TTT ATG AAG AAT TTC CAT GCC AAA CGT
S   G   S   S   S   N   E   K   T   F   M   K   N   F   H   A   K   R

ACC TTG GTT GTG GAT ACC AAC AAA TAC AAC AAG CCA CCT AAC GGT AAA AAC CGT
T   L   V   V   D   T   N   K   Y   N   K   P   P   N   G   K   N   R

GTA AAA CAA TGG GAC ACT CTC GTG GAC ATG GGG AAA ATG AAT CTG AAG AAC GTC
V   K   Q   W   D   T   L   V   D   M   G   K   M   N   L   K   N   V

GAT TCC GAG ATT GCT CAA GTC ACG GAC TGG GAA ATG AAG TAA
D   S   E   I   A   Q   V   T   D   W   E   M   K   *
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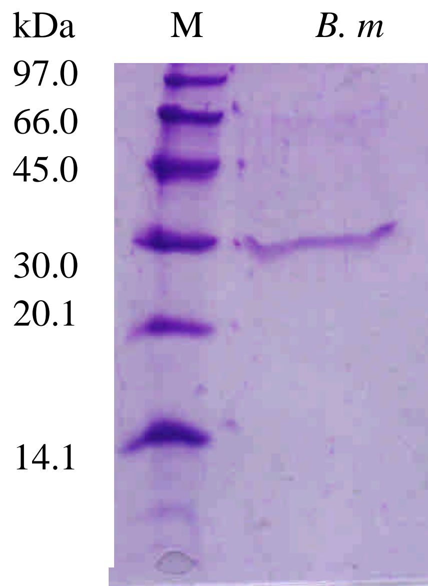
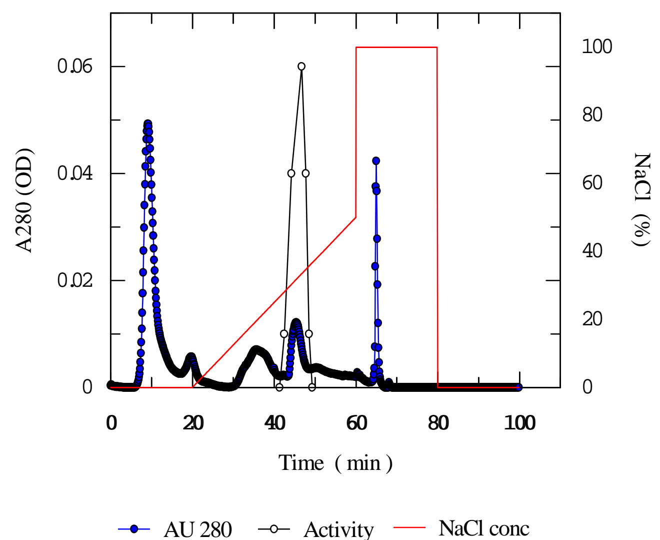
圖一 chito/pET22b(+)與(n)chito/pET22b(+)基因序列

Bacillus expression system 中誘導表現及純化情形

B. megaterium 表現系統利用木糖操縱子 (xylose operon) 為調控因子 (regulatory element)，故必須加入 xylose 誘導，因為此系統沒有在載體上設計訊息胜肽的基因序列，所以重組蛋白質會產生在胞內。在進行了誘導表現之後，經活性測試發現有蛋白質的表現並具有活性，以 HiTrapQ 管柱純化如（圖二）所示。(圖三）為其 SDS-PAGE 的結果，我們雖已成功的利用 *B. megaterium* 系統表 *Bacillus circulans* MH-K1 的 chitosanase，並經純化步驟可得到極佳純度的 chitosanase，可惜其表現量不高，仍有待進一步研究其誘導條件，提高酵素的表現量。



圖三 由 *B. megaterium* 表現幾丁聚醣酵素之純化結果



圖二 Wild type HiTrap-Q 陰離子交換樹脂層析純化圖

E.coli expression system 中誘導及表現情形

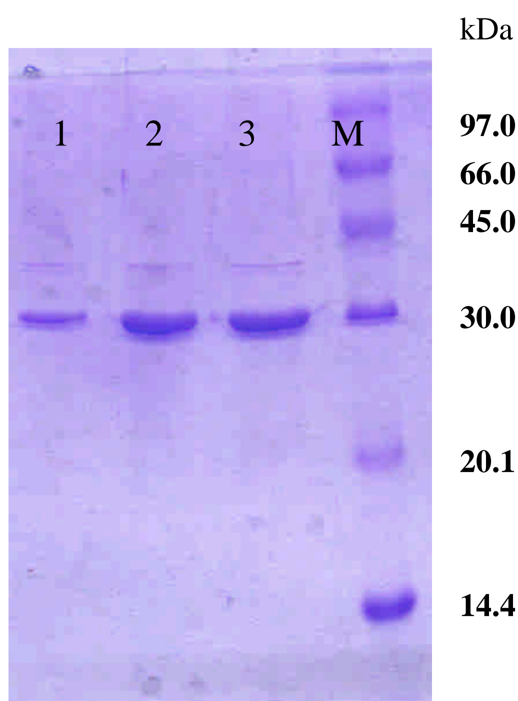
將 chito/pET22b(+) 轉形至大腸桿菌 BL21 (DE3)，並觀察其表現結果，發現沒加入 IPTG 誘導的情況下即有大量蛋白質的表現，此應與培養液中含有半乳糖苷化合物有關，並且與幾丁聚醣反應亦有活性。故培養液的成份很重要，當然在無需 IPTG 誘導下，則可省去找尋最佳的誘導時間。可知所表現出的酵素幾乎分布在胞內，培養時間在 16～20 小時的表現量與活性最佳。另外，也發現培養五天後仍有活性存在。而(n) chito/pET22b(+) 誘導及表現情形也與 chito/pET22b(+) 相同。後續將利用 chito/pET22b(+) 進行基因工程與蛋白質改造的研究。

接著利用 HiTrap SP 離子交換樹脂管柱在 pH 7.0 分離酵素，由 HiTrap SP 的層析圖顯示 (圖四)，在 200～400 mM 的 NaCl 濃度，可將酵素沖洗下來，收集幾丁聚醣酵素活性的部份，經 SDS-PAGE 分析其純度(圖五)，其均質度可達 90 ％以上，分子量約為 29 KDa，結合蛋白質濃度之測定與還原醣之定量，此重組蛋白質純化倍

率及回收率整理如表一。本研究中的所有突變酵素亦利用此純化流程進行純化。

表一：不同步驟下的純化倍率表（unit 定義：1 μmole/min）

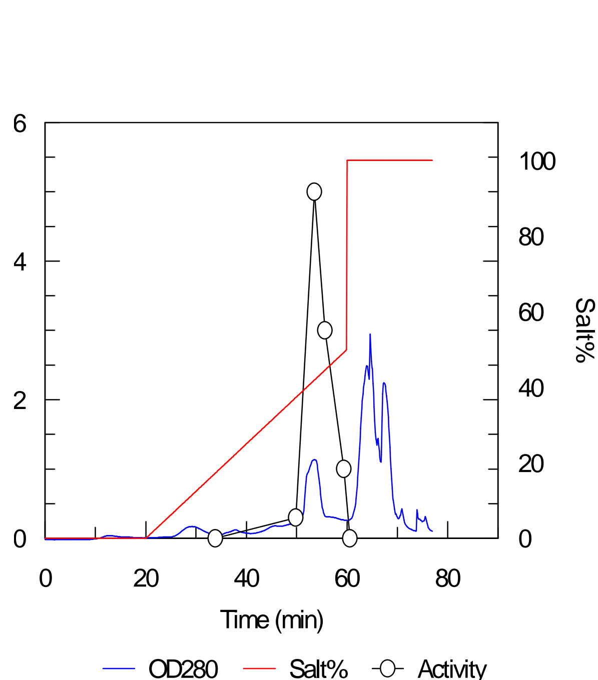
Total protein, (mg)	Step	Tot. activity (unit)	Spec. activity (unit/mg)	Purification fold	Yield (%)
411	Extract	4074	9.90	1	100
287	$(NH_4)_2SO_4$ 0-80 %	3863	13.5	1.4	94.8
3.2	HiTrapSP pH 7.0	959	300.1	30.0	23.5



圖五 Hi-Trap SP 層析結果之 SDS-PAGE 圖

Lane 1：E37C, Lane 2：D55C

Lane 3：wild type, M：Protein marker



圖四 Wild type chitosanase Hitrap-SP 陽離子交換樹酯層析純化圖

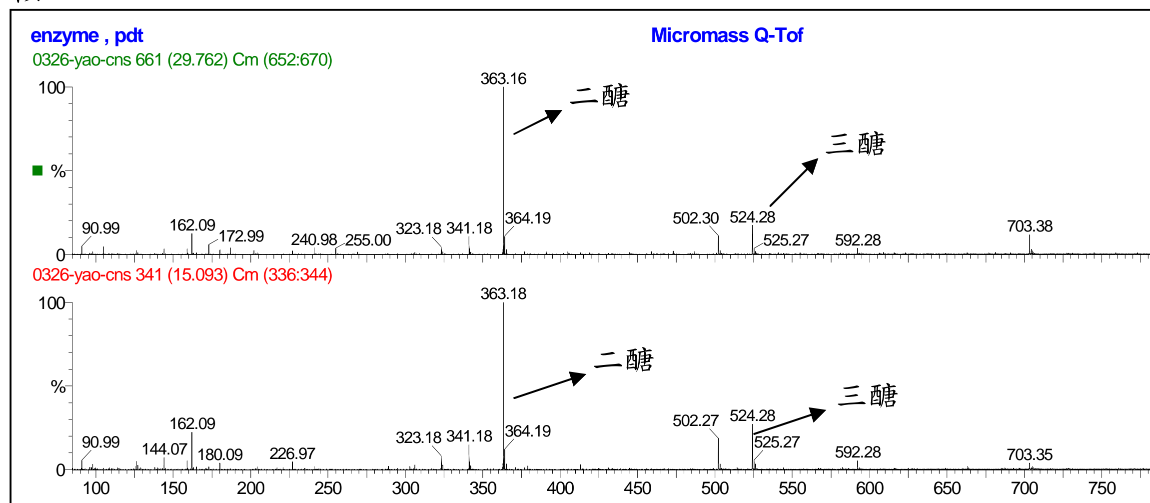
從 1988 年，日本學者由 *Bacillus circulans* MH-K1 發現了 chitosanase 後，即加以研究探討。其重組酵素之表現系統為 *B. brevis* expression system，仍尚未於大腸桿菌系統表現成功。意外的以大腸桿菌系統成功的表現出大量的具活性之重組酵素。因此本實驗仍將利用大腸桿菌表現系統進行 chitosanase 後續相關的研究。

重組幾丁聚醣酵素水解產物之質譜分析

利用重組蛋白質於兩個不同表現系統，所產生的幾丁聚醣酵素，在 37 ℃，pH 6.0

情況下進行幾丁聚醣水解反應，反應初期可得各式長鏈（Dp 2 以上）之寡醣。但隨反應時間增長，其最終產物為幾丁二醣、幾丁三醣。我們再一次證實此酵素為內切型幾丁聚

醣酵素。且對四醣以上之寡醣鏈有較佳之反應性。(圖六)所示、由兩不同表現系統所得之酵素的催化特性與產物沒有明顯的不同，可見不同系統中蛋白質之折疊應相似。



圖六、不同表現系統所得之酵素對幾丁聚醣之催化產物（上圖為大腸桿菌者，下圖枯草桿菌者）幾丁二醣分子量：341、363　幾丁三醣分子量：502、524

表二 ：不同突變點的反應條件

	Annealing Temp	Extension time	Ploymerase	DMSO
E37C	55 ℃	13 min	Pfu	Yes
D55C	60 ℃	13 min	Pfu	No
E37CD55C	61 ℃	13 min	Pfu	Yes

酵素活性催化位置之定點突變

為有利於化學修飾，此酵素之可能胺基酸（essentail amino acid）Glu37 和 Asp55 將先以 Quick change 定點突變法改變成 Cys，即 E37C、D55C 或 E37C/D55C 雙點突變酵素。Quick change 所使用之 PCR 相關條件如表二。

野生株與突變株酵素分子量之測定

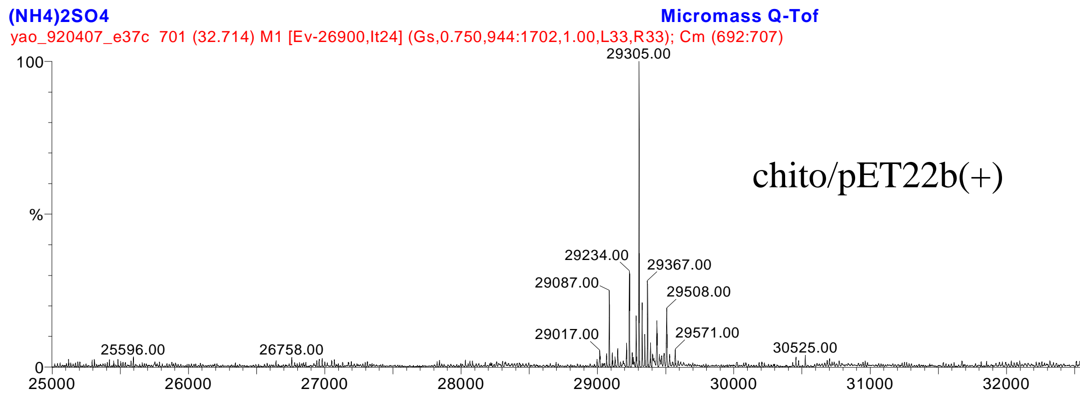
為了確定後續化學修飾的研究，利用軟體推算出野生株(wild type)及各突變株的分子量，再利用質譜儀精確測定純化過後之高純度野生株及各突變株酵素的分子量，以利化學修飾反應後，比較反應前後酵素的分子量變化。由蛋白質質譜儀測定，我們意外的發現含 pelB leader 之 pET22b(+)系統，會出現長度不一定的成熟蛋白質(mature protein)，如（圖七(上)）之質譜所示，m/z 為 29305 之質譜峰較預定蛋白質少一 Met 胺基酸，m/z 為 29234 為少了 Met-Ala 者，m/z 為 29087 則為少了 Met-Ala-Phe

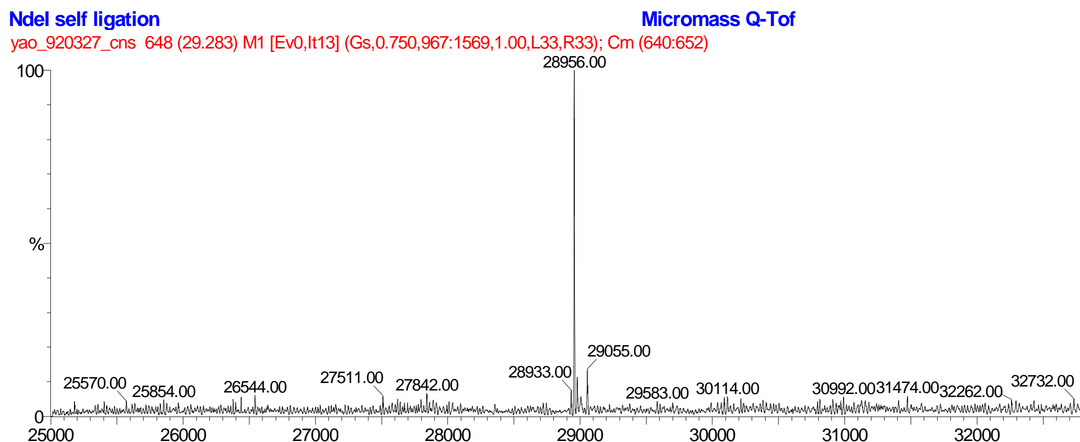
者，可見大腸桿菌之 signal peptidase 對 pelB leader 的專一性不佳。

有趣的是此現象亦發生在無 pelB leader 之 pET22b(+)系統，如（圖七（下））所測得之 m/z 為 28956 質譜峰，比預定之重組蛋白質少了一個 Met 胺基酸，這些質譜結果整理於表三。 此測定所得知質譜與理論值約有 5 amu 之誤差。

表三：大腸桿菌表現統中蛋白質分子量分析表

酵素	Wild type		Mutation in *E.Coli*		
	NO pelB leader	With pelB leader	E37C	D55C	E37CD55C
分子量	-M:28950	-M 29305	29289	29295	29272
		-MA 29234	29222	29227	29201
		-MAF29087	29076	29077	29054



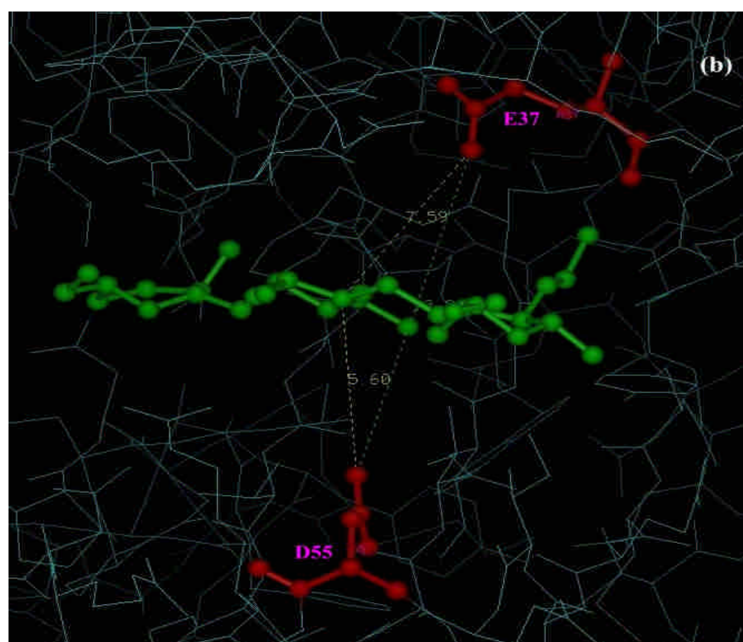


圖七 質譜儀偵測 wild type (n)chito/pET22b(+)及 chito/pET22b(+)分子量的結果

以突變株酵素（E37C & D55C & E37C/D55C）反應，透過特定專一性化合物的標定，進行後續的相關反應機制的研究。首先利用 Iodoacetic acid 標定，結果顯示，Iodoacetic acid 與酵素之標識反應專一性不佳，主要原因可能是酵素之 Lys 和 Cys 均可能與之作用，由於突變株 D55C 及 E37C 皆具有 12 個 Lysine 及 3 個 Cystein，難以得知標定的正確位置，因此無法明確得到高專一性的標定酵素。從質譜分析中，亦可見數個（$-CH_2-COOH$）被標識在酵素上。

檢視 MH-K1 幾丁聚醣酵素之結構，不難發現其催化功能上擔任

general acid (E37) 和 general base (D55)之兩重要胺基酸的文獻推測距離為 10.9 Å。而經由我們利用本實驗室 Insight II package program 發現結果，如圖八，可知 E37 跟反應基質之距離為 7.5 Å，而 D55 則為 5.6 Å。可見兩基因均必須有水分子介入其間協助催化反應之進行，因此我們選擇了 2,2-dithiodiacetic acid、3,3-dithiodipropionic acid 及 4,4-dithiobutanoic acid (見表五) 與突變酵素作用以改變兩催化殘基之相對距離，每一個碳-碳鍵估計可增加為 1.5 Å，但因其長鍵結構之直線距離不易推測，所以使用不同鍵長之化合物特異性的與新植入之 Cys 作用。酵素中雖內含有二個 Cys（Cys50，Cys124），但由結構可知其為雙硫鍵，故相信對本研究之標識反應不會有影響，由實驗中我們以質譜分析亦證實每一個酵素均僅進行單一分子之標識。(見圖九、圖十)。

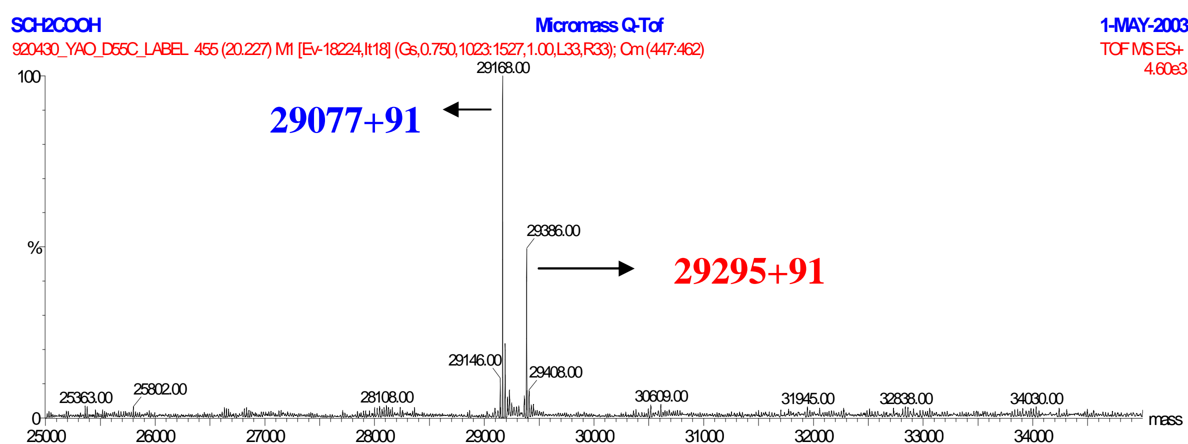


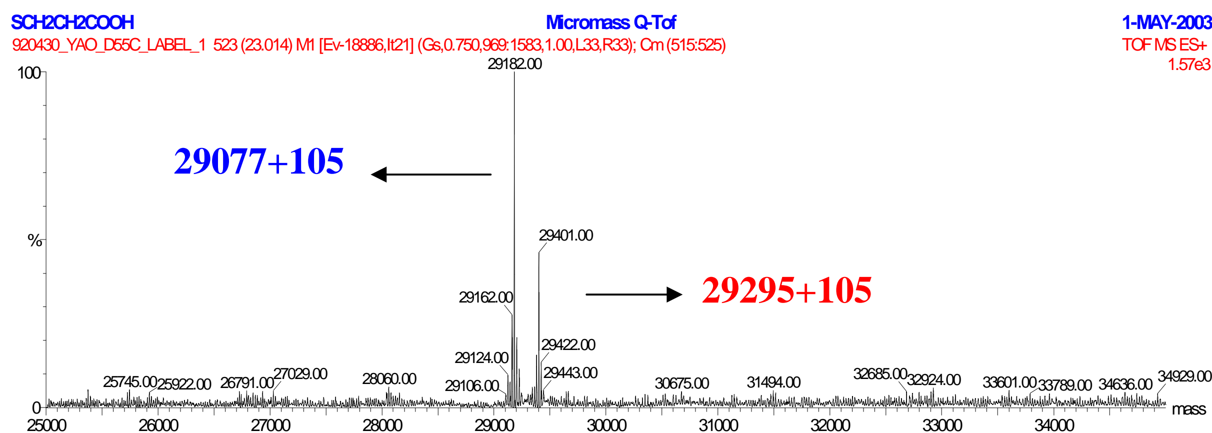
圖八、活化中心相對位置

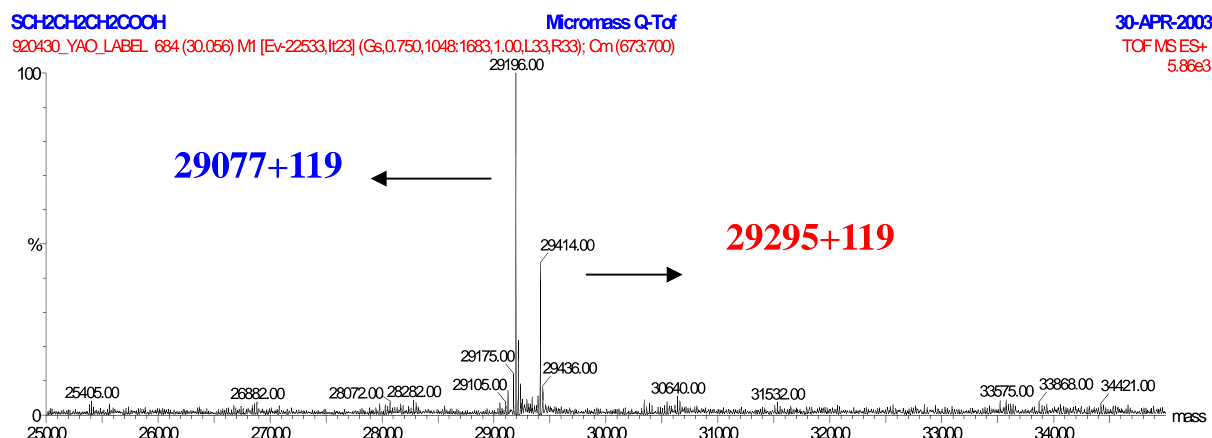
在標定實驗的過程當中，嘗試了許多相關的條件，發現 pH 值及化學標定物的濃度都會影響到反應的結果，分析如下：當 pH 8 時，其標定反應速率優於 pH 7，或標定化學物質濃度較高，標定速率也相對較快，但考慮到酵素在 pH 7 的穩定度及活性較佳，因而以 pH 7 為標識的條件。在反應過程中以質譜儀追蹤反應情形，於反應完成後，利用 Desalting Column 除鹽及純化，可得到高純度之酵素。Wild type 蛋白質的分子量分別為：29295、29077，因前述之不專一成熟蛋白質的問題，圖 4-8 為 D55C 標定後的結果，其以 D-g 標識後之酵素分子量分別為 29386、29168，以

D-p 標識後之酵素之分子量為 29401、29182，以 D-b 標識後酵素之分子量為 29414、29196；圖 4-9 為 E37C 標定後的結果，其未標定前的分子之酵素分子量分別為 29076、29224、29295，以 D-g 標識後之酵素分子量分別為 29289、29380，以 D-p 標識後之酵素之分子量為 29396、29325、29178。

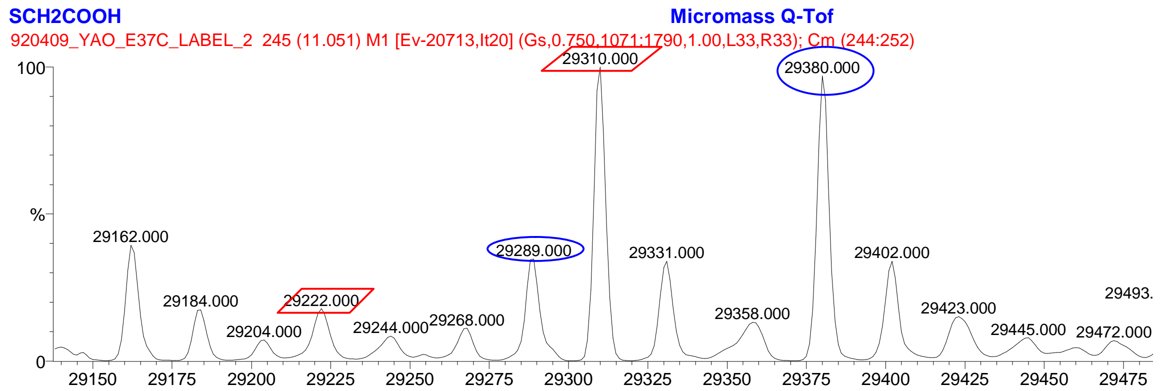
標定化合物(簡稱)	分子式 (標定的分子量)
A. Iodoacetic acid	ICH_2COOH (**MW：59)**
2,2-dithiodiacetic acid (D-g)	$HOOCCH_2SSCH_2COOH$ **(MW：91)**
3,3-dithiodipropionic acid (D-p)	$HOOCCH_2CH_2SSCH_2CH_2COOH$ **(MW：105)**
4,4-dithiobutanoic acid (D-b)	$HOOCCH_2CH_2CH_2SSCH_2CH_2CH_2COOH$ **(MW：119)**

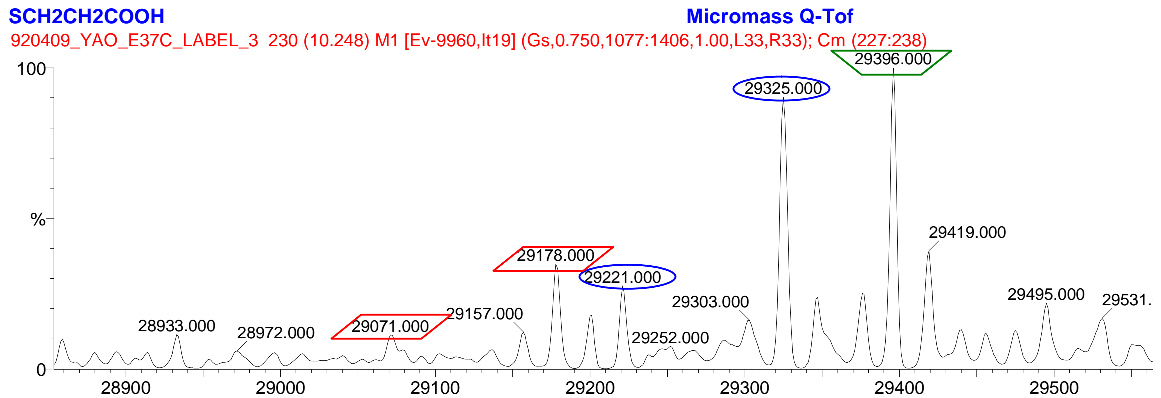


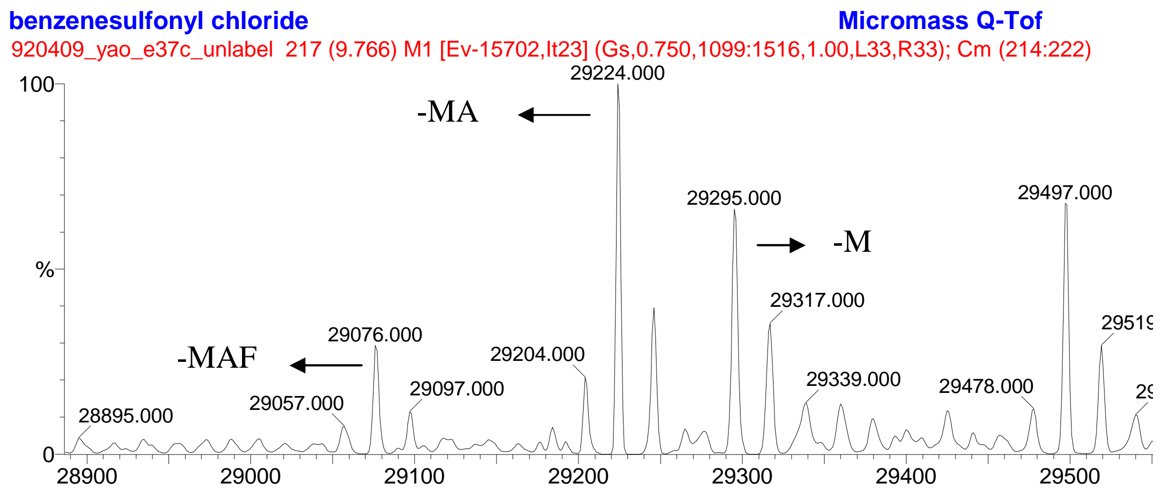




圖九 突變株 **D55C** 與不同 **chemical label** 的結果







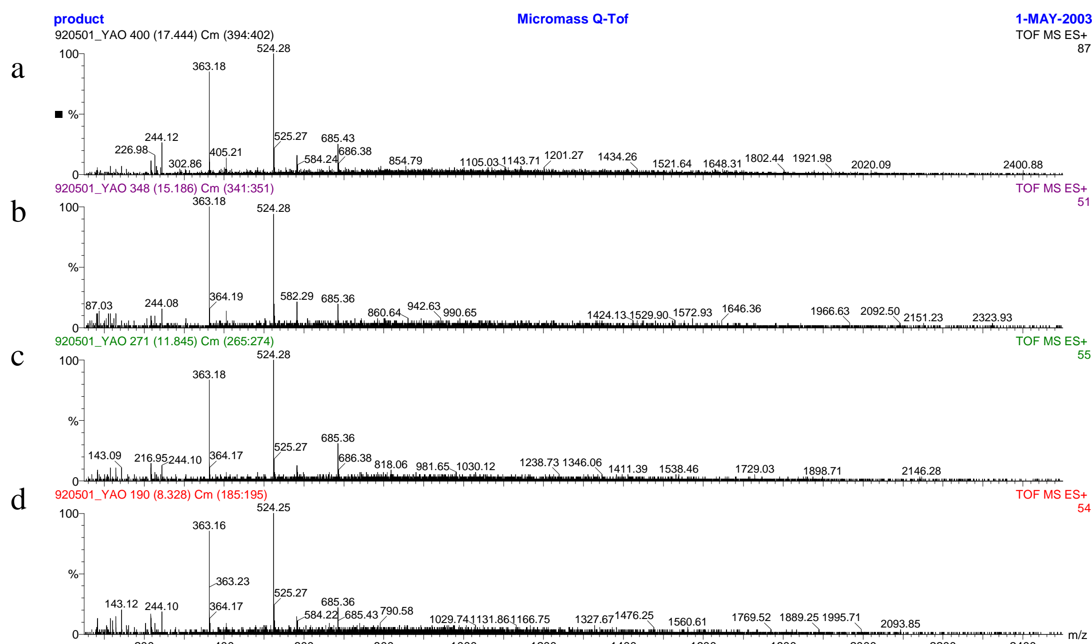
圖十、突變株 E37C 與不同 chemical label 的結果

標定突變株酵素之活性測試及產物分析

標定後的突變株酵素，先利用 DNS 進行初步的活性測試，發現標定後之酵素會具有活性，因此將其與幾丁聚醣作用後，經由質譜儀分析其產物的分子量，發現其水解產物仍以幾丁二醣和幾丁三醣為主，與原本的野生株及突變株相同，故初步無法判斷其產物的不同性。(見圖十一、圖十二)。

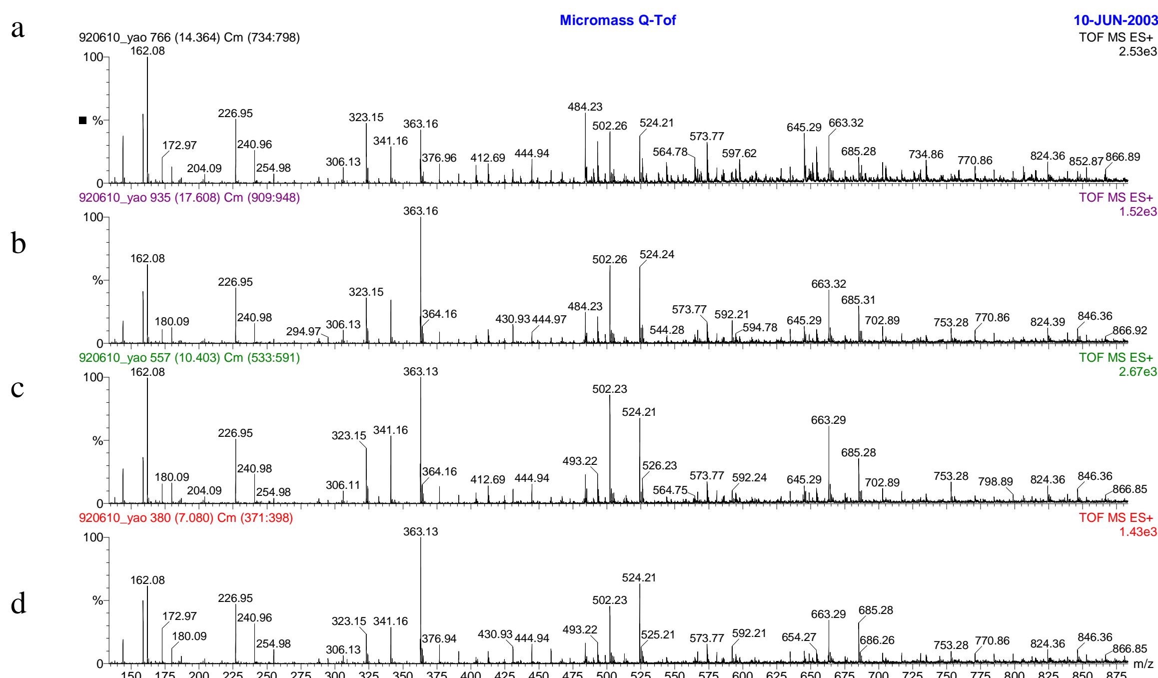
接下來，我們嘗試以轉醣反應來探討標識後之突變酵素的催化位向變化，因為如果在位向保留的機制中，發生轉醣的機率會比較大，因而希望過轉醣的方法，來

做為位向確認的初步推測。可惜在所有突變與經標識之酵素反應中，不論在含 10~20 %各式醇類，均未發現轉醣之現象，但其仍維持活性的存在。



圖十一 (a) D55C-$SCH_2CH_2CH_2COOH$, (b) D55C-SCH_2CH_2COOH, (c) D55C-SCH_2COOH, (d) D55C-unlabel

產物分析： 幾丁二醣：MW : 341、363 幾丁三醣：MW : 502、524

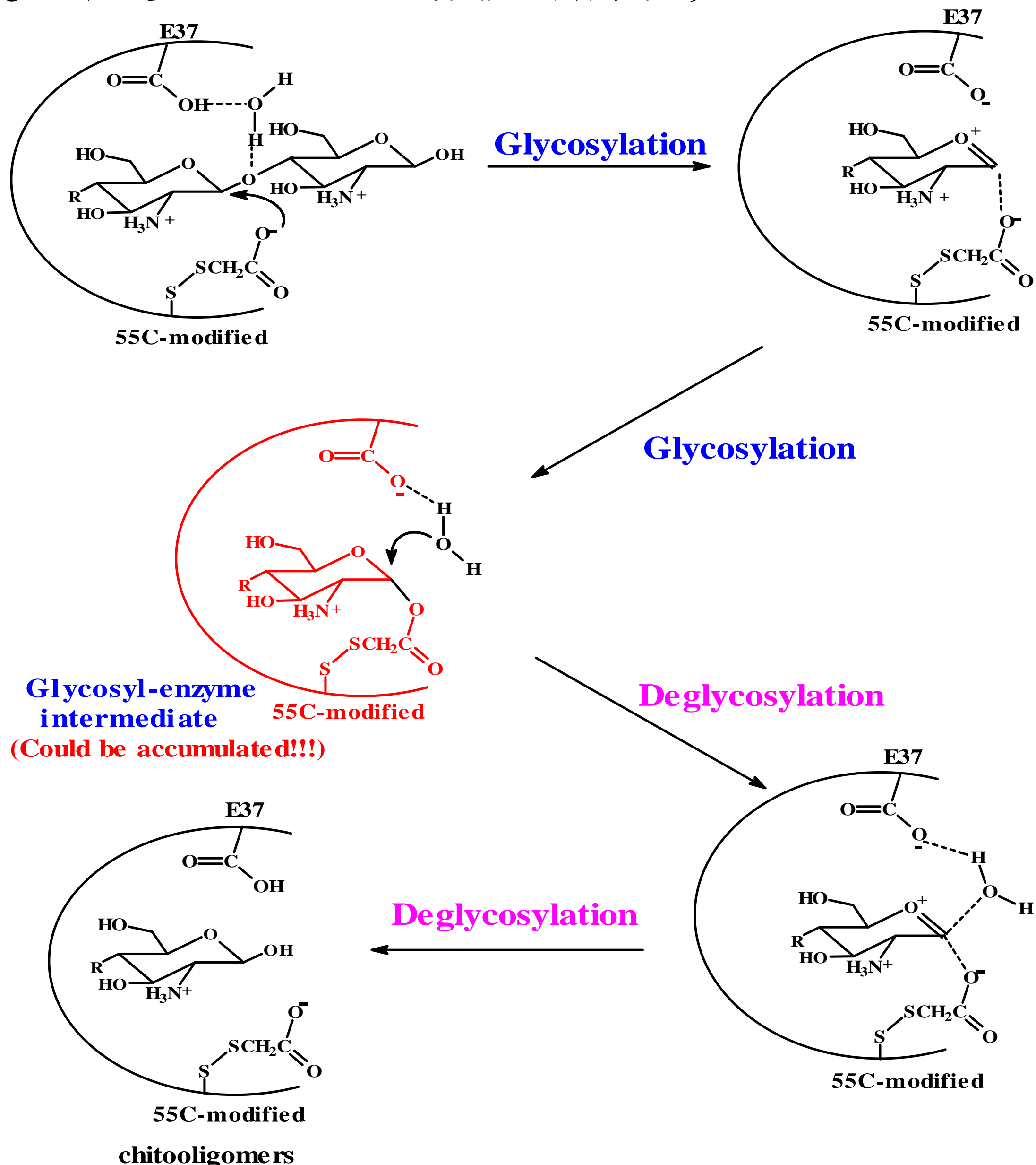


圖十二 (a) E37C-unlabel, (b) E37C-$SCH_2CH_2CH_2COOH$, (c) E37C- SCH_2CH_2COOH, (d) E37C-SCH_2COOH, 產物分析：二醣：MW : 341、363 三醣：MW : 502、524

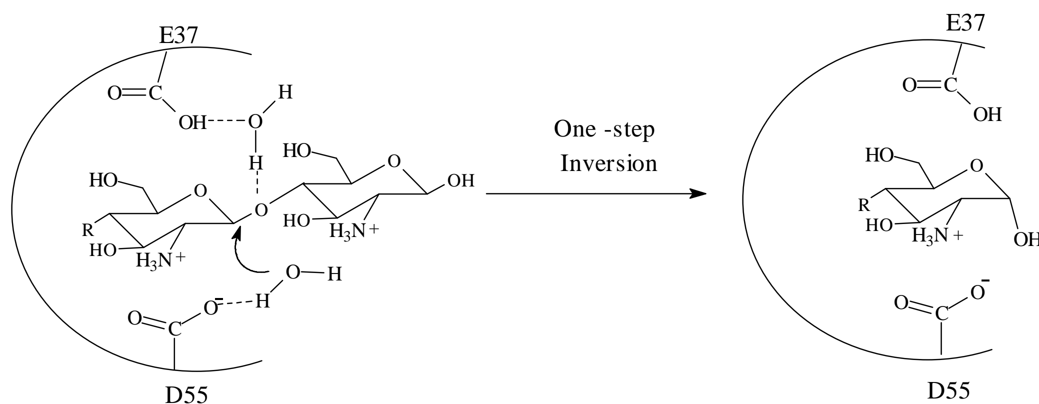
以一般構形保留之反應而言，其機構涉及兩步驟反應。第一步驟為酵素醣化反應 (glycosylation) 而形成酵素--醣基中間體 (glycosyl -enzyme intermediate)、第二步

驟為酵素--醣基中間體之去醣基反應(deglycosylation)。兩步驟之反應過渡態為一帶正電之 carbocation (或其共振態 oxocarbonium ion) （見圖十三），因此若突變酵素之催化機制為保留型，則很可能酵素會於催化反應過程中被醣基標識。又因幾丁醣胺基在 pH 7 以下將被質子化而使得去酵素醣基作用不容易進行，因醣基上攜有正電將使得過渡態(carbocation) 不易生成，因而抑制反應進行。

如前述，雖然我們沒有直接證據顯示其突變酵素或其修飾後的酵素已轉變為保留型之催化機構，但在後續研究將可以直接測定經由寡醣如:幾丁六醣作用過之突變酵素的分子量，應可直接得進一步之資訊。在自然界中，或許因為幾丁聚醣具有易被質子化的特性，因此所有已知之幾丁聚醣酵素之催化反應均為反轉型態進行 (見圖十四)，即酵素之活性位置僅活化水分子，利用活化之水分子與幾丁聚醣作用以避免自己被醣基化而失去活性，但這些推論仍須待進一步驗證。



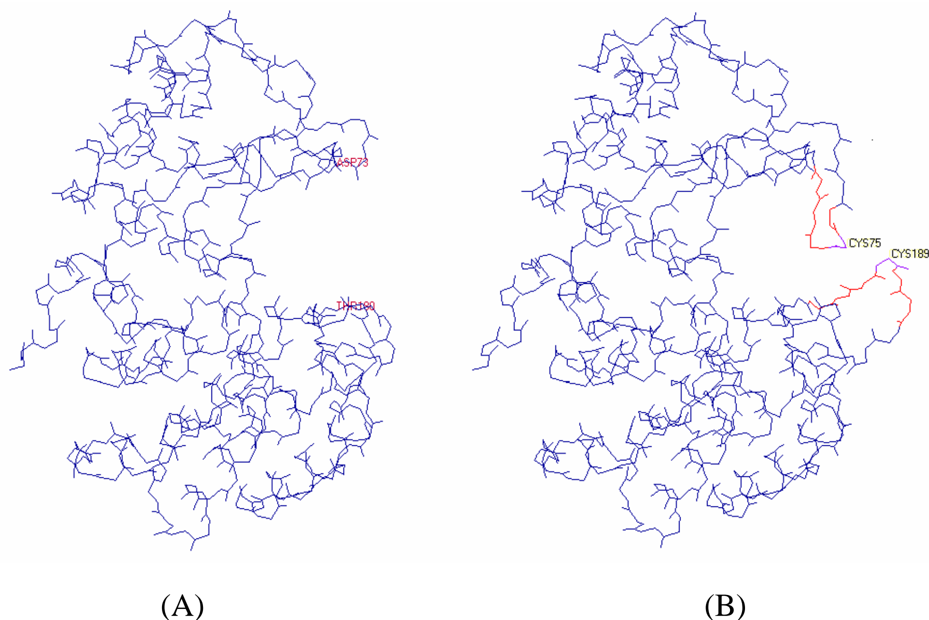
圖十三 化學標示後之位向保留(retention)機制推測圖



圖十四 位向反轉（inversion）機制圖

轉內聚醣胺水解酵素(endo-chitosanase)為外聚醣胺水解酵素(exo-chitosanase)

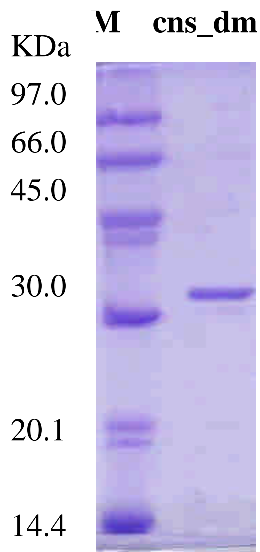
在我們的研究過程中，希望在活性中心，透過突變的方式，加入片段的 peptide，將活化中心的口蓋起來。首先先看原本已只的 Bacillus chitsanase 的結構，如（圖十五.左）， 將透過模擬的方式，在 chitosanse 活化中心得表面得知胺基酸序列為 73 及 180（也就是說先從含 signal peptide 的原本的酵素為胺基酸序列為 116 及 223）為我們將插入小片段的 petide。這兩個表面的 loop 大約為 5aa 及 6aa 長的 peptides。（圖十五.右），加入之後，修飾過得酵素我們稱為 chitosanase-bridge.



(A) (B)

圖十五 Bacillus chitosanase(left) and chitosanase-bridge(right). 兩個 cis 殘基(紫色) 在人工設計 loop（紅色）推測其之間會形成雙硫鍵

這個透過化學修飾過後的酵素，含有兩個人為的 loop，稱為 chitosanase-bridge（CNS_DM）,純化的酵素在 SDS-PAGE 上與 CNS 具有 90% 的相似度，見圖十六 。



圖十六 SDS-PAGE

根據 chitosanase DNA 序列，將其在大腸桿菌中表現並且透過氨基酸序列分析其分子量， chitosanase(cns) 為 29081 Da and chitosanase-bridge(cns_DM)為 30124 Da (圖十七).，同時為了更精準得獲得分子量，也得知這之間差了 1043 daltons，乃是因為加入兩個額外片斷的 peptides. 其質譜分析圖如下：

(A) Chitosanase from Bacillus

MHMSNARPSK SRTKFLLAFL CFTLMASLFG ATALFGPSKA AA ASPDDNFS PETLQFLRNN TGLDGEQWNN IMKLINKPEQ DDLNWIKYYG YCEDIEDERG YTIGLFGATT GGSRDTHPDG PDLFKAYDAA KGASNPSADG ALKRLGINGK MKGSILEIKD SEKVFCGKIK KLQNDAAWRK AMWETFYNVY IRYSVEQARQ RGFTSAVTIG SFVDTALNQG ATGGSDTLQG LLARSGSSSN EKTFMKNFHA KRTLVVDTNK YNKPPNGKNR VKQWDTLVDM GKMNLKNVDS EIAQVTDWEM K

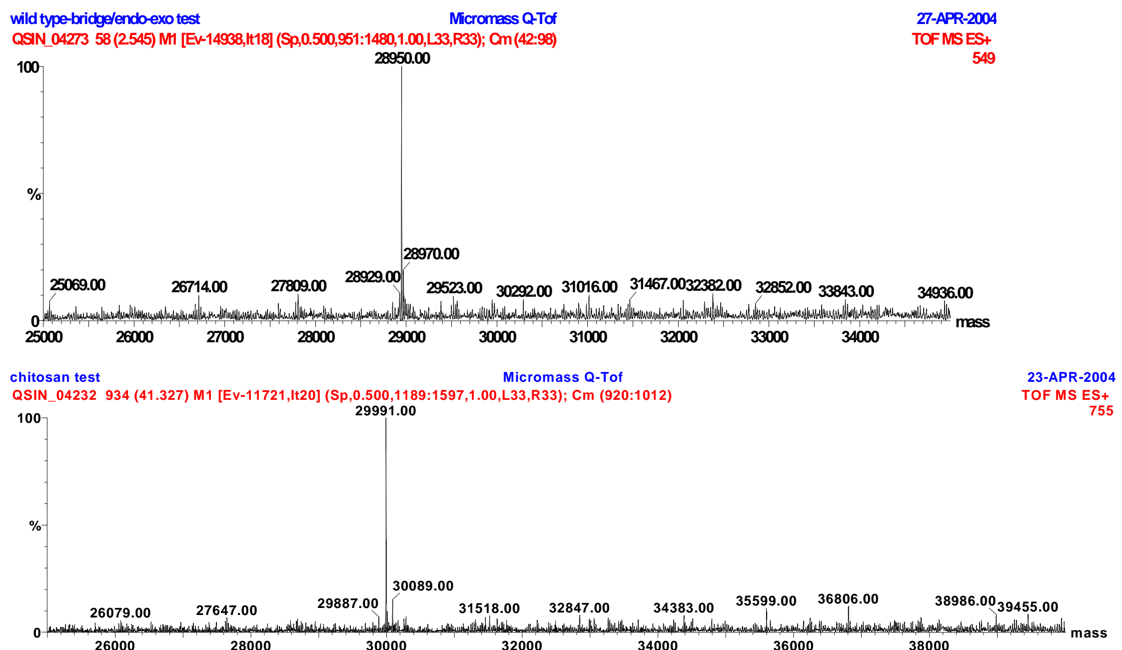
(B) Reconstructed CNS in pet22b(+)

MASPDDNFS PETLQFLRNN TGLDGEQWNN IMKLINKPEQ DDLNWIKYYG YCEDIEDERG YTIGLFGATT GGSRDTHPDG PDLFKAYDAA KGASNPSADG ALKRLGINGK MKGSILEIKD SEKVFCGKIK KLQNDAAWRK AMWETFYNVY IRYSVEQARQ RGFTSAVTIG SFVDTALNQG ATGGSDTLQG LLARSGSSSN EKTFMKNFHA KRTLVVDTNK YNKPPNGKNR VKQWDTLVDM GKMNLKNVDS EIAQVTDWEM K

(C) Bridge_mutant CNS in pet22b(+)

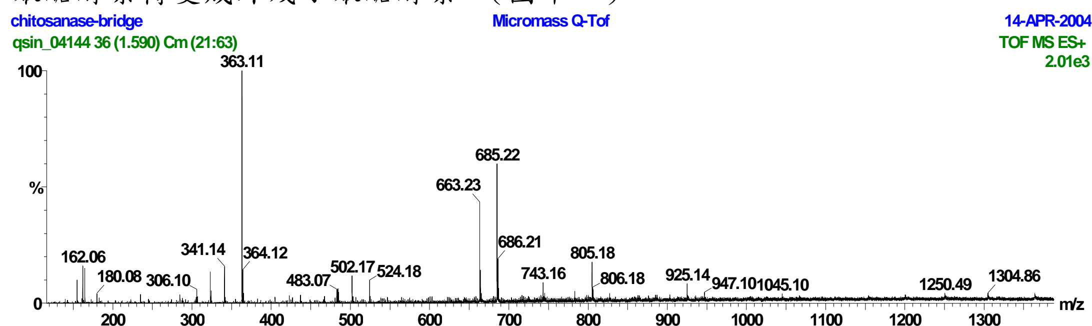
MASPDDNFS PETLQFLRNN TGLDGEQWNN IMKLINKPEQ DDLNWIKYYG YCEDIEDERG YTIGLFGATT GGSRDpclggTHPDG PDLFKAYDAA KGASNPSADG

ALKRLGINGK MKGSILEIKD SEKVFCGKIK KLQNDAAWRK AMWETFYNVY IRYSVEQARQ RGFTSAVTIG SFVDTALNQG ATsrtckpGSDTLQG LLARSGSSSN EKTFMKNFHA KRTLVVDTNK YNKPPNGKNR VKQWDTLVDM GKMNLKNVDS EIAQVTDWEM K



圖十七 質譜分析分子量。wild type (upper) chitosanase and mutant chitosanase with artificial loops (lower), i.e. chitosanase-bridge.

希望夠過利用 chitosan hexmer 為受質來區分 endo 和 exo 的機制。理論上，若為外幾丁聚醣酵素（exochitosanase）的話，自醣類非還原端逐步水解幾丁聚醣產生單一長度的幾丁寡醣，大多數屬短鏈的 1,2 醣（chitobiose,glucosamine）。相對的，內幾丁聚醣酵素（endochitosanase）分解幾丁聚醣產山多種長度的幾丁寡醣。因此，我們利用幾丁六醣為受質，若其為內切型(endo)，可獲得幾丁三醣。但在 chitosanase bridge 與幾丁六醣反應後，所獲得的結果並不能有十足的證據顯示其已經從內幾丁聚醣酵素轉變成外幾丁聚醣酵素，(圖十八)。



圖十八 chitosanase-bridge 與幾丁六醣反應後知產物分析。其主要的產物為二醣(m/z 341, m+Na/z 363) and 四醣 (m/z 663; m+Na/z=685). 及少數的三醣 Only few chitotriose (m/z 502)

為了更進一步了解在 chitosanase bridge 中，兩個在 loop 上的 cys 殘基，是否形成雙硫鍵，我們也與同步輻射中心合作，希望夠過結晶能得更進一步得結果，初

步結晶圖如（圖十九）所示

圖十九 chitosanase-bridge　x-ray 結晶圖。

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計畫結案報告 (2002-2005)
博士後研究員 鄭至玉

題目:Exploration of glycosyl hydrolase family 75: a case study of Chitosanase from *Aspergillus fumigatus*

A powerful chitosanase for the preparation of chitooligosaccharide was previously purified from *Aspergillus fumigatus*. The corresponding gene was cloned and the enzyme was further classified into glycosyl hydrolase family 75. The gene sequence derived from mRNA is composed of 717 bp with a deduced polypeptide of 238 amino acids including the first 17 residues as the signal peptide. By comparing the sequences of genomic DNA and mRNA, we found two introns containing 67 and 82-bp (GenBank AY190324). The recombinant chitosanase was over-expressed in *E. coli* with a form of inclusion body, which was rescued by treating with 5 M urea and subsequently purified by cation-exchanged chromatography. A time-course ^{1}H-NMR experiment on the enzymatic formation of chitooligosaccharides revealed that the mechanism of the enzyme involved an inversion of an anomeric configuration. Through analysis of the products and their corresponding methylated derivatives with LC/MS/MS, the pattern of enzymatic hydrolysis of the GlcNAc-GlcN and GlcN-GlcN linkages in chitosan were unequivocally determined, whereas the GlcNAc-GlcNAc and GlcN-GlcNAc linkages were not digestible. Site-directed mutagenic studies on the ten conserved carboxylic amino acids of the family were performed. Among them, the mutants of D160N and E169Q lost all activity, whereas the other mutants retained > 40 % activity of the wild-type chitosanase. Measurements of circular dichroism of D160N, E169Q, wild-type enzyme and other active mutants yielded similar spectra, indicating that activity loss of the two mutants was not due to the change of protein structure. We conclude that Asp160 and Glu169 are the two essential residues of *A. fumigatus* chitosanase.

Chitosanase (EC 3, 2, 1, 132) is a hydrolytic enzyme acting on the β-1, 4-glycosidic linkage of chitosan obtained from deacetylation of chitin, a linear biopolymer of β-1, 4-linked N-acetylglucosamine (GlcNAc) residues. As such oligomers have versatile biofunctions (1), chitosanase, chitooligosaccharides and their derivatives become attractive to pharmaceutical industries, for food additives, for agricultural immunity controls and for many other prospective applications.

Chitosanase was found in several microorganisms (2). Based on the homology of the amino-acid sequence (3), chitosanase was classified into five glycoside hydrolase families – GH-5, GH-8, GH-46, GH-75 and GH-80. Extensive studies were performed on GH-46, especially on *Bacillus* (4) and *Streptomyces* strains (5). On the basis of the anomeric configuration of the catalytic product, two types of chitosanases, the retaining enzyme and the inverting enzyme, were found. The fundamental catalytic mechanism can be proposed if the type of enzyme is known. In general, the retaining glycosidases are believed to catalyze hydrolysis via a two-step, double-displacement mechanism. Two essential amino-acid residues, one functioning as a nucleophile and the other as a general acid/general base, are involved. In contrast, the inverting glycosidases follow a one-step, single-displacement mechanism with the assistance of a general acid and a general base. The general base polarizes a water molecule to develop a stronger nucleophile for attacking the anomeric carbon, while the general acid protonates the glycosidic oxygen to accelerate the reaction. Among the families containing chitosanase, the retaining configuration of the catalytic mechanism of GH-5 chitosanase was derived from glucanase (6). The inverting configurations were experimentally verified in GH-46 (7, 8) and GH-8 (9). GH-80 was also inferred to be an inverting enzyme. Although several genes of GH-75 chitosanases were cloned including those from *Aspergillus*, *Beauveria*, *Cordyceps*, *Emericella*, *Fusarium*, *Hypocrea*, *Magnaporthe*, *Metarhizium, Nectria* and *Neurospora,* investigations of enzymes of this family are much less reported, presumably

owing to the inaccessible expression of recombinant chitosanase. The artificial gene of *Aspergillus* endo-chitosanase was synthesized in 2004 (GenBank AY787804), presumably owing to the potential application of *A. fumigatus* CSN in large-scale preparation of chitooligosaccharides (10). All GH-75 chitosanase originated from fungus and no other fungal chitosanase has been found in another family so far. Although little work on GH-75 is reported in protein purification, cloning and enzymatic activity (10-13), the digestion pattern toward chitosan, a possible mechanism of reaction and the essential residues involved in catalysis of this family enzyme remain unknown.

In our work, which represents the first extensive investigation on GH-75 enzyme, molecular cloning, recombinant protein expression and re-folding, product analysis and site-directed mutagenesis were performed to explore directly the catalytic features of *A. fumigatus* chitosanase.

MATERIALS AND METHOD

Bacterial Strains, Plasmid, and Culture Condition — The *A. fumigatus* Y2K strain screened from soil in Taiwan with chitosan as the sole source of carbon (10) served as a gene source of cloning. The medium used for cultivation and enzyme induction was M9 medium (composed of Na_2HPO_4 1.3 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4$ 0.24 g, and $CaCl_2$ 0.01 g in 1 L solution) containing chitosan (1 %) at pH 6.0. A spore suspension ($\sim 1x10^{10}$) was transferred to a culture medium (1 L) at 28°C and 120 rpm on a rotary shaker. This five-day culture served for the preparation of the induced chitosanase and chromosomal DNA, but the four-day culture for total RNA extraction. *Escherichia coli* strains TOP10 and pCR 2.1 TOPO-vector (TOPO TA Cloning kit, Invitrogen, USA) were used for DNA cloning, and BL21 (DE3) and pRSET A vector (Stratagene, USA) as the protein expression system.

Isolation of Chromosomal DNA and RNA from Aspergillus fumigutus — The mycelium were collected by filtration and ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen. Chromosomal DNA was then extracted by phenol-chloroform method, and total RNA was isolated through acidic guanidinium thiocyanate-phenol-chloroform extraction (14).

Construction of cDNA library — RNA isolated from the mycelium was used for the construction of a cDNA library. The method of cDNA synthesis was modified from a SMART PCR kit (Clontech Laboratories, Palo Alto, CA) (15). The set of smart primers, pT and pG, were designed and synthesized with the sequences 5'-AAA CAG TGG TAA CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TVN-3' and 5'-AAA CAG TGG TAA CAA CGC AGA GTA CGC GGG-3', respectively, and were used for RT-PCR amplification (ThermoScript™ RT-PCR System, Invitrogen). The reverse-transcription performed at 65 °C for 1 h and subjected to PCR with these cycling parameters for 30 cycles: 94 °C for 30 s, 65 °C for 30 s, 68 °C for 2 min, with an initial denaturing step at 94 °C for 5 min and a final extension at 68 °C for 8 min. The freshly prepared cDNA was purified on elution through a GFX column (Amersham Pharmacia Biotech Inc., USA), cloned into pCR 2.1 TOPO vector and transformed into competent *E. coli* TOP10 for the cDNA library construction.

Amplification of cDNA Fragment Containing csn — According to the N-terminal sequence of the mature CSN (10), two degenerated primers p1 and p2 were designed. The p1 primer, 5'-TAY AAY YTR CCA CCY AAY YTR AAR C-3', was designed on the basis of the first eight amino acids of CSN, whereas the p2 primer, 5'-CNA AYA AYY TNA ARC ARA THT AYG AYG A-3', was synthesized according to the sequence of the fourth to the twelfth amino acid of CSN. These primers were paired with pT for PCR using the cDNA library as template for amplification of the *csn* fragment. These fragments were then cloned into pCR 2.1 TOPO and sequenced.

Amplification of the Genomic DNA of CSN — Genomic DNA of CSN was amplified using p2 and p3 (5'-GGA TCC GTT CTA GTT CGC TAT GCT TTC AA-3') as primers and chromosomal DNA as the template. The amplified DNA fragment was cloned into pCR 2.1 TOPO for sequencing.

Inverse PCR — The upstream nucleotide sequence containing information about the signal peptide was cloned with inverse PCR according to a method described by Ochman *et al*. (16). In general, chromosomal DNA was digested with restriction enzyme. DNA

fragments of suitable size were then purified and self-ligated to form circular DNAs, which then served as the template for inverse PCR. With a set of "outward-going" primers, the circular DNA is amplifiable. In this work chromosomal DNA (~5 ug) was completely digested with *Eco*RI. The digested fragments between 500 and 4000 bp were isolated from the gel of DNA electrophoresis and further self-ligated with T4 DNA ligase (Roche, Germany) at 16 °C, overnight. With a GFX column, the ligated DNA fragments were purified and subjected to serve as a template for inverse PCR. The "outward-going" primers in a pair used to amplify the CSN-related gene were designed as p4 (5'-CTT GTG TTT GTC GTA GAT CTG TTT CAA GT-3') and p5 (5'-GGA AAA TGT TCC AAG GTA CTG GCA AAA G-3'). The cycling parameters of PCR amplification were as follows: 94 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min (30 cycles), with an initially denatured step at 94 °C for 5 min and a final extension at 72 °C for 10 min to complete the unfinished single-stranded products. The inverse PCR product was purified and inserted into pCR 2.1 TOPO for sequence analysis.

Construction of Expression System of Recombinant Chitosanas — For reconstruction of *csn* in an expression vector, p6 primer (5'-CAT ATG TAC AAT TTG CCA AAC AAC TTG AAA C-3') was synthesized with the insertion of NdeI site (CATATG) before the nucleotide sequence of the first eight residues of the mature protein. Use of p3 and p6 as primers and the partial cDNA fragment of *csn* (described in the section of "***Amplification of cDNA fragment containing csn***") as template yielded the mature CSN gene. This PCR product was then cloned into pCR 2.1 TOPO and further transferred into pRSET A expression vector within *Nde* I and *Bam*H I sites. The resulting vector was named pRSET-*csn* and expressed in *E. coli* strains BL21 (DE3).

Activity Assays — Chitosanase activity was analyzed on estimating the amounts of the reducing ends of sugars. The standard assay was performed by mixing chitosan (0.3 mL, 1 %, pH 6.0) and suitably diluted enzyme (0.3 mL). After incubation for 4 h at 37 °C, hydrolysis reactions were terminated and analyzed on adding dinitrosalicylic acid reagent (0.6 mL) (17). The mixture was boiled for 15 min, chilled and centrifuged to remove insoluble chitosan. The resulting adducts of reducing sugars were measured spectrophotometrically at 540 nm. The absorption coefficient of the resulting adducts (at 540 nm) was determined to be 788 $M^{-1} cm^{-1}$ when D-glucosamine was used as sample. One unit of chitosanase activity was defined as the amount of enzyme required to release 1 μmol of detectable reducing sugars at 37 °C in 1 min (18). Alternatively, the products were analyzed by mass spectrometer with electrospray ionization as described in later section.

Protein Expression, Refolding and Purification — *E. coli* strain BL21 (DE3) harboring plasmid pRSET-*csn* was grown overnight in LB medium. This culture (2.5 mL) was freshly inoculated into LB medium (250 mL) supplemented with ampicillin (100 mg/mL). The resulting medium was cultivated at 37 °C for 14 h. The cells were collected and resuspended in phosphate buffer (20 mM, pH 6.0) for sonication. As the recombinant chitosanase formed an inclusion body, the cell debris was collected and incubated in urea (5 M) at 37 °C for 4 h. The supernatant was decanted and kept at 4 °C for at least 3 days to allow protein refolding. Without removing urea, the supernatant was subjected to cation-exchanged chromatography (HiTrap™ 5 ml-SP-Sepharose, Amersham). The column was pre-equilibrated with phosphate buffer (pH 6.0, 20 mM) and eluted with a linear gradient of NaCl (from 170 to 240 mM; 10 mM/min) in the same buffer at a flow rate 2 mL/min. The fractions with chitosanase activity were pooled and concentrated for further experiments. For purification of inactive mutants, SDS-PAGE was employed for analysis of the presence of target protein.

Protein Determination —The protein content of the enzyme preparation was determined either by the bicinchoninic acid (BCA) method as described in the manufacturer's protocol (BCA-1, kit for protein determination; Sigma) or by UV absorption at 280 nm.

NMR Spectra — The stereochemistry of enzymatic hydrolysis was determined by ^{1}H-NMR spectroscopy at 35 °C (Bruker AVANCE 500 spectrometer). For sample preparation, chitosan solution (1 %) was prepared in acetic acid (1 %). The solution was repeatedly lyophilized and exchanged with D_2O. The chitosanase for the NMR measurements was prepared on exchanging the buffer system into one containing deuteriophosphate (phosphate

buffer exchanged with D_2O; 20 mM phosphate; pD 7) via ultrafiltration. After recording the spectrum of the substrate (500 μL), chitosanase (20 μL, 10 μg/mL) was added to the chitosan solution (1 %). Spectra were recorded at 9, 21, 33, 48 and 60 min after the addition of enzyme. All spectra were recorded over a 5000-Hz sweep width with eight scans for a total recording duration 25 s each.

Chemical Methylation of Chitosan Hydrolysate — The chitooligosaccharides were obtained from enzymatic hydrolysis of chitosan with 40 % degree of deacetylation (DDA 40 %). For identification of the structure of these oligosaccharides, a suitable amount of oligosaccharides was chemically methylated at the reducing end of the sugar. The methylation was performed in a large excess of methanol containing perchloric acid (2 %) at 50 ℃ for 10 h. The methylated oligosaccharides were analyzed with a LC tandem mass spectrometer (LC/MS/MS).

Electrospray Ionization Mass Spectrometric (ESI-MS) Analysis — Mass spectra were recorded with a quadrupole time-of-flight mass (Q-TOF, Micromass, UK). This quadrupole mass analyzer was scanned over a ratio of mass to charge in a range 100-2500 units (m/z), with a scan step 2 s and an inter scan of 0.1 s/step. In all ESI-MS experiments, we used the quadrupole scan mode under a capillary needle at 3 kV, source block temperature at 80 °C and desolvation temperature at 150 °C. Proteins used for mass measurement were normally in the range of 5-10 μg with desalted form.

Site-directed Mutagenesis —The site-directed mutagenesis was performed according to the QuikChange method (Stratagene, USA). The basic procedure involved PCR amplification with pRSET-*csn* as template and two synthetic oligonucleotides containing the desired mutation as primer. Table I lists the sequences of primers used for the mutagenic tests herein. The desired mutations were confirmed with DNA sequencing of the full gene.

CD Spectra of Recombinant Chitosanase — Spectra were obtained at 23 ℃ on a spectropolarimeter (JASCO J-715, cell length 1 mm). The concentration of proteins for CD measurement was 1 mg/mL. Sixteen spectra were recorded and averaged.

RESULTS AND DISCUSSION

Molecular Cloning and the Gene Structure of csn — To avoid the interruption of introns in gene, we constructed a cDNA library of *A. fumigatus*. As the time course tests showed that the extracellular chitosanase activity reached a maximum with the five-day culture, a four-day culture of mycelium was used for RNA extraction. The complete molecular cloning of *csn* involved many steps, summarized in figure 1. Step 1 involved amplification of cDNA from RNA. With pT and pG as primers, cDNAs of varied size were synthesized. To obtain the corresponding chitosanase gene, we designed two degenerated primers p1 and p2 according to the N-terminal sequence of the mature CSN (as described in the method). When p1 and pT were used as primers, a major DNA fragment with ~900 bp was amplified (step 2). The amino-acid sequence deduced from the major DNA fragment was further analyzed by the BLAST search on the National Center for Biotechnology Information (NCBI) website; the results indicated that the deduced sequence was highly correlated to a putative conserved domain of the NADH oxidase family (NOF) (data not shown), whereas, when p2 and pT primer were employed, a cDNA fragment corresponding to the *csn* was obtained (step 3). The sequence of *csn* is shown in figure 2. To obtain information about the gene structure of *csn*, we amplified a DNA fragment using p2 and p3 as primer and the chromosomal DNA of *A. fumigatus* as template (step 4). Sequence analysis revealed that two introns with 67- and 82-bp were incorporated in the genomic *csn*. The upstream nucleotides containing the sequence of the signal peptide was unwound by inverse PCR (step 5). The sequence of the full-length genomic *csn* has been published in NCBI GenBank with accession number AY190324.

In conclusion, the gene, open reading frame (ORF), and two introns of *csn* were 866, 717, 67 and 82 bp, respectively. As shown in figure 2, the ORF encoded a polypeptide that comprises 238 amino acids including the first 17 amino acids as the signal peptide. The calculated molar mass of the mature protein fused with a Met at the N-terminus is 23593 Da, further confirmed by ESI mass spectrometry. In addition, the deduced amino-acid sequence showed it to be 90 % identical to that of *Aspergillus oryzae* (Zhang *et al.*, 2000), but much less (<30 %) to that of *Aspergillus nidulans*.

Over-expression, Purification and Refolding of

Chitosanase— When pRSET-*csn* was expressed in BL21 (DE3), weak chitosanase activity was detected in cytosol. In contrast, much protein with molar mass ~25 kDa was observed in cell debris on SDS-PAGE analysis, indicating the formation of an inclusion body of chitosanase (figure 3). Attempts to control the expression level of chitosanase on decreasing the cultivation temperature to 20 ~ 25 °C showed insignificant improvement in obtaining active enzyme in cytosol. The enzyme was therefore purified from the inclusion body *via* the refolding of protein and column-chromatographic purification. In general, the aggregated protein was first resuspended at 5 M (or more) urea. Chitosanase activity was gradually recovered in urea at such high concentration. The urea suspension was kept at 4 °C for at least 3 days before further purification steps were applied. After cation-exchanged chromatographic purification, the purified chitosanase with >90 % homogeneity was obtained. All other mutants (discussed in later section) were purified with the same protocol and attained similar protein homogeneity. SDS-PAGE analysis of the wild-type CSN at various stages of purification is shown in figure 3(a). Although many GH-75 enzymes, which are exclusively fungal chitosanases, have been cloned, we report here the first successful expression, purification of the corresponding recombinant enzyme and investigation of its catalytic function and essential residues.

Catalytic Features of A. fumigatus Chitosanase — The molecular mechanism is conserved within the same family of glycosyl hydrolases (3, 19). A successful investigation of catalytic action of one particular enzyme in the glycosyl hydrolase family is thus valuable for understanding the general features of the family. Insight into the catalytic mechanism is likely best revealed from the study of the x-ray structure. In the absence of such information, kinetic study and the stereochemical outcome of the enzymatic reaction can provide information that enable drawing sophisticated conclusions about the catalytic mechanism (20). Neither the structural information nor the mechanistic action of GH-75 chitosanase is available at present.

^{1}H-NMR spectra have been commonly used to investigate the stereochemistry of various glycosyl hydrolases such as β-galactosidase (21), β-mannanase (22), cellulases, xylanase (23), β-xylosidase and β-1,3-glucanase (24). To understand further the catalytic mechanism of *A. fumigatus* chitosanase, we performed a temporal NMR experiment on enzymatic hydrolysis of chitosan. Figure 4 shows partial ^{1}H-NMR spectra (4.2-6.0 ppm) in a series recorded within 1 h after catalysis was initiated. On inspection of spectrum of chitooligosaccharides, we found three doublets centered at 4.71 ppm (J=7.8 Hz), 4.90 ppm (J=7.8 Hz), and 5.41 ppm (J=3.3 Hz), corresponding to the C1-proton on non-reducing-end sugar, β and α of the anomeric protons, respectively. The ratio of α to β (α/β) anomer was 63/37 when the two anomers approached a state of equilibrium. With the addition of enzyme to chitosan, the doublet, centered at 5.41 ppm, instantly increased, indicating the formation of an α anomer at the reducing end. The corresponding β anomeric sugar (centered at 4.90 ppm) slowly increased over time. At 9 min, the ratio of α-form to β-form was 78:22. At 21, 33, 48 and 60 min, the α/β anomeric signals decreased steadily from 73:27, 69:31, 65:35 to 63:37. Based on these NMR results, we unequivocally identify *Aspergillus* chitosanase as an inverting enzyme. We thus concluded that the catalytic mechanism of GH-75 enzymes involve the inversion of anomeric configuration. With the exception of family 5 chitosanase, chitosanases from GH-8 and GH-46 exhibited an identical stereochemical preference (7-9).

Identifying the Substrate Specificity of CSN— Based on the substrate specificity, chitosanase has been classified into three subclasses (9). Subclass I chitosanase presents a catalytic power on hydrolysis of the β-1,4-linkages of both GlcN-GlcN and GlcNAc-GlcN. Subclass II can split only GlcN-GlcN, and subclass III chitosanases split GlcN-GlcN and GlcN-GlcNAc. To identify the substrate specificity of CSN, we measured the enzymatic hydrolysate of lower DDA chitosan with LC/MS; we found five major oligosaccharides with m/z 341, 383, 502, 544 and 705, corresponding to a chitobiose, a monoacetyl chitobiose, a chitotriose, a monoacetyl chitotriose and a monoacetyl chitotetraose, respectively, shown in figure 5 (a). The structures of these three acetylated chitooligosaccharides were further

determined to be GlcN-GlcNAc, GlcN-GlcN-GlcNAc and GlcN-GlcN-GlcN-GlcNAc by chemical methylation of the reducing end of the oligosaccharides followed by ESI/MS/MS analysis. As shown in figure 5(b), the tandem mass analysis of the signal at m/z 397, the methylated monoacetyl chitobiose, exhibited a fragment with m/z 236 that is derivable only from GlcN-GlcNAc-OCH_3. Similarly, as shown in figure 5(c) and 5(d), the tandem mass analysis of methylated monoacetyl chitotriose (m/z 558) and a methylated monoacetyl chitotetraose (m/z 719) exhibited fragments derived from GlcN-GlcN-GlcNAc-OCH_3 and GlcN-GlcN-GlcN-GlcNAc-OCH_3, respectively. Although other oligosacchrides might be present, they are insignificant. According to these consequences, *Aspergillus* chitosanase was classified to subclass I, which possesses a catalytic power on hydrolysis of the linkages of GlcN-GlcN and GlcNAc-GlcN, but neither GlcN-GlcNAc nor GlcNAc-GlcNAc.

When $(GlcN)_6$ was hydrolyzed with wild-type CSN, chitotrimer $(GlcN)_3$ were released as major product, whereas $(GlcN)_2$ and $(GlcN)_4$ were detected in only trace proportions. As the rate of degradation significantly decreased or even became zero with a shorter chain of substrate, the substrate-binding cleft of CSN can likely accommodate six GlcN residues. The cutting point is predicted to be at the third glycosidic bond. Although structural information about CSN is not yet available, a binding domain of this type (-3-2-1+1+2+3) is discernible in *Streptomyces* sp. N174 chitosanase (8), goose egg-white lysozyme and other glycohydrolases (25,26).

Catalytic Essential Residues of CSN — Although at least five GH-75 chitosanases have been cloned, extensive investigations of these are few. The protein structure, catalytic mechanisms, essential groups and topology of the active site are still unavailable. For locating the possible essential groups and the active site, the conserved amino acids of GH-75 enzymes, especially the commonly seen essential amino acids — glutamate (E) and aspartate (D), were sieved on the basis of amino-acid multi-alignment. Five amino-acid sequences deduced from the available *csn* of *A. fumigatus* (this study; GenBank AY190324), *A. oryzae* (13; GenBank AB038996), *F. solani* (12; GenBank D85388), *B. bassiana* (GenBank AY008269), and *M. anisopliae* (GenBank AJ293219) were aligned by CLUSTALW (27). The result is shown in figure 6. The essential groups of this family are likely to locate on ten potential positions (nine Asp and one Glu), which were also conserved on the alignment of five seeds in Sanger's result (http://www.sanger.ac.uk/cgi-bin/Pfam). In general, glutamate and aspartate were substituted with structurally conservative residues, i.e. glutamine and asparagine, respectively, by site-directed mutagenesis. The primers used for mutational studies and the relative activities of all mutants are summarized in table 1. All mutants were over-expressed as inclusion bodies in *E. coli*. The refolding and purification processes were similar to that of wild-type CSN as described in the experimental section.

Activity assay showed that D59N, D76N, D78N, D80N, D112N, D114N, D194N and D229N retained significant catalytic activity (>60 %) compared with that of the wild-type enzyme. These mutants release $(GlcN)_3$, $(GlcN)_4$, and $(GlcN)_5$ as major products with $(GlcN)_2$ in a minute proportion. The pattern of product distribution is virtually identical to that of native CSN (data not shown). Hence, these eight aspartates are unlikely to function as the essential group of *Aspergillus* CSN. In contrast, the activities of mutant D160N and E169Q were significantly reduced (less than 0.1 %). The residues of Asp160 and Glu169 are likely to be the essential groups of CSN for the catalytic activity if the activity loss is not due to structural collapse. To identify the possible structural alteration of D160N and E169Q, we examined CD spectrometric analyses of these recombinant proteins. As can be seen in figure 7, CD spectra of D160N and E169Q are similar to those of recombinant enzymes with significant chitosanase activity including the wild-type CSN, the D191N/D194N double mutant and many others (data not shown), indicating the presence of structural conservation for all mutants. We further constructed the mutants of D160E and E169D and evaluated their catalytic activities. Both mutants possess at least 40 % activity of that of wild-type CSN. Retaining two carboxylic side-chains is clearly crucial for CSN to be active though the catalytic domain might have been distorted by 1-2 Å apart.

The conserved motif of "DCDID" in GH-75 seems less important than what exists in chitinase, in which the conserved motif of

"DXDXE" is the catalytic center of GH-18 chitinase (28). The mutation in this region of CSN caused only 30 % - 40 % loss of activity. The precise function of this conserved motif in chitosanase is unclear at present.

B. CONCLUSION

Though there are several advantages in recombinant protein production with the formation of inclusion bodies, such as higher-level production, no toxic effect, resistance to proteolysis and highly purified form by simple physical operation, the inclusion body predicament is, however, awkward to resolve, and in only a few cases has the biological activity been restored. The native *Aspergillus* chitosanase is atypically stable with good thermostability and with no influence on its enzymatic activity even by urea (8 M) or guanidium-HCl (0.5 M, data not shown). Its molar mass is about 23.5 kDa, but it can percolate through a 10-kDa cut-off membrane filter. Even though it contains six Cys in its amino-acid sequence, there is no free Cys in its native form that can be modified with DTNB. Moreover, this enzyme is composed of 44 basic amino-acid residues (16 Arg, 8 His, 19 Lys and the N-terminus), but a maximum of only 28+ charges was observed in the ESI mass spectrum (as shown in the inset of figure 2). This phenomenon is explicable through the three-dimensional structure of this enzyme by its disulfide bonds and impacted intensity (29). These intrinsic properties may allow the refolding process to be effective.

From mutational studies on ten conserved carboxylic-acid residues, only D160N and E169Q lost chitosanase activity greatly without structural alteration. Hence Asp160 and Glu169 are suggested to be in the catalytic center and essential for the enzymatic activity. In Sanger's alignment of 14 fungal chitosanase sequences (http://www.sanger.ac.uk/cgi-bin/Pfam), three conserved regions were found; these conserved regions include two essential groups, Asp160 and Glu169 in CSN, and the "DCDID" motif. In addition, Asp160 and Glu169 were recognized as catalytic sites *via* theoretical computation with MuSiCME (http://genome.life.nctu.edu.tw/MUSIC; 30, 31). The remaining question is the specific function, general acid and general base, of Asp160 and Glu169. According to statistical data of the glycosyl hydrolase family, when two distinct carboxylic-acid residues collaborate for catalysis, Asp nearly invariably functions as a catalytic base and Glu as a proton donor in the initial step of the reaction. This feature is present in the retaining enzymes from GH-3 (32), GH-13, GH-33, GH-68, GH-71, and GH-77 and inverting enzymes from GH-8 (23), GH-9, GH-25, GH-46 (7,8) and GH-82. In contrast, the case with Asp functioning as a proton donor and Glu as a catalytic base is rare when both residues are the essential groups of a glycohydrolase. Comparison of the relative acidity of Asp and Glu in a similar environment, such as a catalytic core, can also provide information useful for understanding the natural preference. The pKa of Asp is less than that of Glu according to an empirical relationship (33) or theoretical calculations (34); hence Asp is more readily deprotonated than Glu. Consequently, when a catalytic reaction is performed, the deprotonated Asp functions as a catalytic base (or nucleophile in the retaining enzyme) and protonated Glu as a proton donor. Though the Asp160 and Glu169 are likely to function as the general base and the general acid, respectively, in the catalytic center of *Aspergillus* chitosanase, this prediction still requires proof through extensive investigation such as with active-site affinity labeling or from its crystal structure.

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FOOTNOTE

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FIGURE LEGEND

Figure 1. Exposition of procedure for molecular cloning of *csn*. Step 1: construction of cDNA library; steps 2 and 3: amplification of cDNA fragment containing *csn*; step 4: amplification of gDNA fragment of *csn*; step 5: Inverse PCR; step 6: construction of expression system of recombinant chitosanase. Primer pairs used in each PCR are displayed in parentheses.

Figure 2. Nucleotide sequence and deduced amino-acid sequence of *csn* cDNA from *A. fumigatus*. The first N-terminal 17-amino-acid sequence as a signal peptide is underlined and the N-terminal sequence determined for the native purified enzyme is doubly underlined. The asterisk indicates the stop codon.

Figure 3. Analyses of molecular weight by SDS-PAGE (a) and LC-mass (b). SDS-PAGE analysis of the supernatant of cell lysate (lane 1), cell debris (lane 2), supernatant of cell debris treated with 5 M urea (lane 3), and the purified protein (lane 4). The mass spectrum of the purified chitosanase (inset) and the deconvolution of the

spectrum to give molar mass = 23592±2 amu.

Figure 4. ^{1}H-NMR spectra during hydrolysis of chitosan by chitosanase at 35 ℃. The initial substrate is shown in spectrum A. Spectra were obtained over 9, 21, 33, 48 and 60 min in the presence of chitosanase. The signals at δ= 4.90 ppm (*J*=7.8 Hz), and δ= 5.41 ppm (*J*=3.3 Hz) correspond to α- and β-anomers of C1-H of the reducing end sugar. The ratios of α and β anomeric signals are 63:37, 78:22, 73:27, 69:31, 65: 35 and 63:37 in order.

Figure 5. Mass analysis of chitooligosaccharides obtained from enzymatic hydrolysis of low DDA chitosan. (a) Oligosaccharides with m/z 341, 383, 502, 544 and 705 correspond to a chitobiose, a monoacetyl chitobiose, a chitotriose, a monoacetyl chitotriose and a monoacetyl chitotetraose, respectively. ESI/MS/MS analyses of the chemically methylated chitooligosaccharides (b-d) and illustrations of structural fragmentations (e-g). (b) and (e) for the methylated monoacetyl chitobiose (m/z 397), (c) and (f) for the methylated monoacetyl chitotriose (m/z 558), (d) and (g) methylated monoacetyl chitotetraose (m/z 719 (d) and (g).

Figure 6. **Multi-alignment of five GH-75 fungal chitosanases**. Five available gene sequences from *A. fumigatus* (this study; GenBank AY190324), *A. oryzae* (13; GenBank AB038996), *B. bassiana* (GenBank AY008269), *M. anisopliae* (GenBank AJ293219) and *F. solani* (12; GenBank D85388) are aligned by CLUSTALW (Thompson *et al.,* 1994). Ten prospective candidates to function as catalytic essential groups are revealed and boxed.

Figure 7. CD spectra of recombinant CSN. Mutants of D160N (△) and E169Q (□) exhibit the similar CD spectra as other recombinant proteins with significant chitosanase activity: wild type, solid line; D191N/D194N: dot; D160E, dashed line.

Table 1. Primer pairs of synthetic oligonucleotide designed for mutagenes and their chitosanase activity.

Symbol	**Oligonucleotide sequence**	**Mutation sites***	**CSN Activity**
WT		Wild type	**100 %**
D59N-s	5’CAGTTACTGCGGTAACATTCCGGG	D59N	**96 %**
D59N-a	5’CCCGGAATGTTACCGCAGTAACTG		
D80N-s	5’CCAATATGGACATCGACTGCAACGGC	D76N	**65 %**
D76N-a	5’GCCGTCGCAGTCGATGTTCATATTGG		
D80N-s	5’CCAATATGGACATCGACTGCAACGGC	D78N	**62 %**
D78N-a	5’GCCGTCGCAGTTGATGTCCATATTGG		
D80N-s	5’CCAATATGGACATCGACTGCAACGGC	D80N	**77 %**
D80N-a	5’GCCGTTGCAGTCGATGTCCATATTGG		
D112N-s	5’GAAGTTTGGCATCTCCAACCTGGACGC	D112N	**102 %**
D114N-a	5’GCGTTCAGGTCGGAGATGCCAAACTTC	D114N	**109 %**
E126Q-s	5’GGTGTTTGGAAACCAGGATCACTCTC	E126Q	**108 %**
E126Q-a	5’GAGAGTGATCCTGGTTTCCAAACACC		
D160N-s	5’GGAATCTGGGGTAACACTAACGGTG	D160N	**~0 %**
D160N-a	5’CACCGTTAGTGTTACCCCAGATTCC		
D160E-s	5’GGAATCTGGGGTGAAACCAACGGTG	D160E	**88 %**
D160E-a	5’CACCGTTGGTTTCACCCCAGATTCC		
E169Q-s	5’CGTTTCTACCGGCCAAGCCTCCATTTC	E169Q	**~0 %**
E169Q-a	5’GAAATGGAGGCTTGGCCGGTAGAAACG		
E169D-s	5’CGTTTCTACCGGCGATGCCTCCATTTC	E169D	**40 %**
E169D-a	5’GAAATGGAGGCATCGCCGGTAGAAACG		
D191N-s	5’GGTCACAATCCCAATAATGTCCTCTTC	D191N/D194N	**105 %**
D191N-a	5’GAAGAGGACATTATTGGGATTGTGACC		
D229N-s	5’CGATCGGTAACAAACTGGTTGCTGG	D229N	**80 %**
D229N-a	5’CCAGCAACCAGTTTGTTACCGATCG		

Note: The nucleotides indicated in bold font and underlined are the mutation sites and silent mutation sites, respectively. *: all mutational sites are conserved in family 75 except E126 and D191.

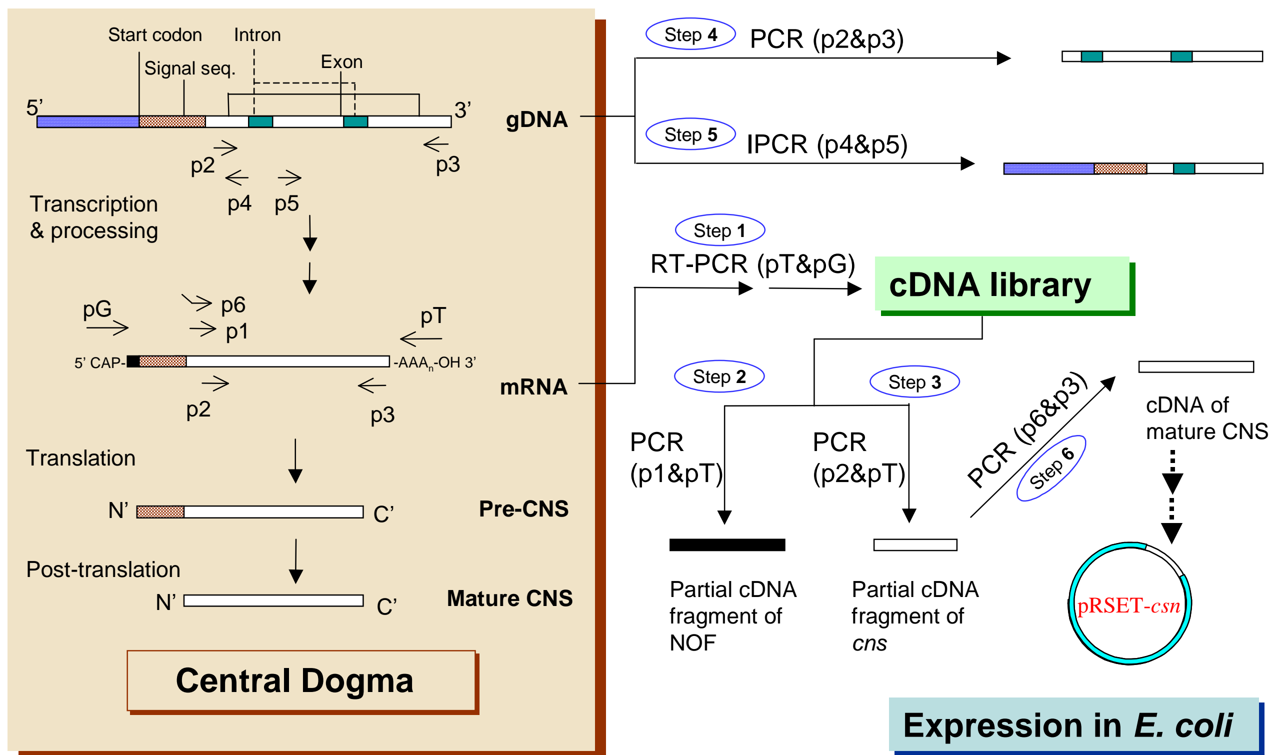

Figure 1.

ATG CGT CTC TCT GAA ATT CTT ACT GTT GCT CTG GTC ACT GGG GCC ACT GCT TAT 54

M R L S E I L T V A L V T G A T A Y

AAT TTG CCC AAC AAC TTG AAA CAG ATC TAC GAC AAA CAC AAG GGA AAA TGT TCC 108

N L P N N L K Q I Y D K H K G K C S

AAG GTA CTG GCA AAA GGG TTC ACC AAT GGT GAT GCT AGC CAA GGC AAG TCT TTC 162

K V L A K G F T N G D A S Q G K S F

AGT TAC TGC GGC GAC ATC CCG GGT GCC ATT TTC ATC TCC TCC TCC AAG GGG TAC 216

S Y C G D I P G A I F I S S S K G Y

ACC AAT ATG GAC ATT GAC TGC GAC GGC GCC AAC AAC TCC GCC GGC AAG TGC GCC 270

T N M D I D C D G A N N S A G K C A

AAC GAC CCG TCC GGC CAG GGC GAG ACT GCC TTC AAG TCC GAC GTG AAG AAG TTT 324

N D P S G Q G E T A F K S D V K K F

GGC ATC TCC GAC CTG GAC GCC AAC ATC CAC CCC TAT GTG GTG TTT GGA AAC GAG 378

G I S D L D A N I H P Y V V F G N E

GAC CAC TCT CCC AAG TTC AAG CCC CAG TCA CAT GGC ATG CAG CCA TTG AGT GTT 432

D H S P K F K P Q S H G M Q P L S V

ATG GCT GTC GTG TGC AAT GGC CAA CTG CAT TAC GGA ATC TGG GGT GAC ACC AAC 486

M A V V C N G Q L H Y G I W G D T N

GGT GGC GTT TCT ACC GGC GAA GCC TCC ATT TCT TTG GCC GAC CTT TGC TTC CCC 540

G G V S T G E A S I S L A D L C F P

AAC GAG CAT CTC GAT GGC AAC CAT GGT CAC GAT CCC AAT GAT GTC CTC TTC ATT 594

N E H L D G N H G H D P N D V L F I

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G F T S K D A V P G A T A K W K A K

AAT GCG AAA GAA TTC GAG GAC AGT ATC AAG TCG ATT GGT GAC AAG CTG GTT GCT 702

N A K E F E D S I K S I G D K L V A

GGT TTG AAA GCA TAG CGA ACT AGA ACA GAT CGA AGA TAG CTG TGG CAG GGT CTT 756

G L K A *

GCT GTT GCA ACC ATA TTT TCG TGC ACA CAA TCG GAG GCG CAG TAC ATA GGA GTG 810

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AAA AAA AAA AAA 3'

Figure 2

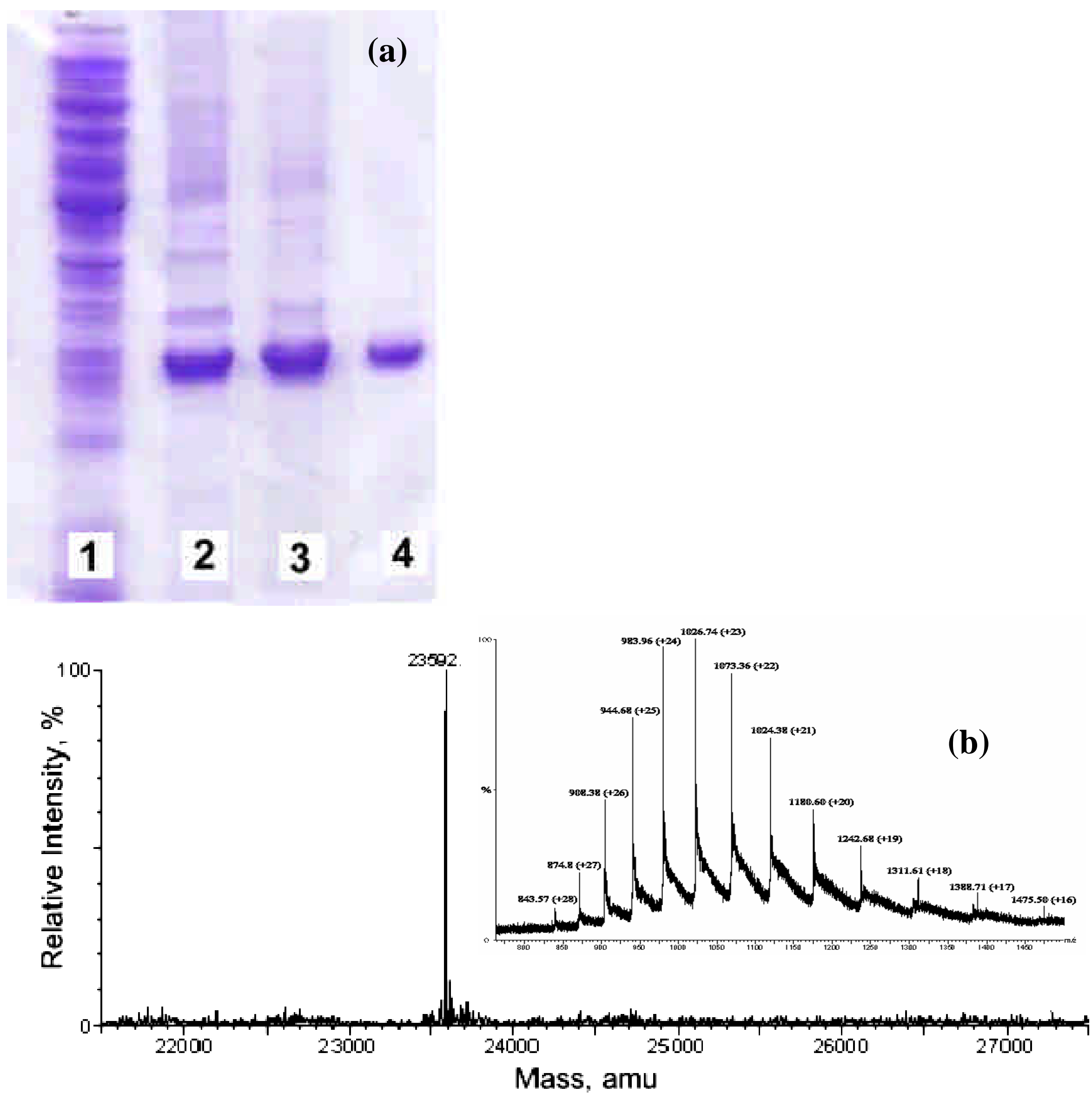


Figure 3

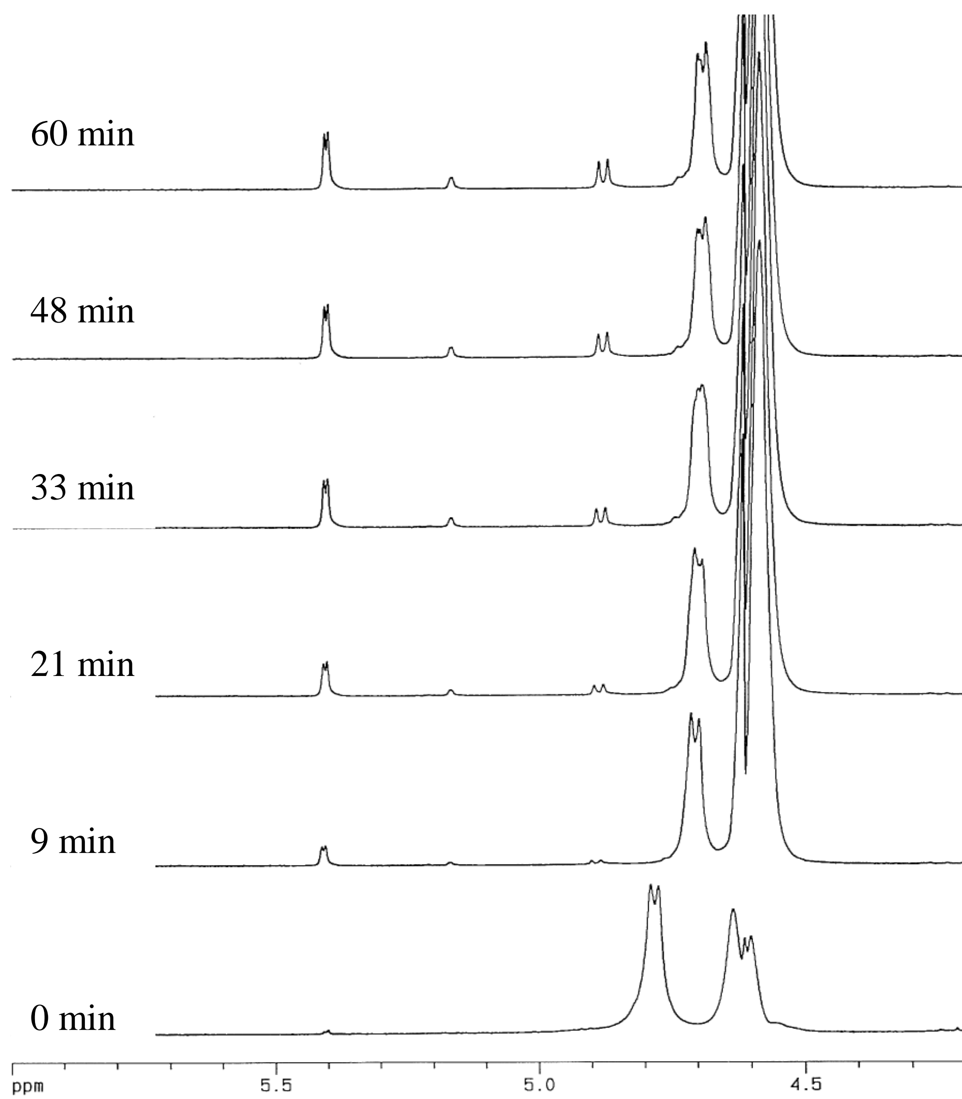
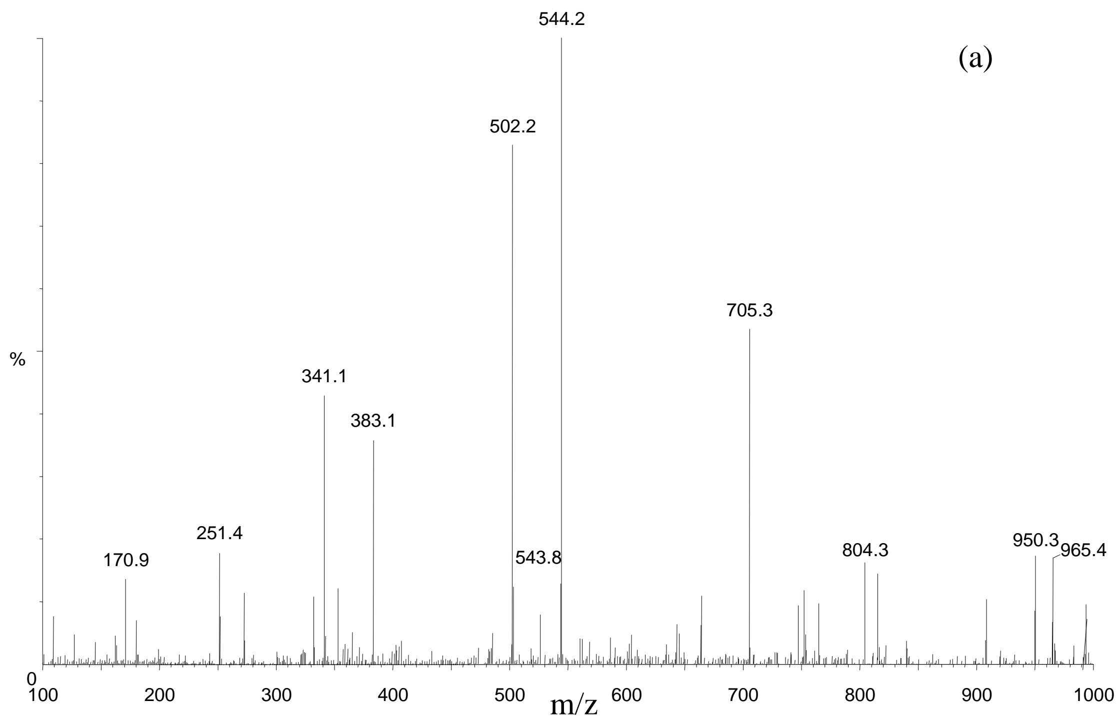


Figure 4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705.3
341.1
383.1
251.4
170.9
543.8
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%
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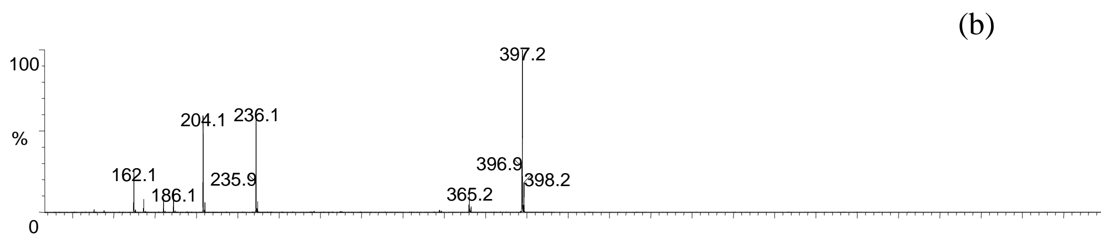
100
200
300
400
500
600
700
800
900
1000
m/z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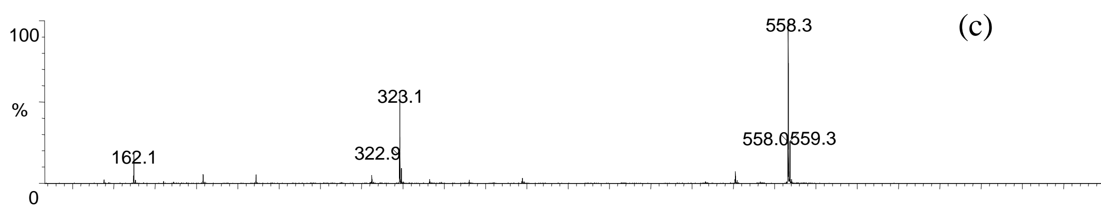
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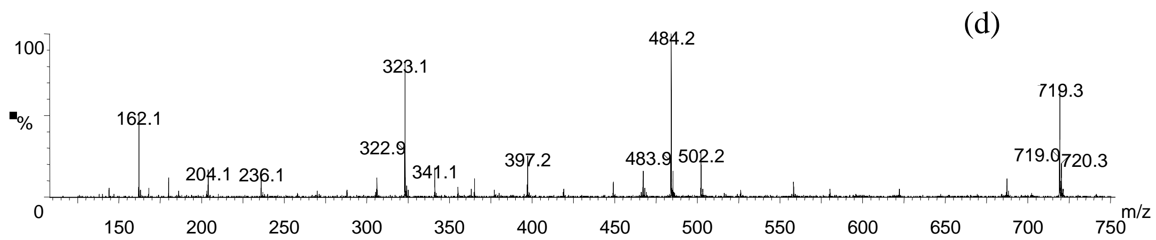
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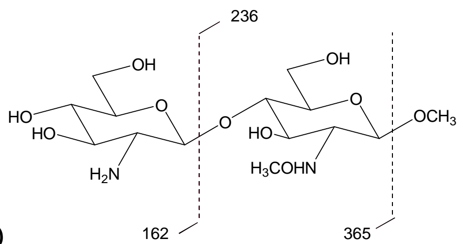
(e)



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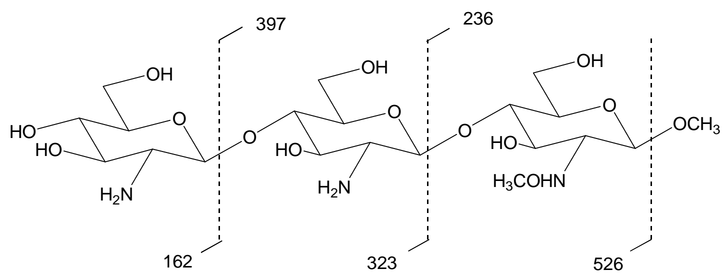

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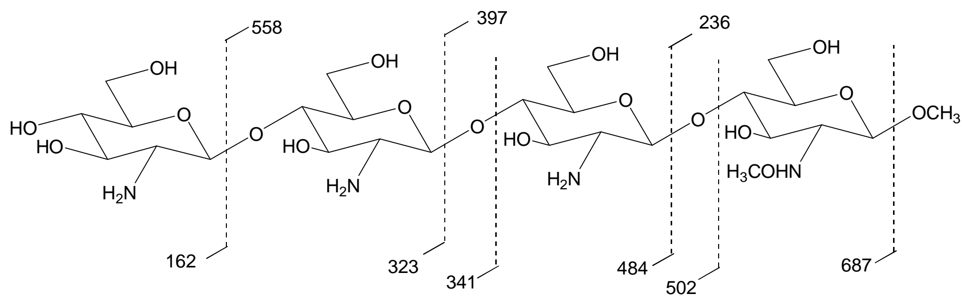

Figure 5

[*A. fumigatus*] 47 DASQGKSFSYCGDIP---GAIFISSSKG---YTNMDIDCDGANNS---AGIS--DLDANI 117
[*A. oryzae*] 48 HSHDGKSFSYCGDIP---NAIYLHSSKNGGQYADMDIDCDGANRH---AGID--DLDANI 121
[*B. bassiana*] 50 AS-------YCGDIP---NAIFLKG-NG--TYDNMDIDCDGVNRS---AGIP--DLDANV 113
[*M. anisopliae*] 49 AT-------YCGDLP---NAIFLKGSNG--NYDNMDIDCDGANNS---AGIP--DLDANL 113
[*F. solani*] 51 DGDSG-TYSYCGDHVKDYNVIYLQGKNG--KLVNMDIDCDGVQGSPADDGTSQKDLDANI 129

[*A. fumigatus*] 158 WGDTNG----GVSTGEASISLADLCFPNEHLDGNHGHDPNDVLFIGFTSKDAVPGAT-AKW 213
[*A. oryzae*] 165 WGDTNG----HTATGEASLSMAELCFPEEDPSGDSGHEPNDVLYIGFTGKEAVPGKS-ADW 220
[*B. bassiana*] 154 WGDVNG----GVLTGEASLSMAKLCFPDEPLSGDNGHDAKDVMYIAFTGNDTVPGKDGADW 210
[*M. anisopliae*] 154 WGDTNG----FTSTGEASLALGKLCFPNEGLSGDNGHDPKDVHYIGFTEGDTVPGKSGANW 210
[*F. solani*] 173 WGDENGDDGDQPMVGEASISLATACFG-KSMNGNFGHSDDDVLYIAFPGADAVPGAKGAKW 232

[*A. fumigatus*] 214 KAKNAKEFEDSIKSIGDKLVAGL 236
[*A. oryzae*] 221 KADSTESFEESIKELGDKLVAGL 243
[*B. bassiana*] 211 SAKNTEKFAKSIKCLGDKLVDRL 233
[*M. anisopliae*] 211 KAKKTADFEASIKALGDKLVARL 233
[*F. solani*] 233 NAKNFDEFQTSITSLGDKLIKRI 255

Figure 6

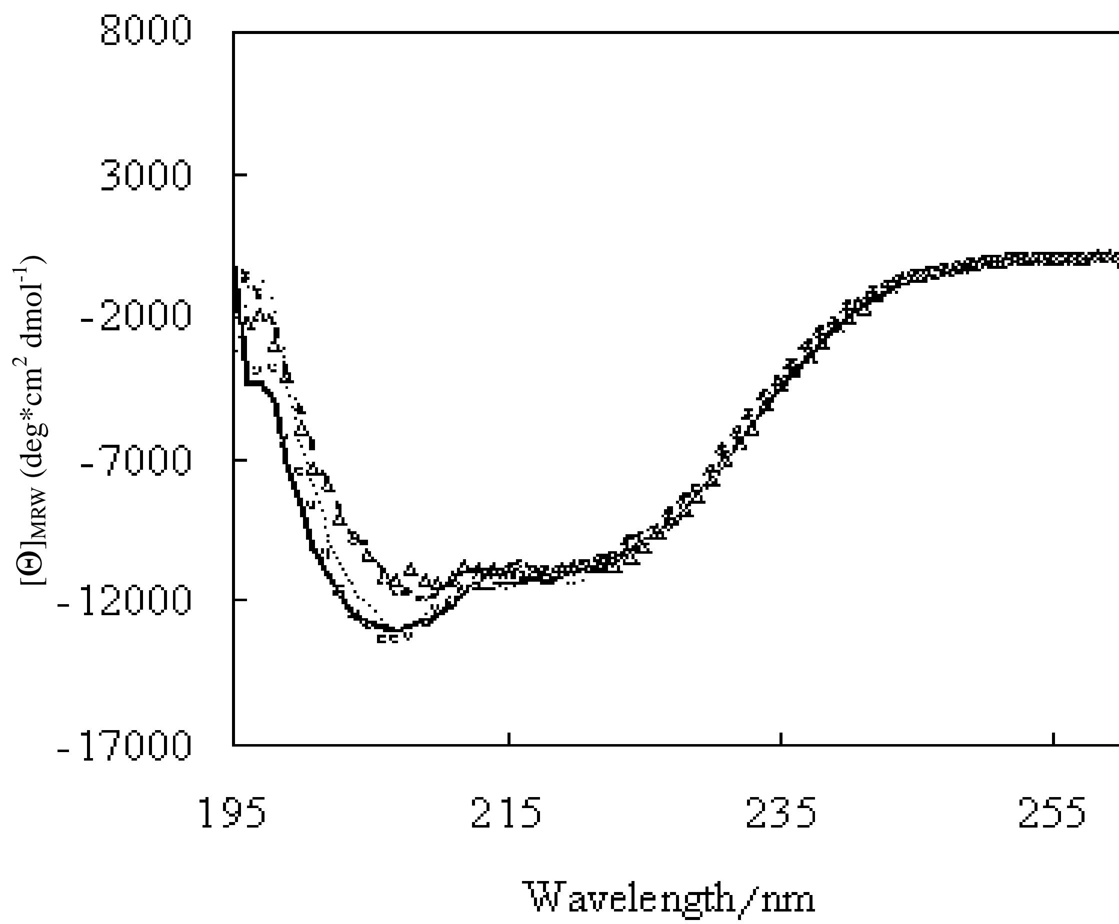

Figure 7