摘要

幾丁聚醣水解酵素(chitosanase)是煙麴菌*(Aspergillus fumigatus)*中擁有極高工業應用性的酵素，屬於黴菌幾丁聚醣水解酵素所屬的 GH-75 家族。煙麴菌的幾丁聚醣水解酵素基因被克隆出且置於大腸桿菌中表現並定性。水解產物利用核磁共振光譜以及質譜定序方法，證實此酵素是以反轉機制水解以及切 GlcNAc-GlcN 和 GlcN-GlcN 鍵而不切 GlcNAc-GlcNAc 和 GlcN-GlcNAc linkages 鍵。重組的酵素在大腸桿菌中以內含體(inclusion body)形式表現，利用 5M 尿素可救回 35%的活性。另外，此酵素也在另一訊息胜肽表現系統中以胞內可溶蛋白質形式表現。將 GH-75 家族中五條幾丁聚醣水解酵素序列作比對，篩選十個保留度最高的胺基酸做定點突變。因為 D160N 與 E169Q 明顯失去活性且其他突變點仍保有 40%以上活性。而利用 circular dichroism 證明不是因為二級結構的改變造成。另外，由理論計算方式也的到相同預測結果。而表面電漿共振儀證明 D160N 與 wild type 對於幾丁聚醣的三醣與四醣有相同的親和特性。這些結果證明 Asp^{160} 與 Glu^{169} 在此酵素中扮演重要的催化角色。最後，由多方資料整合歸納出 Asp^{160} 是 catalytic base 而 Glu^{169} 是 proton donor。

Abstract

A powerful chitosanase for the preparation of chitooligosaccharide was previously purified from *Aspergillus fumigatus*. The corresponding gene was also cloned and the enzyme was further classified into glycosyl hydrolase family 75. 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. Alternatively, the recombinant enzymes were also expressed in a pRSET_SP system containing a signal peptide. The recombinant chitosanases were found to produce as soluble protein intracellularly. 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. Surface plasma resonance (SPR) studies revealed that the binding properties of D160N and the wild type enzyme with either chitotetramer or chitotrimer are comparable. We conclude that Asp160 and Glu169 are the two essential residues of *A. fumigatus* chitosanase.

Content

Chart Content

Chapter 1 Introduction

1-1 Chitosan oligosaccharide

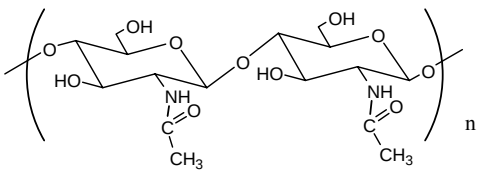
1-1-1 Chitosan/chitin

Chitosan, a linear polymer composed of β-1,4-linked glucosamine (GlcN) residues with various degrees of N-acetylated residues, is obtained by deacetylation of chitin. Chitin, a linear, cellulose-like polymer of N-acetylglucosamine (β-1,4 glucosidic linkage of 2-deoxy-2-acetoamino-D-glucose, GlcNAc) is estimated at approximately 10^9 tons annually and is the second most abundant biopolymer (Jeon *et al.,* 2000).

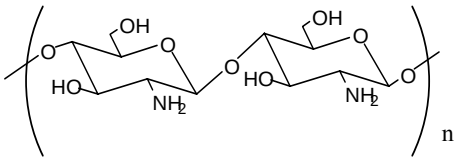
Chitosan is obtained by removing N-acetyl groups of chitin and becomes soluble in some dilute acids. There are two ways to obtain chitosan. One is deacetylation of chitin, this deacetylation process releases amino group and gives the chitosan a cationic character. The other is extraction from natural sources (Suntornsuk *et al.,* 2002).

Chitosan is not only inexpensive, non-toxic, and biodegradable, but it also possesses bioactivity and antibioticity.

Application of the materials and derivatives prepared from chitin/chitosan is very promising in various fields including medicine, pharmacology and food industry as a result of their biological activity, biocompatibility and biodegradability in combination with its low toxicity.



Chitin

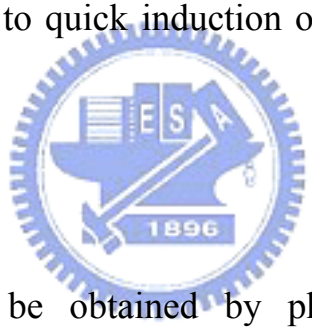


Chitosan

1-1-2 Chitosan oligosaccharide

Introduction

Chitosan has developed as new physiological materials. Though having promising properties in many fields, the applications of chitin/chitosan polysaccharides are somewhat limited for their high molecular weight, which results in low solubility in neutral aqueous media (Jeon *et al.,* 2000; Ilyina *et al.,* 2000). However, increasing attention has aroused in converting chitin and chitosan to their oligosaccharides (Zikakis, 1984). Especially in medicine and food industry, for example, a low molecular weight (LMW) chitosan with high solubility in acid-free solution and low viscosity enables efficient absorbability in the intestine, and leads to quick induction of systematic medicinal effect on organisms.



Preparation

Chitosan oligomers can be obtained by physical decomposition method, chemical hydrolysis, enzymatic methods or fermentation (Zikakis, 1984). The most prevalent method is enzymatic hydrolysis from chitosan/chitin polymer. As shown in literatures, several commercial enzymes, such as lipase (Ilyina *et al.,* 2000), protease (Zhang *et al.,* 1999), papain, cellulase, lysozymes (Rhoades *et al.,* 2000), α-amylase, glucanase, and bromelaid (Hung *et al.,* 2002), have been found to possess chitosanase activity. Though the chitosanase activities of these enzymes are low, they are occasionally used for chitooligosaccharides prepared from chitin oligomers, which is obtained from the enzymatic hydrolysis of chitin by chitinase, following by the catalysis of chitin deacetylase (Aiba, 1994).

Compared with the enzymes above, chitosanase is believed to be the most suitable candidate for chitooligosaccharides preparation. It specifically catalyzes the hydrolysis of chitosan to yield oligosaccharides with higher degree of polymerization (DP). For lowering the enzyme cost, immobilized-enzyme is often used in industrial processes.

Many investigations had been reported for preparation of specific chitosan oligosaccharide by enzymatic hydrolysis. For example, a powerful chitosanase for the preparation of chitooligosaccharide was previously induced and purified from *Aspergillus fumigatus* (Cheng *at el.,* 2000) that will be described in 1-4.

Properties

The functional properties of chitooligosaccharides are greatly dependent upon their molecular sizes (Jeon *et al.,* 2000). Generally speaking, comparing with their original polymers, the significant benefits of oligosaccharides include: (1) lower molecular weights; (2) simple component which makes it simple to identify the accurate executed motif; (3) extensible material which allows many kinds of modifications to get various derivative matter to be adequate for diverse applications.

Application

Amino sugars and their derivatives represent an important group of biologically active compounds influencing the immune system of mammals and the protection system of plants. Also, glucosaminyl- oligosaccharides possess many biological functions. One of the most interesting properties is anti-tumor effect (Suzuki *et al.,* 1986) (Zhang *et al.,* 1999) against murine cancer cells (Seo *et al.,* 2000), sarcoma-180 (Suzuki *et al.,* 1986), MM-46 and Meth-A solid

tumors in mice and afterward cancer chemopreventive potential (Zhang *et al.,* 1999). Other important specific biological activities of chitosan oligosaccharides include anti-microbial (anti-fungi/bacteria) activity (Jeon *et al.,* 2001; Choi *et al.*, 2001; Rhoades *et al.,* 2000; No *et al.,* 2002), disease-resistance response and as phytoalexin elicitor in higher plant (Zikakis *et al.,* 1984; Muzzarelli *et al.,* 1985), immune-stimulating function, antimutagenic activities (Nam *et al.,* 2001), intestine functions and enhancement of protective effects against infection with some pathogens in mice (Jeon *et al.,* 2000). The other important biological activity is a hypo-cholesterolemic effect that makes chitosan oligosaccharides prophylactic agents for liver diseases (Kim *et al.,* 1998-1) (kim *et al.,* 1999), atherosclerosis and hypertension. These properties permit chitooligosaccharides and their derivatives to make new contributions to pharmaceutical industries, food application, agricultural bio-control, cosmetic and other related fields. For example, chitosan oligomers have been used as moisturizing agents, food and feed additives, waste water treatment agents, fertilizers, seed coating fungi stat (Rivas *et al.,* 2000), wound-healers, blood anticoagulants, hemostatic materials, alcohol intoxication reducer (patent No. KR 142373, March 31, 1998) and diabetic mellitus.

With these tremendously valuable applications, chitooligosaccharides and its related degrading enzyme – chitosanase are proved to be very promising for development.

1-2 Chitosanase

1-2-1 Introduction

Chitosanase (EC 3.2.1.132), hydrolytic enzyme, acts on β-1,4-glycosidic linkage of chitosan. By the way, chitosanase is more restrictively defined as the enzyme attacks chitosan but not chitin (Kimoto *et al.,* 2002). Since the hydrolytes of chitosan have high biological activities and value, hydrolysis method in chitooligosaccharides industry, especially hydrolysis by chitosanase, is worthy more and more attention and exploitation.

Source

Many organisms have been found to possess chitosanase activity (Somashekar *et al.,* 1996) as diverse as viruses (Monaghan *et al.,* 1973; Sun *et al.,* 1999; Yamada *et al.,* 1999), bacteria, fungi and plants (Osswald *et al.,*1994). Molecular weight of this kind of enzymes is between 10~50 kDa, and monomer and dimer conformations have been observed. Most of chitosanase in fungi and bacteria are extra-cellular enzymes (Grenier *et al.,* 1991); and those in plants are intra-cellular (Shimosaka *et al.,* 1993). Some species secrete not only chitosanases but also one or more chitosan-degrading-related enzyme, such as chitinase and exo-β-D-glucosaminidase.

Functions of chitosanase in ecological system

Since chitosan acts as protective supporting material in some organisms, this will be the clue to establish the physical bio-function of chitosanase. These functions can be summarized as defensive equipment, a digesting subsistence, and a weapon for intrusion, in plants, marine bacteria and virus, respectively.

Paradoxically, some fungi have chitosan-like cell walls and simultaneously possess the chitosanase activities. The explanation of this phenomenon is that fungi use chitosanase to decompose their own cell walls when cell division and autolysis occur.

Additionally, chitosanases are pathogenesis-related proteins with lytic activity against fungal spore in some plants (Grenier *et al.,* 1990). Pathogenesis-related (PR) proteins are host-encoded proteins induced by some pathogens or certain types of stress (for review, see Carr and Klessig 1989). The potential antifungal activity of the various forms of chitosanases from intercellular fluids (IFs) of barley leaves, cucumber cotyledons, and tomato leaves had been evaluated with purified enzymes against fungal pathogens.

Since there is no report shown the existence of chitosanase in mammalian cells so far, we can expect that chitosan-degrading enzymes or bacteria may be present in part of the digestion system, especially for those animals who subsist on insects. Besides the digestive function of chitosanase, a few peculiar questions about the physical functions of this enzyme are remained of interest.

Applications

Because of the powerful catalyst for chitosan degradation, chitosanase can be used as an agent for generation of fungal protoplasts, as a fungicide (Selitrennikoff, 2001), as an insecticide for biological control of plant pathogens, and as a pathogenesis-related protein (Zikakis *et al.,* 1984; Muzzarelli *et al.,* 1985). Currently main application of this enzyme is focused on the chitooligosaccharide preparation, for which the down stream application, such as carbohydrate drug development, can be evolved.

Some other uses, such as medicine, will be developed in the future. It is because of the prevalence of immuno sufficient disease that the diseases caused by fungus become serious. Therefore, to utilize chitosanase as an anti-fungal drug can be excepted soon.

Moreover, gene of chitosanase is also useful in science and agriculture. Since some pathogenesis-related proteins are chitosanase in some plants, chitosanase gene cloned from those plants can be transfected into other economical crops to against fungal spores.

Endo- and exo-chitosanase

According to the acting position of substrates and the proportion of DP of products, chitosanase can be divided into two main classifications.

Endo-chitosanase randomly hydrolyzes chitosan into many different lengths of chitooligosaccharides. Endo-chitosanase hydrolyzes slowly when the substrate is smaller than chitosan heptamer.

Exo-chitosanase gradually degrades chitosan from one side, often from the non-reducing end; and releases single length of chitooligosaccharides, chitosan monomers or dimers. Actually, in paper research we still did not find the example of exo-chitosanase which releases chitosan dimers. However, there is one specific type of exo-chitosanases, exo-β-D-glucosaminidase, which is capable of cleaving glucosamine from chitosan dimer or oligomer (Nanjo *et al.,* 1990). Besides, there are some exo-β-D-glucosaminidases have been reported to possess the transglycosylation activity (Shuichi *et al.,* 1999). Transglycosylation is defined as transferring chitosan dimer onto tetramer or

pentamer. In the initial stage of transglycosylated hydrolysis, producing higher DP of chitooligosaccharides will be obtained.

1-2-2 Families

Families of glycohydrolases (GH family)

International union of Biochemistry classifies and sets up the nomenclature according to catalysis types and specificity to substrates of enzyme. Hydrophobic cluster analysis and amino acid sequence relatedness of the catalytic domain are used initially to classify glycohydrolases into families. Amino acids sequence relatedness of its catalytic domain is usually sufficient now to assign an enzyme to a family (Warren, 1995). Up to date, glycohydrolases are classified into 99 families by the related catalytic domain of amino acid sequences (Coutinho *et al.*, 1999).

Family distribution of chitosanase

Although chitosanase are widely distributed in many organisms, only less than 50 chitosanase genes in microbes have been cloned and sequenced. According to their amino acids similarities, these chitosanase are classified into GH 5, 8, 46, 75, and 80. The classification results of study these families are shown as table 1-2-1.

Table 1-2-1 Family distribution of chitosanase

Family	Mechanism	Catalytic Nucleophile / base	Catalytic proton donor	3D structure
5	Retaining	Glu (experimental)	Glu (experimental)	Available Fold $(\beta/\alpha)_8$
8	Inverting	Asp (inferred)	Glu (experimental)	Available Fold $(\alpha/\alpha)_6$
46	Inverting	Asp (experimental)	Glu (experimental)	Available Fold α/β
75	Inverting	Unknown	Unknown	Not available
80	Inverting	Unknown	Unknown	Not available

1-2-3 Mechanism and substrate specificity

Inversion and retention

In general, glycohydrolases cleave the glycosidic bond of the substrate by two different mechanisms: retention and inversion of the anomeric configuration. The well-known mechanisms of glycosidases are using illustrated in figure 1-2-1. Besides, by inspecting and comparing the x-ray structures of these two types of enzymes, the different distances between two essential groups and substrate are observed.

Inverting catalysis is a one-step, single-displacement mechanism with the participation of a general acid and a general base. The general base polarizes an H_2O molecule to develop a stronger nucleophile for attacking the anomeric

carbon, while the general acid protonates the glycosidic oxygen to accelerate the reaction. The distance between two essential groups in the activity cavity is about 9.5~10.5 Å. The larger cavity in the active site permits an H_2O molecule to allocate near the general base residue and to be activated so that the H_2O molecule attacks the anomeric center of substrate directly. The enzymatic products with inverting configuration, β-form to α-form or α-form to β-form, are obtained.

Retaining catalysis is via two-step, double-displacement mechanism. Two essential amino acid residues, one functioning as the nucleophile and the other as the general acid/base, are involved. In the first step (glycosylation step), the nucleophile attacks at the anomeric carbon of glycoside, whereas the general acid/base catalytically protonates the glycosidic oxygen, thereby assisting the leaving of the aglycon moiety. In the second step (deglycosylation step), the breakdown of the glycosyl enzyme intermediate proceeds from a general base-catalyzed attack of H_2O at the anomeric center to release glycol moiety with the anomeric configuration similar to that of reactant. Transglycosylation activity is often seen with the catalysis of retaining enzyme, whereas, it is absent in the case of inverting enzyme reaction. The distance between the essential groups of the activity cavity with substrate shown in figure 1-2-1 is about 5.5~6.5 Å. The enzymatic products with retaining configuration, β-form to β-form or α-form to α-form, are obtained.

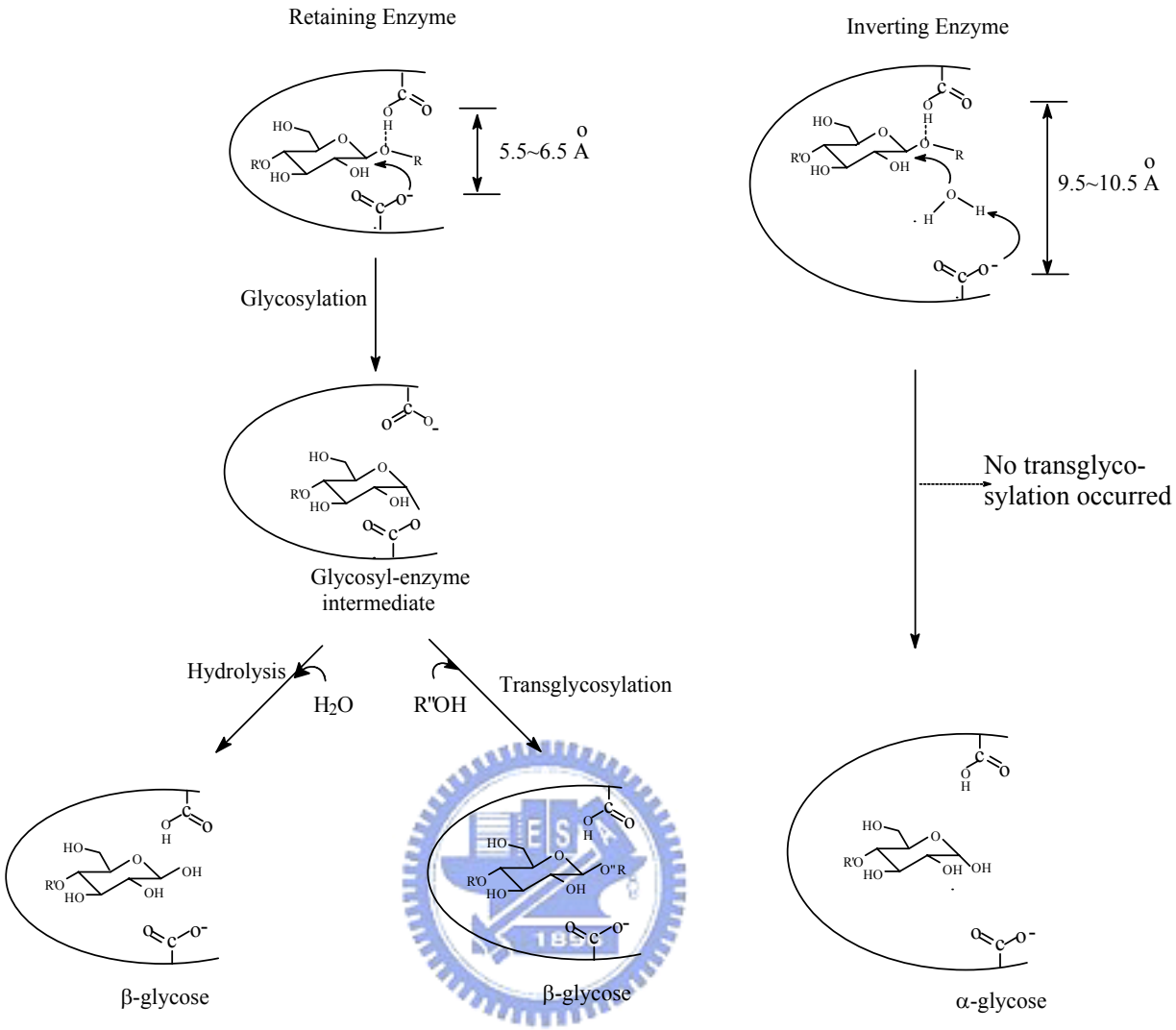


Figure 1-2-1 The mechanistic action of glycohydrolases cleaving the glycosidic bond of the substrate in retention and inversion of the anomeric configuration.

Subsite

The subsite nomenclature is proposed by Davies *et al.* (Davies *et al.,* 1997). It is because the rapid growth of known three-dimensional structures of glycosyl hydrolases that has been accompanied by a diverse and disparate array of nomenclature for the labeling of their sugar-binding subsites. Two criteria are essential: (1) indication of the position of the subsite relative to the point of cleavage; (2) no change in the subsite labeling when new complexes, with extra sugar units at either the reducing or the non-reducing end.

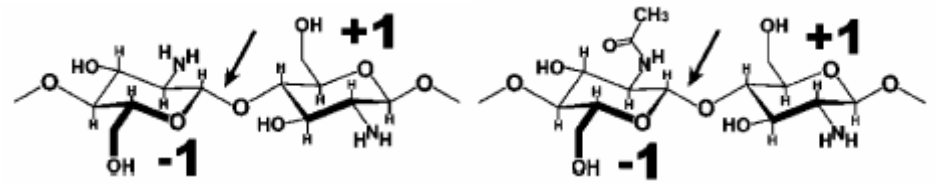
The structural-biology community are adopted the -*n* to +*n* subsite nomenclature widely used by molecular enzymologists. Subsites are labeled from -*n* to +*n* (where *n* is an integer). The -*n* represents the non-reducing end and the +*n* the reducing end, with cleavage taking place between the -1 and +1 subsites.

The subsite character of endochitosanase can be identified by interacting with chitosan hexamers and by their hydrolytes if there is no X-ray crystal structure (Ueda *et al.,* 2003). There are two main types, [(-3), (-2), (-1), (+1), (+2), (+3)] and [(-2), (-1), (+1), (+2), (+3), (+4)] (Fukamizo *et al.,* 2000). The former is acting on the center of chitosan hexamer and releases only chitosan trimers; the latter binds to the first two units of chitosan from the non-reducing end and releases chitosan dimers and tetramers.

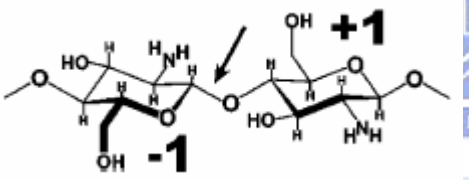
Subclass

Based on the substrate specificity, chitosanases have been classified into three subclasses (Adachi *et al.,* 2004). In each subclass, chitosanases commonly hydrolyze the β-1,4-linkage when both -1 and +1 sites are occupied by glucosamine residues.

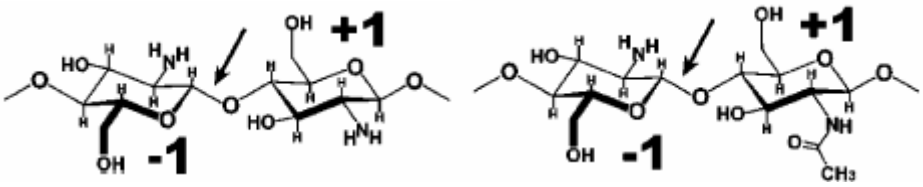
Subclass Ⅰ chitosanase presents a catalytic power on hydrolysis of the β-1,4-linkages of both GlcN-GlcN and GlcNAc-GlcN



Subclass Ⅱ chitosanase can split only GlcN-GlcN



Subclass Ⅲ chitosanase can split GlcN-GlcN and GlcN-GlcNAc.



None of these three subclasses can split GlcNAc-GlcNAc.

1-3 *Aspergillus fumigatus*

Chitosanase described in this study originates from *Aspergillus fumigatus*. The molds such as *Aspergillus fumigatus* are filamentous fungi. They are especially prevalent growing on the nonliving organic materials in the soil. They disperse their non-sexual spores called conidia in the air. Up to now, all chitin/chitosan related enzymes in *A. fumigatus* discovered are endo-chitosanase (cheng *et al.,* 2000), exo-β-D-glucosaminidase (Kim *et al.,* 1998-2; Eom *et al.,* 2003), exo-chitinase and endo-chitinase (Xia *et al.,* 2001). These enzymes have roles during growth and morphogenesis in *A. fumigatus* and other fungi.

As one of the most ubiquitous of airborne saprophytic fungi, conidia of *A. fumigatus* are continuously inhaled by humans but rarely have any adverse effects as they are eliminated efficiently by the innate immune response (Latgé *et al.,* 2001). However, exposure to *A. fumigatus* still can cause an allergic response in sensitive individuals. More importantly, *A. fumigatus* is an opportunistic pathogen of bone marrow transplant patients, AIDS patients, and other immune compromised individuals caused by disease, drug therapy or genetic conditions.

A. fumigatus is the most common mold causing infection worldwide. The first infection described in human, an aspergilloma, was reported in 1842 (Bennett, 1842) and then many cases of invasive disease in non-immunocompromised patients were reported. These cases and more recent epidemiological data emphasize that *A. fumigatus* is a primary, albeit rare, pathogen of human. Allergic disease due to *Aspergillus* was first described in 1952 (Hinson *et al.,* 1952) and the first invasive (and fatal) infection in an immunocompromised patient was described in 1953 (Rankin, 1953).

There are drugs to treat fungal infections. These drugs can have some nasty side-effects because they are often toxic to people as well as to fungi. Also, several fungi are now resistant to the available drugs and there is a desperate need for new and better antifungal agents. As a result, several investigations on enzymes secreted from *A. fumigatus* have been carried out to understand the pathogenesis associated with *A. fumigatus* infection (ex: Xia *et al.,* 2001; Jaques *et al.,* 2003). These enzymes will become the candidates of anti-fungal drugs in the future. Moreover, chitin is a major structural component of the cell wall of all fungi pathogen for humans and absent from mammals. Therefore, enzymes of chitin metabolism are thought to be potential targets for novel antifungal agents. However, it is believed that chitosanase can also serve as a candidate of antifungal drug.

1-4 *Escherichia coli* expression system of recombinant chitosanase from *Aspergillus fumigatus*

Chitosanase described in this study originates from *Aspergillus fumigatus* isolated from soil by our lab and screened by using chitosan as a sole carbon source (Cheng *et al.,* 2000). To distinguish and simplify, the native type of chitosanase investigated in this study is called *Aspergillus* chitosanase for short; the chitosanase which is reconstructed into *E. coli.* and then mutated by gene engineering is called recombinant chitosanase for short.

Aspergillus chitosanase, sequence shown in figure 1-5-1, is classified into Family 75, with a calculated molecular weight of 23462 Da and is confirmed to act as an inverting configuration by previous work in our lab (Cheng, 2003).

No matter incubation, purification and investigation an enzyme which secretes from fungi are very complicated and difficult. Therefore, a cDNA library of *Aspergillus fumigatus* was constructed and the correspondent chitosanase gene was cloned and sequenced in our lab by Dr. Cheng (Cheng, 2003). In summary, the genomic *Aspergillus* chitosanase consists of 866 base pairs with 2 introns (67 and 82 bp), and the 717 base pairs of ORF containing a 17-amino acid signal peptide leading to a mature protein comprised of 221 amino acids with calculated molecular weight of 23462 Da. The genetic information had been published in GenBank with the accession number *AY190324*. The gene was further cloned into pRSET A vector within the *Nde* Ⅰ and *Bam*H Ⅰ cutting sites, and named as pRSET/csn. The recombinant chitosanase is expressed in *E. coli.*, but secreted and aggregated as inclusion bodies. Subsequently, to avoid the folding controversy in inclusion bodies some important mutants' plasmids

are inserted a 23 amino acids signal peptide sequence before the start codon of chitosanase. Afterward, chitosanase is secreted as extra- or intra-cellular protein in different *E. coli.* strains.

```
5'  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
    GGC TTC ACT AGC AAG GAC GCC GTG CCT GGA GCG ACT GCC AAG TGG AAG GCA AAG  648
     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
    GGT AGT GTA GAA GAA TCT TAC TTT TTC TGT ACT TCA AAA AAA AAA AAA AAA AAA  864
    AAA AAA AAA AAA 3'
```

Figure 1-3-1 The nucleotide and the deduced amino acid sequences of *csn* from *A. fumigatus* Y2K. The first N-terminal 17-amino-acid sequence (underlined) is a signal peptide. The asterisk indicates the stop codon.

1-5 The objective of this study

Many potential applications of chitosanase will largely rely on the research progress on the subject of the genetic-engineering modification of chitosanase for improving its specific product-splitting pattern and transglycosylation activity. Therefore, we attend to make a thorough inquiry of *Aspergillus* chitosanase including finding out its essential amino acids and to identify them. After that, we also employ essential genetic-engineering methods to create slightly different chitosanase to set up an enzymatic method of producing specific chitosan oligosaccharide, especially chitosan hexamer.

In the future, chitosanase might be utilized in clinical treatment because of the infection disease caused by fungi.

Chapter 2 Material and Method

2-0 General description

Material and chemical

1. *Escherichia coli* strains

   XL1-Blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*)
   (Stratagene, USA)

   BL21-DE3 (*E. coli* B, F^-, *ompT, hsdS*B(r^- m^-), *gal dcm* (DE3))
   (NOVAGEN, USA)

   ECOS21 (*E. coli* B, F^+, *dcm, omp*T, *hsd*S($r_B^-r_B^-$), *gal*λ (DE3))
   (Yeastern, Taiwan)

   JM109 (e14–(McrA$^-$), *recA1, endA1, gyrA96, thi-1, hsdR17*(r^- m^+)
   supE44 relA1.(*lac-proAB*))
   (Stratagene, USA)

2. The chemicals of incubation media were purchased from pronadisa and Difco.
3. The chemicals of activity analysis and modification were Purchase from Sigma and Aldrich.
4. Buffer for purification and dialysis were purchased from Showa and Merck.
5. Enzyme for molecular biology experiment including restriction enzymes, polymerase, and ligase were purchased from Merck, NEB, and Roche.
6. The chemical for SPR were purchased from BIAcore and Sigma.
7. HiTrap Desalting column (Pharmacia)
   HiTrap Sp column (Pharmacia)
   HiTrap Q column (Pharmacia)
8. VIVASPIN 20mL concentrator (VIVASCIENCE)

Instrument

1. Shaking incubator (Firstek, Scientific, orbital shaking incubator Model S302R)
2. High speed centrifuge (Kubata 7820)
3. FPLC system (Waters 650 E advanced protein purification system)
4. Fraction collector (ISCO)
5. Sonicator (MISONIX)
6. Centrifugal evaporator CES-series (PANCHUM)
7. Freeze dryer CT-series (PANCHUM)
8. Pump
9. PCR (PROGRAM TEMP CONTROL SYSTEM PL808) (ASTEK)
10. DNA electrophoresis (MP-100) (MAJOR SCIENCE)
11. ESI-Q-TOF (Micromass)
12. SPR (BIAcore X)
13. UV spectrum (HP 8452A)
14. Ultra microplate reader (EL 808) (BIO-TEK INSTRUMENTS,INC)
15. Fluorescence spectrophotometer (F-4500) (HITACHI)
16. Circular Dichroism spectrometer (Jasco J-715 spectropolarimeter)
17. NMR (VARIAN UNITYINOVA 500 NMR)

2-1 Site-directed mutagenesis

Principle

The site-directed mutagenesis is performed by QuikChange™ method which is developed by Stratagene. Using PCR with the parental vector as template, and two synthetic oligonucleotides containing the desired mutation as primers, site-directed mutant can be amplified. The set of two oligonucleotides primers each is complementary to opposite strands of the template, contained mutated nucleotides and extended by DNA polymerase. In general, two primers in one set are complement. However, some pairs of primers in this study are different from the ordinary. We designed two different mutations respectively on the opposite primers in one set. Therefore, we will obtain four types of plasmids in PCR products (shown in figure 2-1-1). After transformation and proliferation, three types of *E. coli.* can be obtained. The products should be sequenced to make sure the accuracy of mutants.

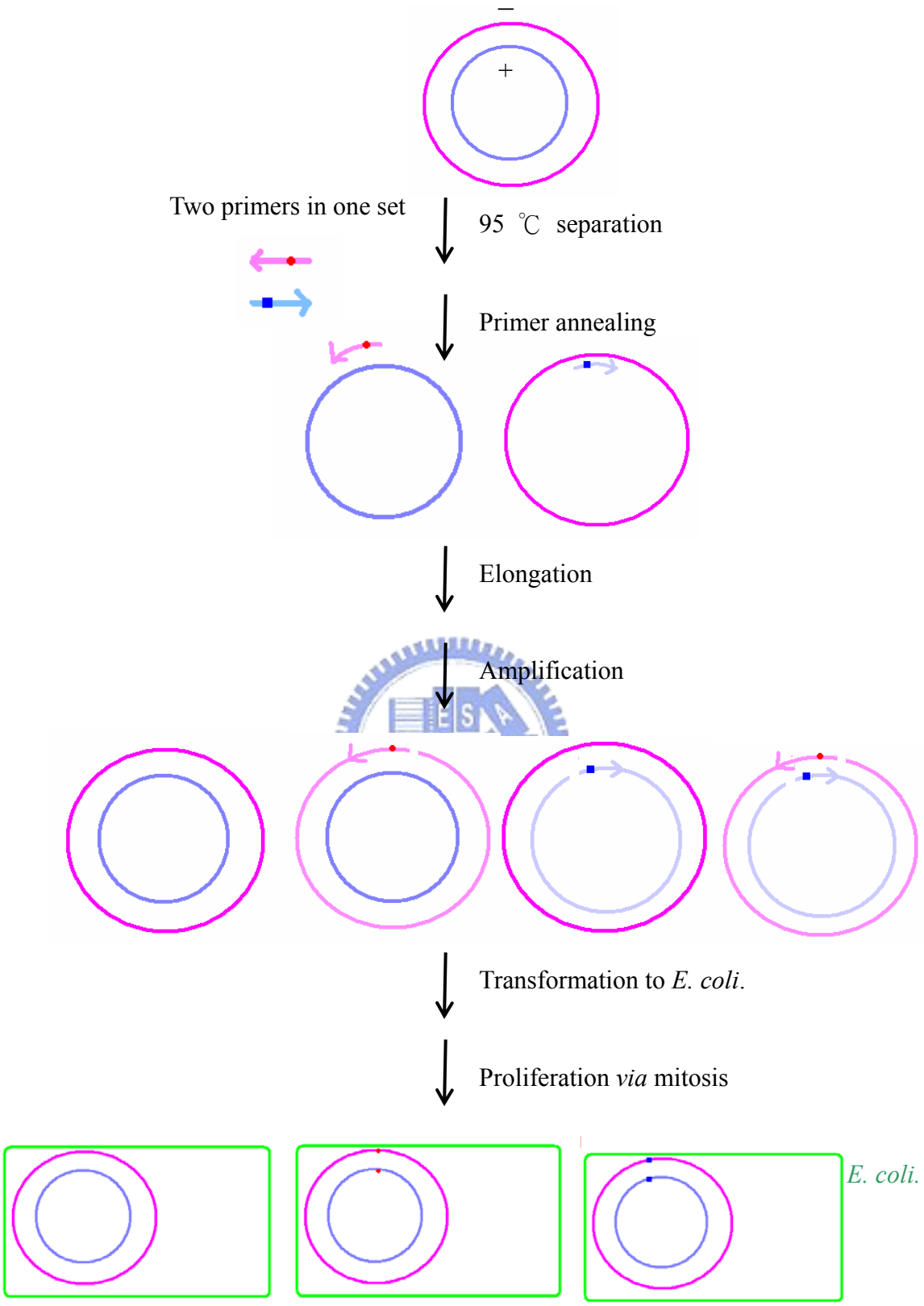


Figure 2-1-1 QuikChange procedure with two different primers in one set

Procedure

1. Reaction solution :

Reagent	Concentration	Volume
Template (pRSET/csn)	80 ng/μL	1μL
Primer (sense)	10 pmol/μL	1μL
Primer (anti-sense)	10 pmol/μL	1μL
dNTP	10 mM	1μL
Mg^{2+}	15 mM	1μL
Reaction buffer	10 X	5μL
Pfu polymerase	2U/μL	1μL
ddH_2O		39μL

2. The general conditions of these 11 mutants are summarized and described as figure 2-1-2. Depending on the set of primers, the annealing temperature in the stage Ⅱ is from 54 ℃ to 57 ℃, and this stage is repeated for 25 cycles to amplify the mutated DNA fragment.

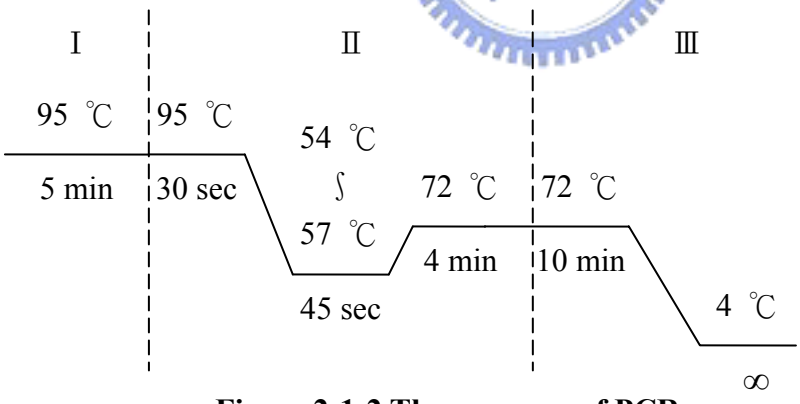


Figure 2-1-2 The program of PCR

3. The PCR products are then treated with *Dpn* Ⅰ to digest the parental DNA template.
4. The resulting mixture containing the nicked vector DNA incorporating the desired mutations is transformed into XL1-Blue cells to joint and multiply the plasmids which are then sequenced.

2-2 Expression and purification of chitosanase

2-2-1 Inclusion body expression system

A. Expression and rescution

1. *E. coli* strain ECOS 21 harboring plasmid pRSET/cns is grown in LB medium overnight. The overnight culture (1mL) of is freshly inoculated to 100 ml LB medium supplemented with 100 μg/mL ampicilin and 5 mg/mL glucose at 37 ℃ for 10 hours and then added IPTG to the final concentration of 100 μg/mL and then incubated for four more hours.
2. The cells are collected by centrifugation, resuspended in phosphate buffer, and lysed by sonication. The soluble intracellular protein and the insoluble factions are separated by centrifugation.
3. The insoluble debris containing inclusion body of recombinant chitosanase is solubilized in 5 M urea at 37 ℃ for 4 hours and then centrifuged.
4. The supernatant is decanted and put in 4 ℃ refrigerator.

B. Purification

1. The resulting solution is applied to the cation-exchange (HiTrap SP) column, which is pre-equilibrated with 20 mM, pH 6.5 phosphate buffer.
2. Elution is performed with NaCl gradient from 0 to 400 mM (40 mM/minute) in 20 mM, pH 6.5 phosphate buffer. Then the column is washed by pH 6.5, 1 M NaCl, 20 mM phosphate buffer for 10 minutes and then pH 6.5, 20 mM phosphate buffer for 10 minutes. Elution is monitored at 280 nm and the flow rate is 1 mL/minute.

2-2-2 The pRSET_SP (PSP) expression system

A. Establishment

Principle

The signal peptide is often used to localize proteins to specific regions within the cell or to be secreted. It is usually at the N terminus and normally absent from the mature protein. Normally, its function refers to the sequence (more or less 20 amino acids) that interacts with signal recognition particle and directs the ribosome to the endoplasmic reticulum where co translational insertion takes place. The signal sequence is normally removed from the growing peptide chain by signal peptidase, a specific protease located on the cisternal face of the endoplasmic reticulum.

The signal peptide expression system used in this study was established in our laboratory by Mr. Wu with pRSET_SP vector (shown as figure 2-2-1). The signal peptide sequence shown is cloned from *Serratia marcescens* chitinase gene and inserted into pRSET A vector behind ribosome binding site.

The signal peptide expression system of *Aspergillus* chitosanase is obtained by substituting original insert (chitinase) by chitosanase gene, wild type and mutants.

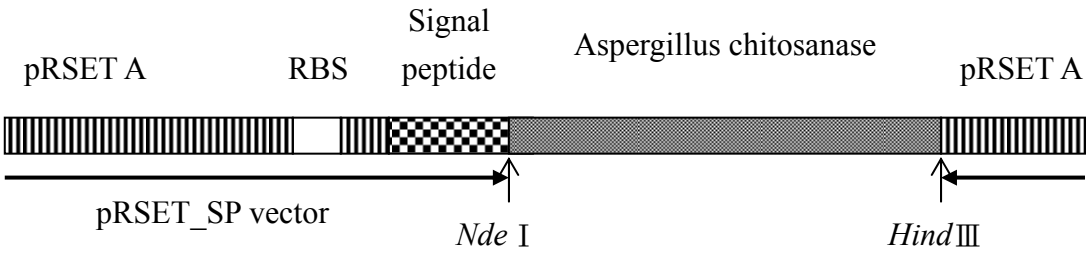
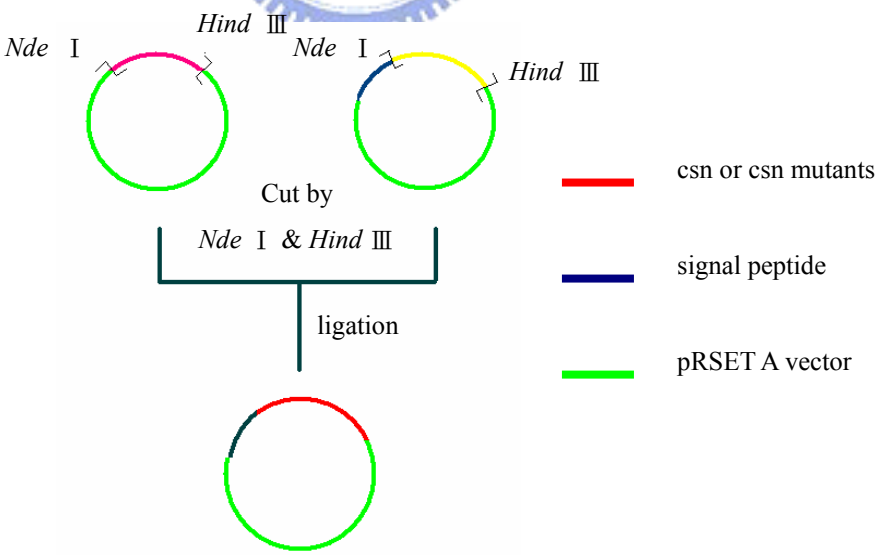


Figure 2-2-1 Illustration of pRSET_SP/csn

Procedure

1. The pRSET/csn containing chitosanase gene (wild type or mutated chitosanase) and the pRSET_SP vector are both cut by the restriction enzyme – *Nde* Ⅰ and *Hind*Ⅲ.
2. The desired fragments, chitosanase gene and pRSET_SP vector, are obtained by using Gel elution Kit after DNA electrophoresis.
3. The ligation process link the inserts fragment with the signal vector in proportion of 1: 3 (insert : vector), 1μL T4 DNA ligase, 1μL γATP, 2μL 10X ligase buffer, and adding ddH_2O to 20μL. The solution reacts at 16 ℃ for 24 hours.
4. The ligation products will be amplified by transforming into XL1-Blue cells and then sequenced to confirm the sequences.



B. Production

1. *E. coli* strain JM109 harboring plasmid pRSET_SP/*csn* was grown in LB medium overnight. The overnight culture (100 μL) of was freshly inoculated to 1 L LB medium supplemented with 100 μg/mL ampicilin at 37 ℃ for 14 hours.

C. Purification

1. The cells are collected by centrifugation, resuspended in phosphate buffer, and lysed by sonication. The soluble intracellular protein and the insoluble debris are separated by centrifugation.
2. The soluble intracellular protein was poured into the anion-exchange (HiTrap Q) column, which is pre-equilibrated with 20 mM, pH 6.5 phosphate buffer.
3. First, the column with protein sample inside is eluted by 20 mM, pH 6.5 phosphate buffer for 30 minutes. Then, the buffer is immediately changed by pH 6.5, 1 M NaCl, 20 mM phosphate buffer for 40 minutes to elute out the remained binding protein. Finally, the column is washed by pH 6.5, 1 M NaCl, 20 mM phosphate buffer for 40 minutes. The flow rate is 2 mL/min and the eluting solution is monitored at 280 nm. The fractions are collected every 1 minute and the fractions containing chitosanase are collected and used for next step.
4. The collected solution is further purified by cation-exchange (HiTrap SP) column, which is pre-equilibrated with 20 mM, pH 6.5 phosphate buffer.
5. First, the column with protein sample inside is eluted by 20 mM, pH 6.5 phosphate buffer for 20 minutes. Then, elution is performed with a gradient of NaCl from 0 to 1 M at the rate of 16.6 mM/min. Finally, the column is

washed by pH 6.5, 1 M NaCl, 20 mM phosphate buffer for 30 minutes and then pH 6.5, 20 mM phosphate buffer for 40 minutes. The flow rate is 2 mL/min and the eluting solution is monitored at 280 nm. The fractions are collected every 2 minutes and analyzed by activity test and SDS-PAGE.

2-3 Enzyme activity test by DNS

Principle

Chitosanase activity is analyzed by estimating the amounts of the reducing ends of sugars using DNS reagent. (Miller *et al.*, 1959)

Procedure

1. Suitably diluted enzyme (0.3mL) is mixed with 0.3 mL of 1% chitosan, pH 6.0. The mixture is incubated for 4 hours at 37 ℃.
2. Hydrolysis reaction is terminated and analyzed by adding 0.6 mL of dinitrosalicyclic acid reagent.
3. The mixture is boiled for 15 minutes, chilled and centrifuged to remove insoluble chitosan.
4. The resulting adducts of reducing sugars, D-glucosamine, are measured spectrophotometrically at 540 nm. The extinction coefficient of D-glucosamine (at 540 nm) is 788 $M^{-1}cm^{-1}$.
5. Alternatively, the products are analyzed by Mass Spectrometer with electrospray ionization as described above. One unit of chitosanase activity was defined as the amount of enzyme required to release 1 μmole of detectable reducing sugars at 37 ℃ in 1 minute (Fink *et al.*, 1991).

2-4 Mass analysis

Principle

The accurate molecular weight will give some clues of the composition and sequence of protein or oligosaccharides. We also design a succession of devices, such as methylation and tandem mass, to understand the pattern of enzyme hydrolytes. Peptide mapping can determine the sequence of amino acids after digesting protein and selecting a specific peptide. Moreover, correct molecular weight spectrum can confirm that the enzyme which we got has the exact amino acids composition.

Procedure

A. Sample preparation - enzyme

Before injecting into mass spectrometer, protein samples have to eliminate small molecular components. Therefore, enzymes are dialyzed against 20 mM ammonium acetate at 4 ℃ overnight, and then dried through lyophilization. The dried enzymes are stocked in 4 ℃. Sample will be re-dissolved to optimal concentration and then analyzed by mass.

B. Sample preparation – chitosan hydrolyte and its chemical methylation

The chitosan oligosaccharides are obtained from lower DDA (40%) chitosan hydrolyzed by chitosanase for a long time reaction (2 week). For identifying the sequence of these oligosaccharides, a suitable amount of oligosaccharides are chemical methylation at the reducing-end of sugar. The methylation is performed in a large excess of methanol containing 2% of perchloric acid at 50

℃ for 10 hours. The methylated oligosaccharides are analyzed by LC tandem mass spectrometry (LC/MS/MS).

C. Instrument set up and operation

1. Instrument and set up :

   Mass spectra are recorded by using a quadrupole time-of-flight mass spectrometer (Q-TOF, Micromass). In all ESI-MS experiments, the quadropole scan mode under capillary needle of 3 kV, source block temperature at 80 ℃ and desolvation temperature at 150 ℃.

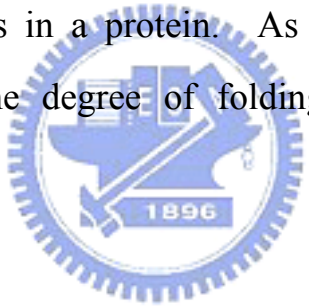
2. Sample used for mass measurement are normally in the range of 5~10 μg and measured directly at desalted form in 10% acetonitrile containing 0.1% formic acid.

2-5 Investigation of secondary structure

2-5-1 Fluorescence spectroscopy

Principle

Protein fluorescence is generally excited at 280 nm or at longer wavelengths, usually at 295 nm. Proteins contain three aromatic amino acid residues (tryptophan, tyrosine, phenylalanine) which may contribute to their intrinsic fluorescence. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Therefore, changes in intrinsic fluorescence can be used to monitor structural changes in a protein. As a result, it is assumed that the secondary structure or the degree of folding can be roughly revealed by fluorescence spectra.



Procedure

1. The optimum concentration (255 μg/mL) protein is put into the quartz cell which is 1 cm in length of its light path.
2. Set the program and scan the spectra, excitation from 260 nm to 400 nm and emission from 280 nm to 500 nm.

2-5-2 Circular dichroism spectroscopy

Principle

Circular Dichroism (CD) is observed when optically active matter absorbs left and right hand circular polarized light slightly differently. The instrument needs

to be able to measure accurately in the far UV at wavelengths down to 190-170 nm. CD spectra for distinct types of secondary structure present in peptides, proteins and nucleic acids are different. The analysis of CD spectra can therefore yield valuable information about secondary structure of biological macromolecules. Current CD spectrometers measure CD in terms of ellipticity Θ, usually expressed in millidegrees. Ellipticity Θ is the standard acquisition scale used today.

Procedure

1. The optimum concentration (150~250 μg/mL) protein sample in 20 mM phosphate buffer was put into the circular cell which is 0.1cm in length
2. Set the program and scan for 36 times.
3. The data is presented in ellipticity (**Θ**) and then transformed into mean residues molar ellipiticity ($\mathbf{[\Theta]_{MRW}}$).

   $\mathbf{[\Theta]_{MRW} = \Theta/(10*C_r*l)}$

   $\mathbf{C_r = (n*1000*c_g)/M_r}$

   Where **Θ** is ellipticity in mdeg;

   $\mathbf{C_r}$ is the mean residue molar concentration

   l is the cell path in cm

   n is the number of peptide bonds (residue)

   $\mathbf{c_g}$ is the macromolecule concentration (g/mL)

   $\mathbf{M_r}$ is the molecular weight of the protein

   [Θ] is expressed in deg*cm^2*decimole^{-1}

2-6 Investigation of anomeric configuration by NMR

Principle

The mechanism studies of glycoside hydrolysis enzyme are described at chapter 1. Investigation of anomeric configuration can be done by NMR by measuring the growth and decline of α and β proton with time.

Procedure

The stereochemistry of hydrolysis reaction was determined by ^{1}H-NMR at 35 ℃.

All spectra are recorded over a 500 Hz sweep width with 32 scans.

1. One mL of 1% chitosan solution is prepared in 1% acetic acid, then exchanged buffer by lyophilisation in 500μL D_2O trice, finally dissolved in 500μL D_2O and then placed in a dried 5 mm NMR tube.
2. 240 μg/mL D160E chitosanase (in 20 mM phosphate buffer pH 6.5, 200 mM NaCl) is pre-exchanged buffer against by sodium acetate at 4 ℃ overnight, dried by lyophilization, and then also lyophilized in D_2O using the same procedure as the substrate, and dissolved in 20μL D_2O. The dried enzymes activity is confirmed.
3. After recording the spectra of the substrate (500μL), 20μL chitosanase is added to the NMR tube which is immediately placed back in the spectometer.
4. The spectra are recorded every 10 minutes for 1 hour.

2-7 Characterization of enzymes by SPR

2-7-1 Introduction

The surface plasmon resonance (SPR) phenomenon occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index: the glass of a sensor surface (high refractive index) and a buffer (low refractive index).

A wedge of polarized light, covering a range of incident angles, is directed toward the glass face of the sensor surface. Reflected light will be detected

An electric field intensity, known as an evanescent wave, is generated when the light strikes the glass. This evanescent wave interacts with, and is absorbed by, free electron clouds in the gold layer, generating electron charge density waves called plasmons and causing a reduction in the intensity of the reflected light.

The resonance angle at which this intensity minimum occurs is a function of the refractive index of the solution close to the gold layer on the opposing face of the sensor surface.

A binding analysis SPR changes occur as a solution is passed over the surface of a sensor chip. To perform an analysis, one interactant is captured on a sensor surface. The sensor surface forms one wall of a flow cell. Sample containing the other interactant(s) is injected over this surface in a precisely controlled flow. Fixed wavelength light, in a fan-shaped form, is directed at the sensor surface and biomolecular binding events are detected as changes in the particular angle where SPR creates extinction of light. This change is measured continuously

to form a **sensorgram** (Figures 2-7-1 and 2-7-2), which provides a complete record of the progress of association or dissociation of the interactants.

When the sensor is exposed to a sample containing that analyte, the binding of the ligand and the analyte causes a change at the metal surface, within the plasmon field, and the shift in the resonant wavelength of the incident light is measured. The size of the shift is proportional to the quantity of the analyte in the sample. Because of the very specific relationship between the ligand and analyte, no other molecules in the sample can be mistakenly measured by the sensor.

Analyte binds to the surface-attached ligand during sample injection, resulting in an increase in signal. At the end of the injection, the sample is replaced by a continuous flow of buffer and the decrease in signal now reflects dissociation of interactant from the surface-bound complex. A response of 1000 RU (resonance units) corresponds to a change in surface concentration of 1 ng*mm^{-2}.

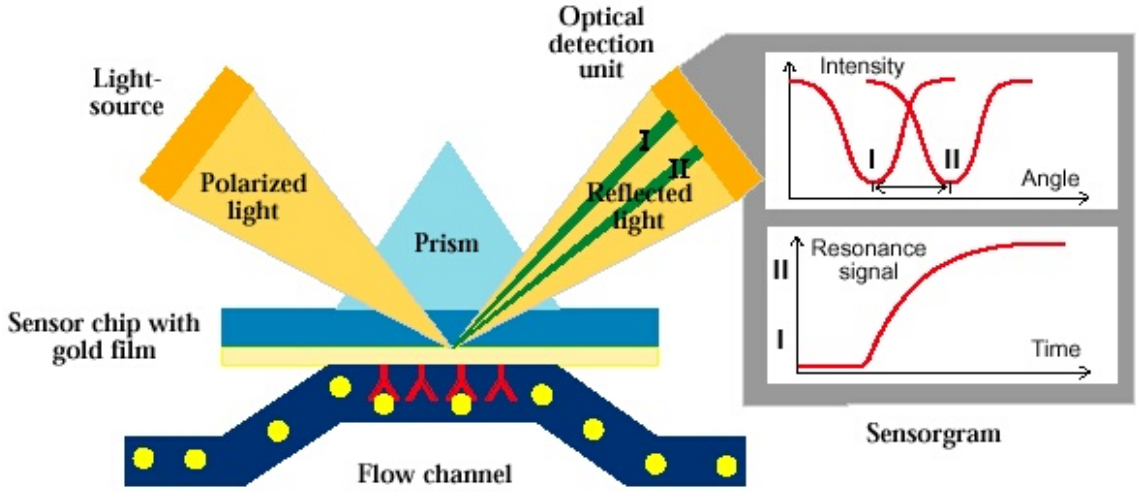


Figure 2-7-1 Principal of SPR system

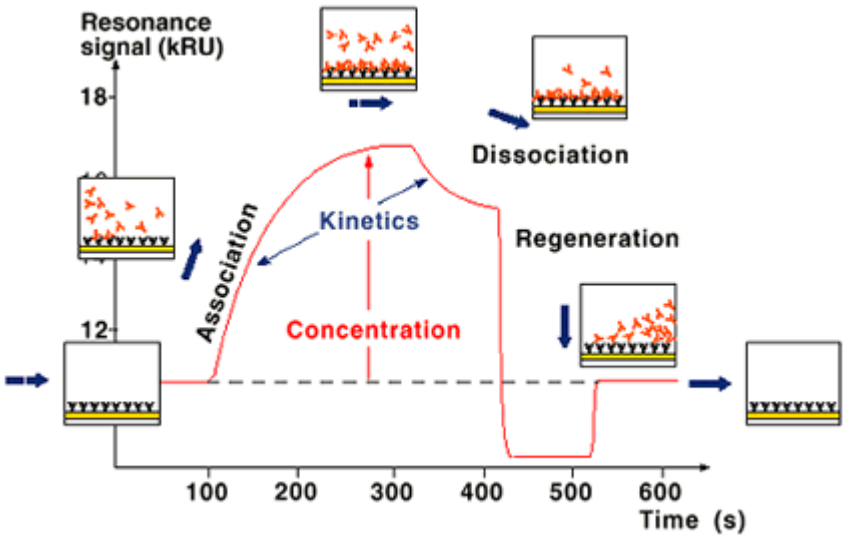


Figure 2-7-2 Sensorgram.

*** The pictures in this page are adapted from BIAcore co.**

BIAcore

BIAcore systems utilize the natural phenomenon of surface plasmon resonance (SPR) to perform protein interaction analysis. BIAcore's technology is non-invasive, label free and delivers high quality results in real time. Complete profiles of recognition, binding and dissociation are generated in real time.

Data

SPR system can provide data in form of RU value and give the relationship between ligand and analyte including:

- Specificity
- Kinetics: rates of reaction
- Affinity: the strength of binding
- Concentration

- Multiple interactions during complex formation

Applications

The phenomenon of SPR is completely non-specific. It cannot distinguish between different chemical changes. While this may appear to be a limitation, it is really a powerful advantage. Specificity depends upon selection of pairs of molecules which react only with each other. One member of the pair is the detector and the other is the target analyte (i.e. the substance we wish to detect and quantitate). Another member of pairs is named ligand. Any pair of molecules which exhibit specific binding can be adapted to SPR measurement. These may be an antigen and antibody, a DNA probe and complementary DNA strand, an enzyme and its substrate, an oil and a gas or liquid which is soluble in the oil, or a chelating agent and metal ion. For most applications, a dextran matrix covering the gold layer enables molecules to be immobilized to a sensor surface and provides a hydrophilic environment for interactions.

2-7-2 SPR in this study

2-7-2-1 Objective and design

SPR is applied in comparison the interaction between all recombinant chitosanase and chitosan tetra- or tri-mers. The study expects to acquire the enzyme characteristics of specificity, kinetics...and so on.

In this experiment, we design to use two methods, chitosan substrate or enzyme as ligand, to get more precise data.

2-7-2-2 Chip preparation – immobilization procedure

Au chip activation

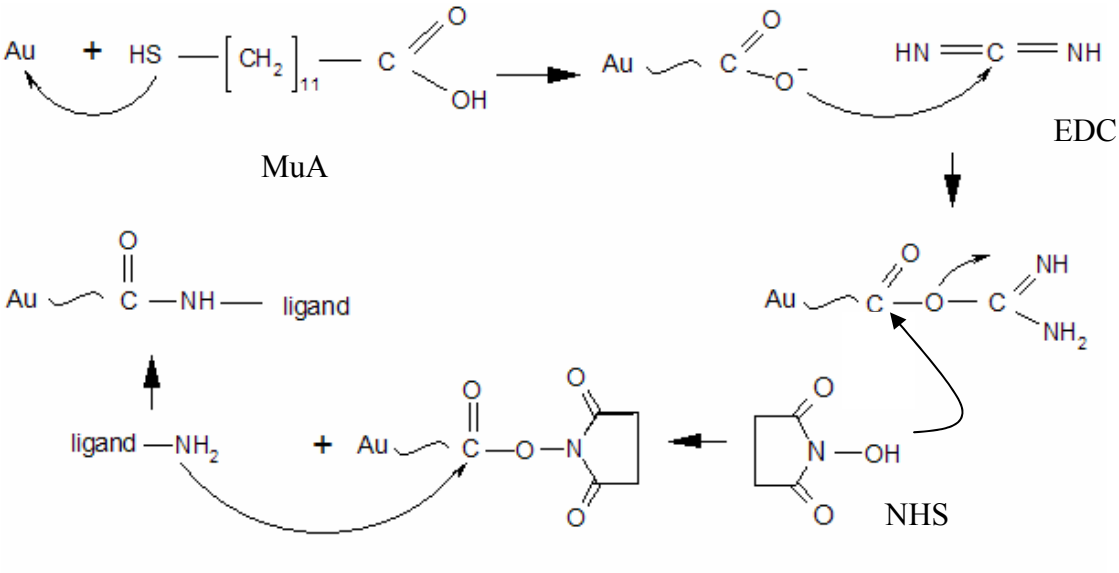


Figure 2-7-3 Au chip activation

Chemicals :

MuA : muramic acid

EDC : 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)

NHS : 2-[4-[(2-amino-4-hydroxy-5,6,7,8-tetrahydropteridin-6-yl methylamino]benzoyl]aminoglutaric acid

1. The Au chip is washed with 1mM HCL twice and then with dried THF.
2. The Au chip is self-assemble with 10% MuA in dried THF overnight and then washes with dried THF.
3. The chip is immersed in 0.02 g/mL carbondiemide or EDC in dried THF overnight.
4. The chip is washed by dried THF for several times and then immersed in 0.23 g/mL NHS in dried THF for 2~4 hours.
5. The activated chip is then coupling with ligand as soon as possible.

Immobilization chitosanase as ligand

It is because of the trace amount of ligand, for example: enzyme, that the coupling steps are processing in BIAcore system. The activated chip is put into BIAcore system and has to avoid contacting to water; otherwise the active groups will be removed.

1. The enzyme solution is flowing through the activated chip by injecting into BIAcore flow cell at the rate of 1 μL/ minute to couple with the activated chip.
2. The excess ligand is washed away by injecting coupling buffer at the rate 10μL/ minute.
3. The remaining active groups is blocked by injecting 1M, pH 8, ethenolamine for 1 hour at the rate of 10μL/ minute.
4. The chip is washed by at least 3 cycles of alternating pH by 0.1 M, pH4, acetate buffer containing 0.5 M NaCl and 0.1 M, pH 8, Tris-HCl buffer containing 0.5 M NaCl by injecting at the rate of 10μL/ minute.

2-7-2-3 Operation of BIAcore X

Materials and preparations

- BIAcore X (The machine has to warm up for 30 min.)
- BIACORE CONTROL and BIAEVALUATION 3.1 software
- The actived or labeled Au chip (The chip have to put in the RT for 30 minutes)
- 20 mM, pH 7, phosphate buffer
- All solutions have to degas by using ultrasonic vibrator and go through the 0.22 μm filter.

Operation

Before doing the analysis the system have to execute the maintain process: once "DESORB" and twice "PRIME". Start sensorgram through multichannel (Fc1 and Fc2) with 20 mM, pH 7 phosphate buffer at the rate of 20 μL/minute until smoothing the signal curve.

1. BSA and 0.1% SDS are alternatively injected into the flow cell. In order to block the remaining non-coupling site of the chip, BSA is employed here to occupy the site which may possible bind by analyte nonspecifically and then wash the excess BSA away by SDS. These steps should repeat until the RU value of the end equal to the beginning (at least not higher than 50 RU) of the same cycle.
2. Inject analyte through FC2 (FC1 as blank reference) by using the delay mode for 60 seconds.
3. After one analysis cycle, the system is washed by 0.1% SDS to reduce the RU value to the beginning (at least not higher than 50 RU).
4. Then inject next sample (repeat step 2 and 3).

2-8 Chemical label

Many enzyme properties can be identified through labeling chemical on specific site in enzyme, for example, estimation of disulfide bond and free thiol, modification of important amino acid, and trapping the intermediate of catalytic pathway.

The most important use of chemical label is trapping the intermediate and then getting the direct clue and evidence of catalytic pathway. The reversible character of the steps involved in enzyme-catalyzed reactions makes the determination of each substrate-binding site less then straightforward. At steady-state, a constant amount of enzyme-substrate complex is known to be present, but if an attempt is made to isolate this and hydrolyte it so as to identify the amino acid to which the substrate is attached, the effort will not usually be rewarded with success; this is because the substrate will dissociate from the enzyme during the procedures involved. However, if the enzyme-substrate complex can be trapped in a modified form by some chemical process so that the substrate is no longer able to dissociate from the enzyme, then it may be possible to identify the substrate-binding site. In many investigations of enzyme characteristics, labeling an organic compound on enzyme is a direct evidence to make sure the site of essential group. On the other hand, chemical label also can be applied as a modification method in order to get new enzyme which act in different way and produce different product. In fact, the concept of labeling can be performed at many different experiments of enzyme research after sophisticated design.

2-8-1 Estimation of disulfide bond

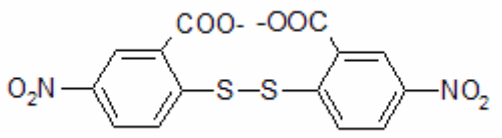
Principle

Estimation of disulfide bond in *Aspergillus* chitosanase is employed DTNB (5,5'-dithionitrobenzoic acid) which will interact and bind with free thiol of cysteine in protein. In addition, DTT (dithiothreitol; Cleland's reagent) which is used to reduce disulfide bonds quantitatively and maintain monothiols in the reduced state will be employed in contrast experiment.

All the free thiol in cysteine of heated enzymes which disintegrate the protein folding will expose and enable to interact with DTNB. The contrast experiment is breaking all disulfide bond in the enzyme by adding DTT and enable to confirm that all the cysteine are interacting with DTNB.

On the other hand, DTNB attack the free thiol in cysteine of enzyme and then break apart into thionitrobenzoate anion. The thiol molar concentration will increase and its absorbance at 412 nm can be monitored. The molar absorbance of the thionitrobenzoate anion is $\varepsilon_{412} = 14{,}290\ M^{-1}\cdot cm^{-1}$.

The enzymes used here are purified from intra-cellular soluble protein and changed to pH 8. The enzyme is heated in 95 ℃ for 30 minutes to disintegrate the folding.



DTNB

Procedure

1. Normal enzymes contained 3 mM DTNB and 3 mM DTNB only which serves as blank are prepared.
2. The UV spectrometer is set to detect 412 nm every 20 seconds for 7200 seconds and read the two samples in turn every time. The data is subtracted blank from sample with enzyme by instrument.
3. Repeat step 1 and 2 and use heated enzyme which replace the normal one.

2-8-2 Recovery of activity

Principle

The hypothesis of this experiment is that D160C and E169C will recover their activity if DTNB is linked on cysteine in the enzyme active domain. Because DTNB has carboxyl group that can replace and act as the original amino acid-Asp or Glu. Moreover, the modified enzyme may hydrolyze chitosan into different products by different mechanism.

Procedure

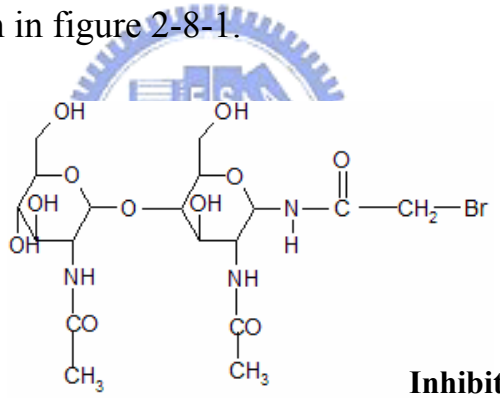
1. D160C, E169C, wild type enzyme are separately mixed with pH 8.0, 10 mM DTNB in proportion of 1 : 1 at 37 ℃ overnight.
2. After incubating, the molecular weight changes of products are checked by Mass and then compare the activity variations.

2-8-3 Labeling inhibitor onto enzyme

Principle

In the general essential amino acid investigation, an inhibitor which is analogue of enzyme substrate is designed and labeled on enzyme to demonstrate the exactly catalytic amino acid. The labeled enzyme will be digested by protease and analyzed by peptide mapping.

The inhibitor - chitobiose (chitin dimer) derivative is synthesized by Mr. Shrestha in our lab. First, amination of chitobiose is carried out by using excess $(NH_4)_2CO_3$ solution at room temperature for 5 days, which give β-1-aminochitobioside. Then, bromo acetic anhydride is added to get the inhibitor product shown in figure 2-8-1.



Inhibitor

Procedure

1. Wild type enzyme mixed with inhibitor in proportion of 3 : 1 is incubated at 37 ℃ overnight.
2. The labeled enzyme is confirmed the molecular weight by mass and then compared the activity with the non-labeled enzyme.
3. Peptide mapping is further investigation procedure to confirm that which amino acid is exactly labeled by the inhibitor. The labeled enzyme is digested by protease-trypsin and then the fragments are analyzed by mass.

Chapter 3 Results and Discussion

Part Ⅰ Production of enzyme

3-1 Site-directed mutagenesis

3-1-1 Multi-alignment

Although many GH-75 chitosanases have been cloned, extensive investigations of this family enzyme are rare. For instance, the protein structure, catalytic mechanism, essential groups and topology of the active site remined 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 available gene sequences of GH-75 enzyme from microbial strains including *A. fumigatus* (Cheng *et al.*, 2003; GenBank AY190324), *A. oryzae* (Zhang *et al.*, 2000; GenBank AB038996), *F. solani* (Shimosaka *et al.*, 1996; GenBank D85388), *B. bassiana* (Berretta *et al.*, 2001; GenBank AY008269), and *M. anisopliae* (Screen *et al.*, 2000;GenBank AJ293219) were aligned by CLUSTALW (Thompson *et al.*, 1994). Ten conserved residues (9 Asp and 1 Glu) were found to be Asp^{59}, Asp^{76}, Asp^{78}, Asp^{80}, Asp^{112}, Asp^{114}, Asp^{160}, Glu^{169}, Asp^{194}, and Glu^{229} (see figure 3-1-1). In general, glutamate and aspartate were substituted with sterically conserved residues, i.e. glutamine and asparagine, respectively. The 11 pairs of primers were designed and their sequences were summarized in table 3-1-1. The site-directed mutations were performed by QuikChange mutation, pRSET/csn as template.

3-1-2 Further design

Preliminary activity assays of all 11 mutants, showed that Asp^{160} and Glu^{169} are likely to be the essential amino acids (discussed in 3-3-1). For further investigating the roles of Asp^{160} and Glu^{169}, four mutants, D160E, E169D, D160C, and E169C, were constructed. The first two mutants were designed based on the idea of functional substitution (E/D or D/E), whereas, the latter two were replaced with a thiol residue. The primers used for mutagenesis were shown in table 3-1-2.

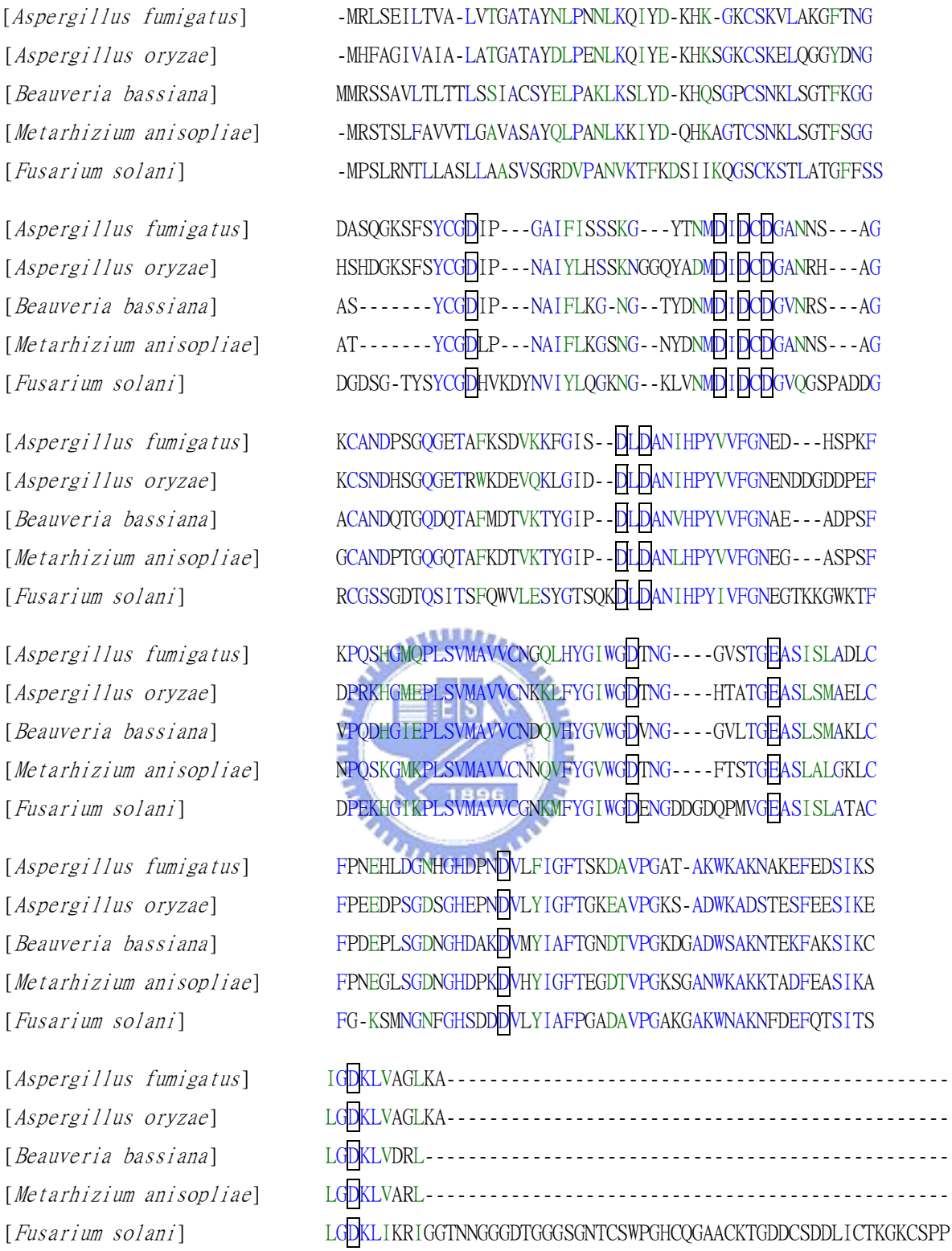


Figure 3-1-1 The multiple sequence alignment of GH-75. Five available gene sequences are aligned by CLUSTALW. It reveals 10 potential candidates to function as catalytically essential groups as indicated in box.

Table 3-1-1 Primer pairs of synthetic oligonucleotide designed for essential amino acid candidates.

Mutation site	symbol	Oligonucleotides sequence
D59N	D59N-s	5′CAGTTACTGCGGT**A**ACATTCCGGG
	D59N-a	5′CCCGGAATGT**T**ACCGCAGTAACTG
D76N	D80N-s	5′CCAATATGGACATCGACTGCAACGGC
	D76N-a	5′GCCGTCGCAGTCGATGT**T**CATATTGG
D78N	D80N-s	5′CCAATATGGACATCGACTGCAACGGC
	D78N-a	5′GCCGTCGCAGT**T**GATGTCCATATTGG
D80N	D80N-s	5′CCAATATGGACATCGACTGC**A**ACGGC
	D76N-a	5′GCCGTCGCAGTCGATGTTCATATTGG
D112N	D112N-s	5′GAAGTTTGGCATCTCC**A**ACCTGGACGC
D114N	D114N-a	5′GCGT**T**CAGGTCGGAGATGCCAAACTTC
E126Q	E126Q-s	5′GGTGTTTGGAAAC**C**AGGATCACTCTC
	E126Q-a	5′GAGAGTGATCCT**G**GTTTCCAAACACC
D160N	D160N-s	5′GGAATCTGGGGT**A**ACACTAACGGTG
	D160N-a	5′CACCGTTAGTGT**T**ACCCCAGATTCC
E169Q	E169Q-s	5′CGTTTCTACCGGC**C**AAGCCTCCATTTC
	E169Q-a	5′GAAATGGAGGCTT**G**GCCGGTAGAAACG
D191/194N	D191N-s	5′GGTCAC**A**ATCCCAAT**A**ATGTCCTCTTC
	D191N-a	5′GAAGAGGACAT**T**ATTGGGAT**T**GTGACC
D229N	D229N-s	5′CGATCGGT**A**ACAAACTGGTTGCTGG

The nucleotides indicate in bold and underlined are the sites of mutation and silent mutation, respectively.

Table 3-1-2 Four Pairs of primers for further mutation of D160 and E169.

Mutation site	symbol	Oligonucleotides sequence
D160E	D160E-s	5′GGAATCTGGGGTTGCACCAACGGTG
	D160E-a	5′CACCGTTGGT**T**TCACCCCAGATTCC
D160C	D160C-s	5′GGAATCTGGGGT**TG**CACCAACGGTG
	D160C-a	5′CACCGTTGGTTTCACCCCAGATTCC
E169D	E160D-s	5′CGTTTCTACCGGCTGTGCCTCCATTTC
	E160D-a	5′GAAATGGAGGC**A**TCGCCGGTAGAAACG
E169C	E160C-s	5′CGTTTCTACCGGC**TGT**GCCTCCATTTC
	E160C-a*	5′GAAATGGAGGCATCGCCGGTAGAAACG

The nucleotides typed in bold are the mutated sites.

3-2 Inclusion body expression system

3-2-1 Expression and purification

Expression

The wild type and mutated *cns* were first constructed in pRSET (designated as pRSET/csn), which were further transformed into ECOS 21 competent cell. Unfortunately, chitosanase protein were produced and aggregated as an inclusion body. The SDS-PAGE analysis was shown in figure 3-2-1. In general, some advantages, such as higher-level production, no toxic effect, resistant to proteolysis and high purity by simple physical operation, were found. However, the inclusion bodies predicament is awkward to resolve, and only very limited cases were shown to restore the biological activities. Since the native *Aspergillus* chitosanase is unusually stable, the protein structure of this enzyme is believed to be very hardy and consequently allows the refolding process to be effective. Wild type and all mutants were successfully overexpressed in ECOS 21 as inclusion body shown as figure 3-2-2.

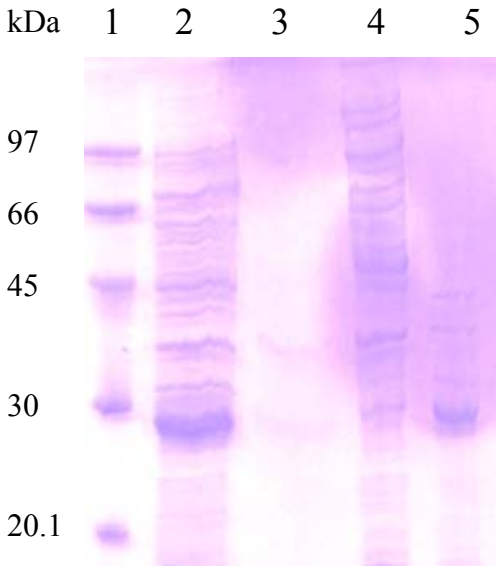


Figure 3-2-1 SDS-PAGE analysis of Protein. Lane 1, protein marker; Lane 2, whole cell; Lane 3, extra-cellular protein; Lane 4, intra-cellular soluble protein; Lane 5, intra-cellular insoluble protein (inclusion body).

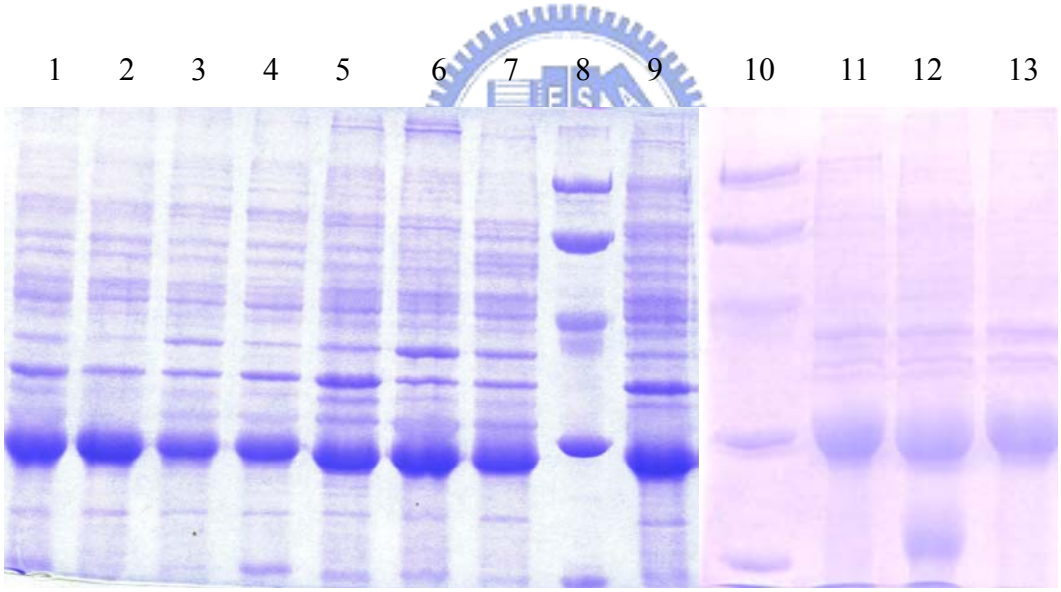


Figure 3-2-2 Inclusion bodies of wild type and all mutants. Lane 1, wild type; Lane 2 D59N; Lane 3, D112N; Lane 4, D114N; Lane 5, E126Q; Lane 6, D160N; Lane 7, E169Q; Lane 8, protein marker (from top to bottom are 97, 66, 45, 30, 20.1 kDa, respectively); Lane 9, D191/194N; Lane 10, protein marker; Lane 11, D76N; Lane 12, D78N; Lane 13, D80N.

Purification

The enzyme was 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 (figure 3-2-3), the purified chitosanase with >90 % homogeneity was obtained using 200-300 mM NaCl as eluent. All other mutants (discussed in later section) were purified with the same protocol and attained similar protein homogeneity (figure 3-2-4). Although many GH-75 enzymes, exclusively fungal chitosanases, have been cloned, we report here the first successful expression, purification on the corresponding recombinant enzyme and investigation of its catalytic function and essential residues.

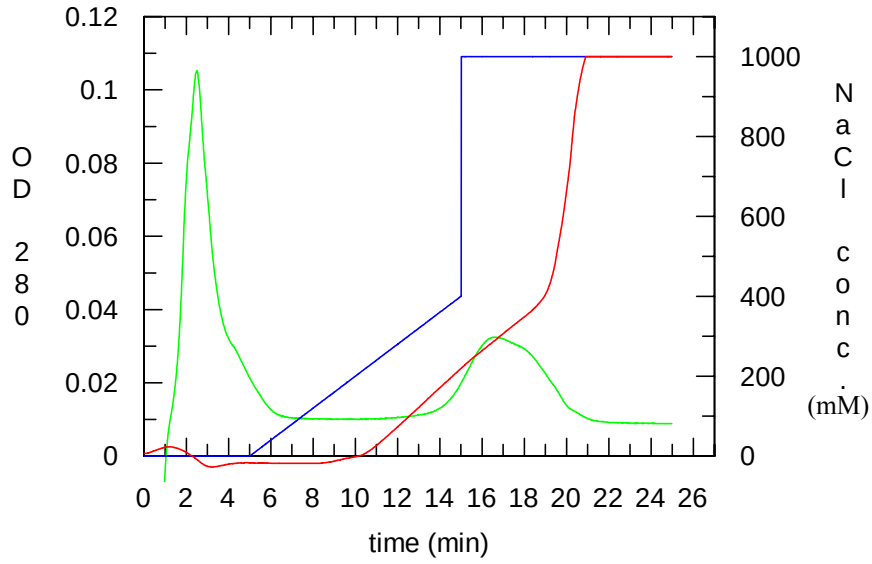


Figure 3-2-3 HiTrap-SP chromatography of wild type chitosanase derived from inclusion body. The blue line is concentration of NaCl in program; and the red line is actual concentration of NaCl in column. The green line is the UV absorption at 280 nm.

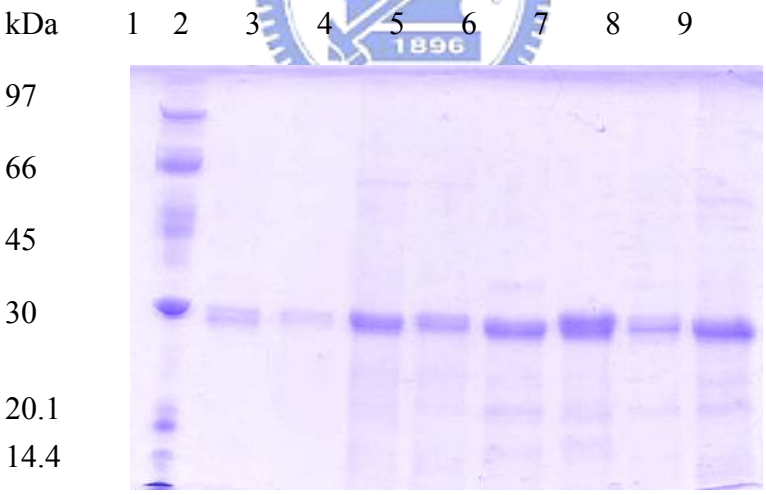


Figure 3-2-4 Purified wild type and mutants. Lane 1, protein marker; Lane 2 and 3, native chitosanase; Lane 4, wild type; Lane 5, D191/194N; Lane 6, D160N; Lane 7, E169Q; Lane 8, D160E; Lane 9, E169D.

Molecular weight determination

In addition to SDS-PAGE, ESI-MS was employed to determine the molecular weight of recombinant chitosanase. All mutants were analyzed by MS to ensure the mutational change. Figure 3-2-5 exhibited the mass analysis of the recombinant wild type chitosanase (23593 Da = 23462 Da + Met). Note that the MW of the native enzyme is 23462 Da (Cheng, 2003). For constructing the expression system in pRSET, an additional methionine was incorporated to the N-terminus of polypeptide chain.

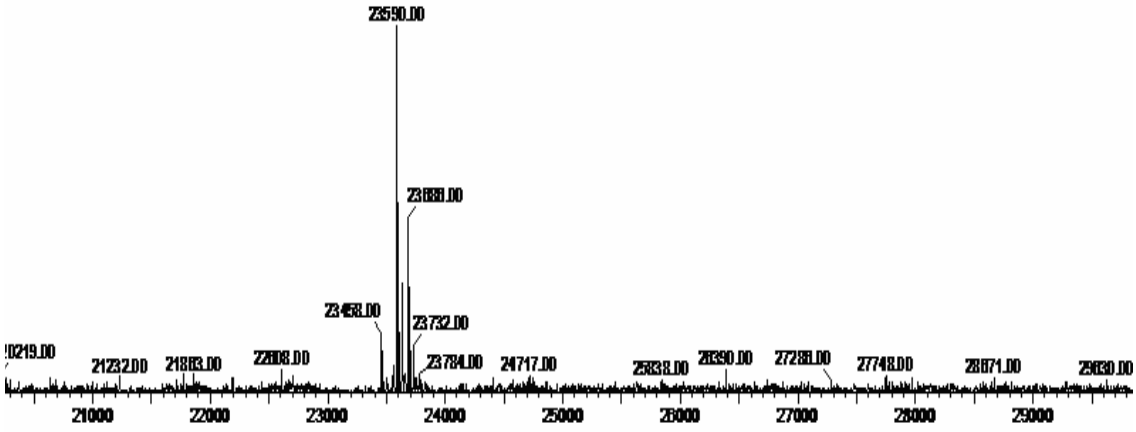


Figure 3-2-5 Mass spectrum of recombinant chitosanase purified from inclusion body. The molecular weight of recombinant chitosanase is 23593±4 Da.

3-2-2 Discussion of refolding problem

The 3D fluorescence spectrum of enzyme may reveal structural information when the conformation of enzyme has changed. The fluorescence spectra of the active and denatured recombinant chitosanase (heated inactivated at 95 ℃ for 1 hour) were shown in figure 3-2-6 (a) and (b), respectively. As can be seen, the center of contour was red-shifted as the enzyme was denatured. The highest spikes of the spectra were observed with excitation at 284 nm and emission at 333 nm and 337 nm for active and denatured chitosanase, respectively.

This observation may be useful for tracing the refolding progress of the chitosanase. Further study will be performed in near future.

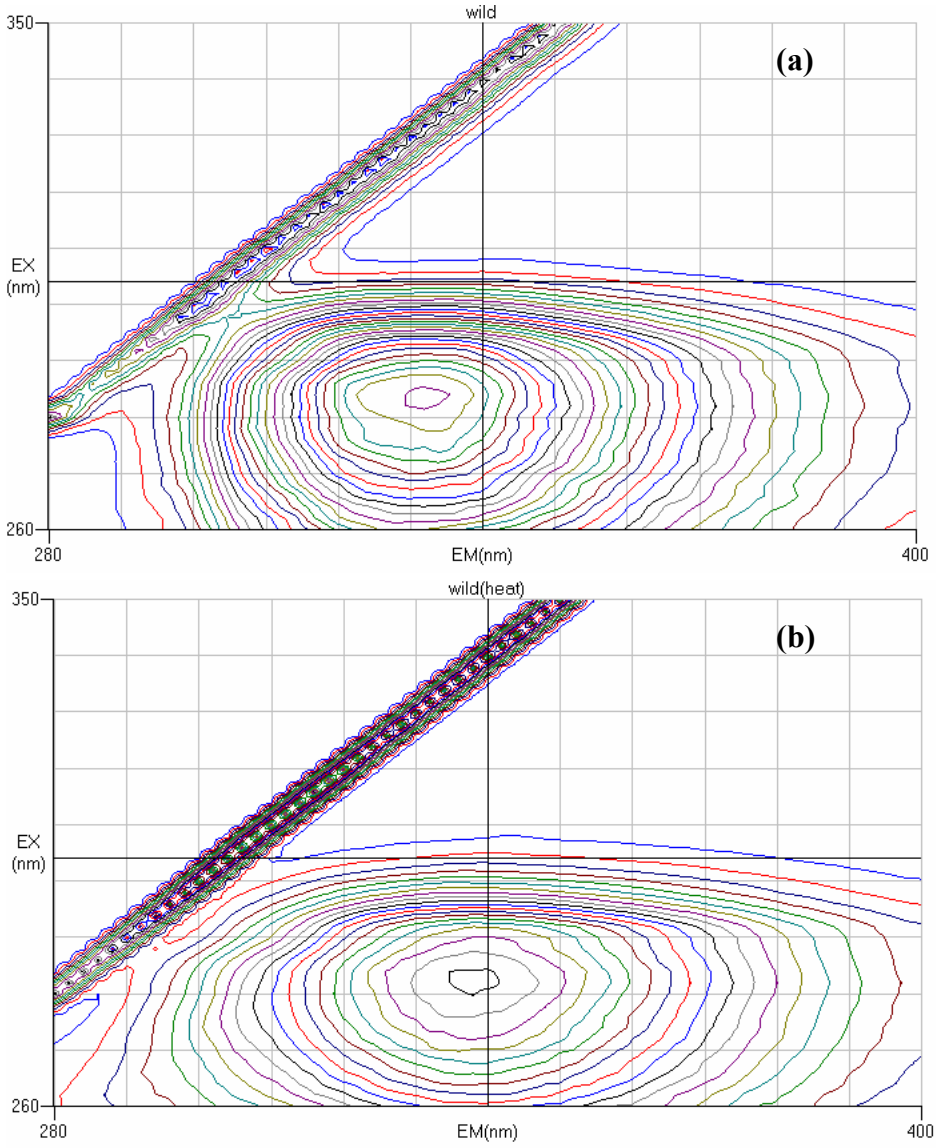


Figure 3-2-6 Fluorescence spectra of active (a) and denatured (b) wild type chitosanase.

The highest point of (a) is located at EX 284 nm and EM 333 nm; that of (b) is located at EX 284 nm and EM 337 nm.

3-3 The pRSET_SP (PSP) expression system

3-3-1 Exploration

Since the protein rescue from inclusion body was tedious and time-consuming, a pRSET_SP expression system (PSP system), containing a signal peptide directly derived from *Serratia marcescens* chitinase signal peptide (sequence shown in figure 3-3-1), was constructed and used for protein expression. Interestingly, with this signal peptide, some important recombinant proteins were expressed and form an intracellular and soluble protein. The new recombinant plasmid was named as pRSET_SP/csn. Owing to the gene manipulation, *Nde* I cutting site (CATATG) was inserted between signal peptide and the *cns*. Thus, the molecular weight of the recombinant proteins will become 23730 Da [23462 Da (native) + 131 Da (met) + 137 Da (His)]. The signal peptide contains 23 amino acids, including a hydrophobic central core followed by the peptidase recognition sequence Ala-X-Ala (Perlman *et al.*, 1983) (Wang *et al.*, 2001). In PSP system, the recombinant protein was expressed as extra- or intra- cellular enzyme in different strains of host cell (Wu, 2002). In this study, we used three different strains of host cell (XL1-Blue, BL21(DE3), and JM109) to express pRSET_SP/csn. The results were summarized in table 3-3-1.

1 Met Arg Lys Phe Asn Lys Pro Leu Leu Ala Leu Leu Ile Gly Ser
1 ATG CGC AAA TTT AAT AAA CCG CTG TTG GCG CTG TTG ATC GGC AGC

16 Thr Leu Cys Ser Ala Ala Gln Ala His Met Arg Leu Ser Glu Ile
46 ACG CTG TGT TCC GCG GCG CAG GCC CAT ATG CGT CTC TCT GAA ATT

Figure 3-3-1 Sequence of signal peptide and part of *Aspergillus* chitosanase. First 23 double underlined amino acids are signal peptide; the waved underlined amino acids are first part of *Aspergillus* chitosanase. The shadowed amino aicds are AXA motif; the correspondent nucleotide sequence of amino acids typed in bold are the position of *Nde* I cutting-site.

Table 3-3-1 Summarization of all expression systems

***coli.* strain**	**Plasmid**	**Expression position**	**Expression form**	**Molecular weight**	**Feature**
ECOS21	RSET/ csn	Intra-cellular	clusion body	23462	clusion body
XL1-Blue	SET_SP/c sn	Extra-cellular	Soluble-protein	---	Extra-llular protein
BL21-DE3	SET_SP/c sn	Intra-cellular	Soluble-protein	---	Lower expression
JM109	SET_SP/c sn	Intra-cellular	Soluble-protein	23730/ 28472	

T7 promoter and DE3 system

The vector, pRSET A which is controlled by T7 promoter will only express protein in *E. coli.* strain encoded T7 RNA polymerase, i.e. DE3 genotype. Hence, we usually utilize XL1-Blue (not DE3 strain) *E. coli.* strain as cloning host but not expression host. However, in our experiments, XL1-Blue can express protein if the target gene is inserted in the appropriate position of pRSET_SP vector. For example, a soluble *Aspergillus* chitosanase can be found in medium and in cell of XL1-Blue or JM109 harboring pRSET_SP/csn plasmid. The "leakage" phenomenon may be due to the *bla* promoter present at the up-stream region of the inserted gene. Consequently, T7 DNA polymerase is not required for expression. Interestingly, the expression feature (intra-cellularly or extra-cellularly) of our signal peptide expression system seems to be cell-line dependent. The detail relationship and the secretion mechanism between the exogenous signal peptide and the expression host remain unknown.

Molecular weight of protein

The correct molecular weight of recombinant wild-type chitosanase secreted from signal peptide expression system is 23730 Da. However, in few cases, an intra-cellular recombinant chitosanase with larger molecular weight (28472 Da) was purified (shown in figure 3-3-2). The excess molecular weight (4742 Da) was probably due to the non-excision of signal peptide. However, the extra 23-aa of the signal peptide could not explain the mass discrepancy. Perhaps the translation did not stop at TAG stop codon (amber codon) of recombinant *Aspergillus* chitosanase. JM 109 and XL1-Blue strain can translate amber codon into glutamine and elongate the translation until next recognizable stop codon. To explore minority cases of the larger molecular weight enzyme, the sequence after stop codon in frame and the possible stop codons were listed in figure 3-3-3. We proposed that the first TGA functions as the stop codon. In sum way, we propose that the excess molecular weight is probably the original enzyme plus 23 amino acids signal peptide and 20 amino acids after amber codon. This argument can be verified through N'-terminal sequencing in the future.

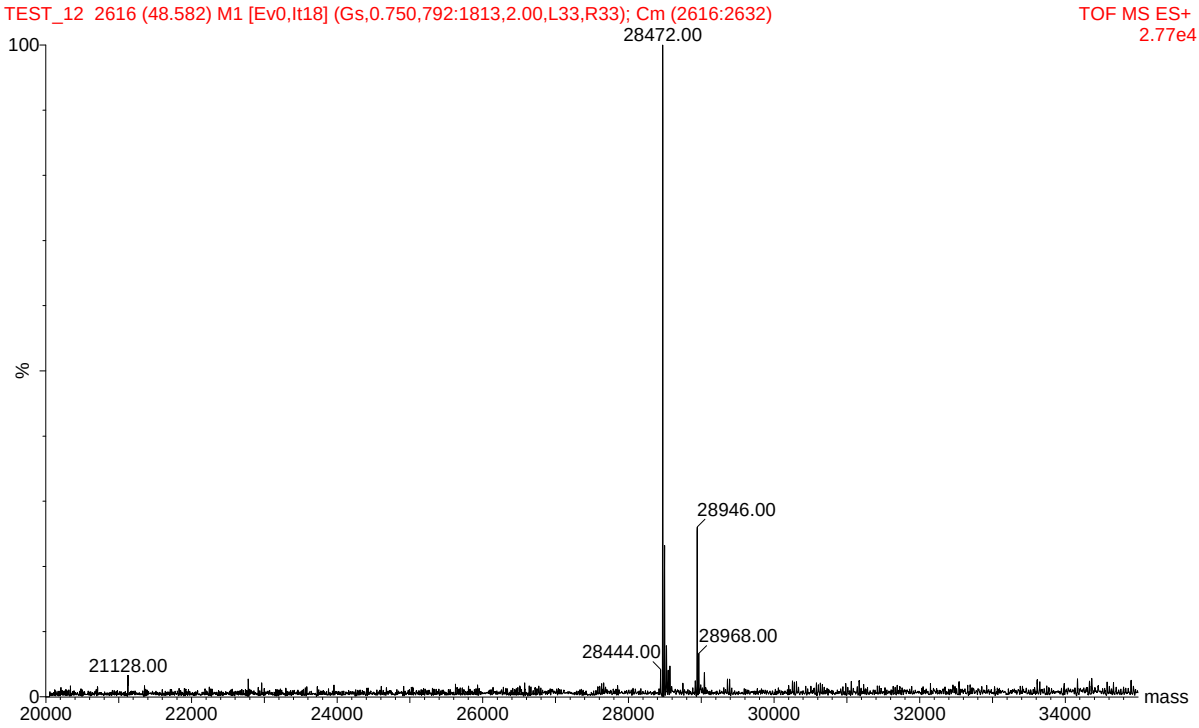


Figure 3-3-2 Mass spectrum of intra-cellular soluble chitosanase from JM109. The molecular weight-28472 are larger than theoretical value.

GTTGCTGGTTTGAAAGCA TAG CGA ACT AGA ACG GAT CCG AGC TCG AGA TCT GCA GCT GGT ACC ATG GAA TTC GAA GCT TGA TCC GGC TGC TAA CAA AGC CCG AAA GGA AGC TGA GTT GGC TGC TGC CAC CGC TGA GCA ATA ACT AGC ATA ACC CCT TGG GGC CTC TAA ACG GGT CTT GAG GGG TTT

Figure 3-3-3 Sequence after the stop codon of pRSET/csn. The shadowed nucleotides are *Aspergillus* chitosanase stop codon. The underlined sequence is the end part of *Aspergillus* chitosanase; after the underlined sequence the nucleotides are the pRSET A sequence after *Bam*H Ⅰ cutting site. The double underlined codons are indicated the possible stop codons and the first one match with experiment.

Secondary structure

The CD spectra of native chitosanase and wild type recombinant enzymes purified from inclusion body, extra- and intra-cellular soluble proteins were measured. As can be seen in figure 3-3-4, though the spectra of soluble proteins are slight different from that of native chitosanase, the catalytic activities are comparable. Interestingly, the CD spectra of chitosanase purified from inclusion body (kept at 4 ^{o}C in 5M urea for 28 days prior to purification) are superimposed. Unfortunately, the relative activities of both chitosanases are currently unavailable.

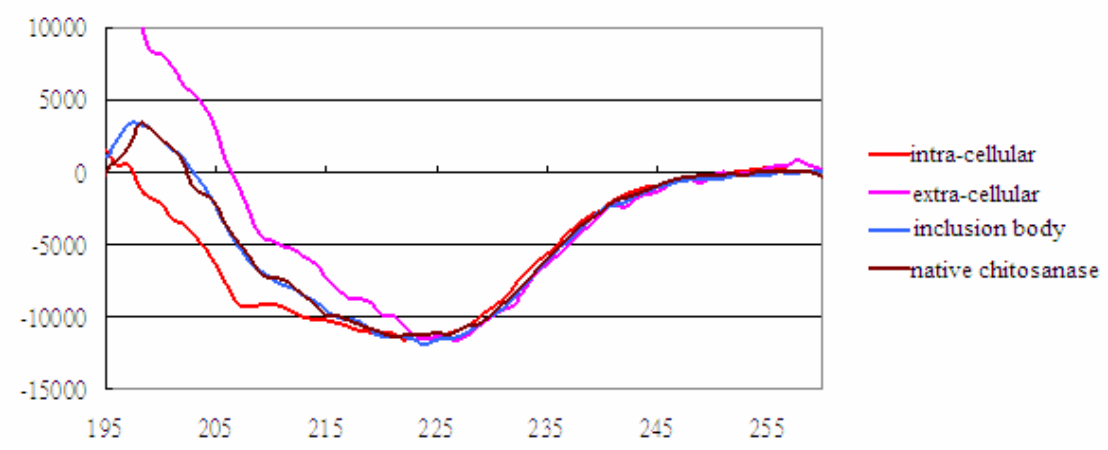


Figure 3-3-4 CD spectrum of chitosanases. Wild type recombinant chitosanases from intra-cellular, extra-cellular, and inclusion body are compared with native chitosanase. The intra-cellular soluble chitosanase is purified from JM109 and the extra-cellular chitosanase is purified from XL1-Blue. The inclusion body is purified from ECOS21 and put at 4 ℃ for 28 days.

3-3-2 Expression and purification

Expression

Our previous study has shown that the incorporation of a bacterial signal peptide in expression vector, the recombinant protein was expressed as either extra-cellular or intra-cellular enzyme depending on the strains of *E. coli* (Wu, 2002). For avoiding the tedious and time-consuming process of protein refolding, we used this secretion system in JM109. Wild type, D160N, D160E, E169Q, E169D, and D191/194N were expressed through this system.

Purification

The recombinant chitosanase was purified in two steps by using two different columns, HiTrap Q column and HiTrap SP column. First, intra-cellular crude enzyme was applied onto HiTrap Q column and the chromatography was shown as figure 3-4-5. And then, the non-bonding protein eluted at the very early stage (0 mM NaCl) was collected and applied onto HiTrap SP column. The chromatography was shown as figure 3-4-6. The fractions eluted with 200~300 mM NaCl were found to containe chitosanase with ~90% homogeneity.

SDS-PAGE analysis of the purified recombinant enzymes were shown in figure 3-4-7. The mass of the recombinant enzymes were also confirmed by mass. Figure 3-4-8 showed that the mass spectrum of D160E with expected molecular weight of 23730±4 Da.

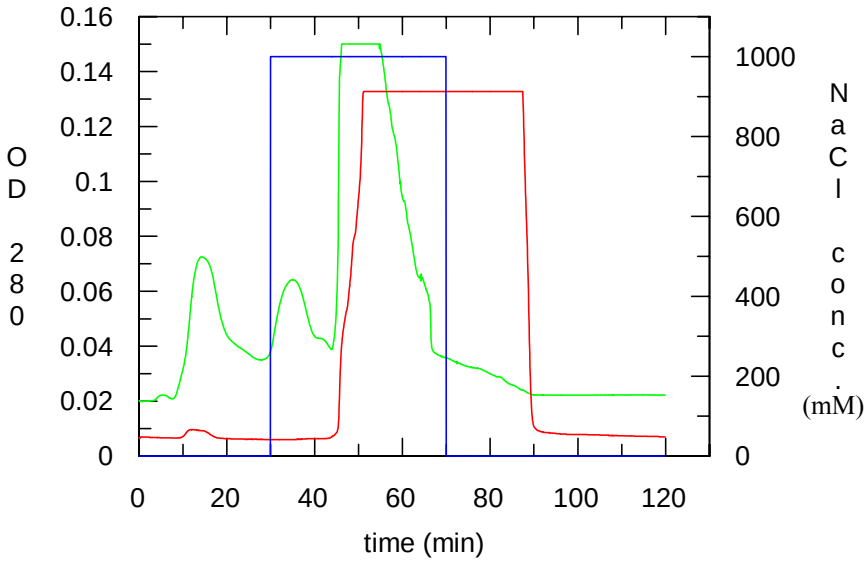


Figure 3-3-5 HiTrap Q column chromatography of intra-cellular chitosanases.

The blue line is concentration of NaCl in program; and the red line is actual concentration of NaCl in colunm. The green line is UV absorption at 280 nm.

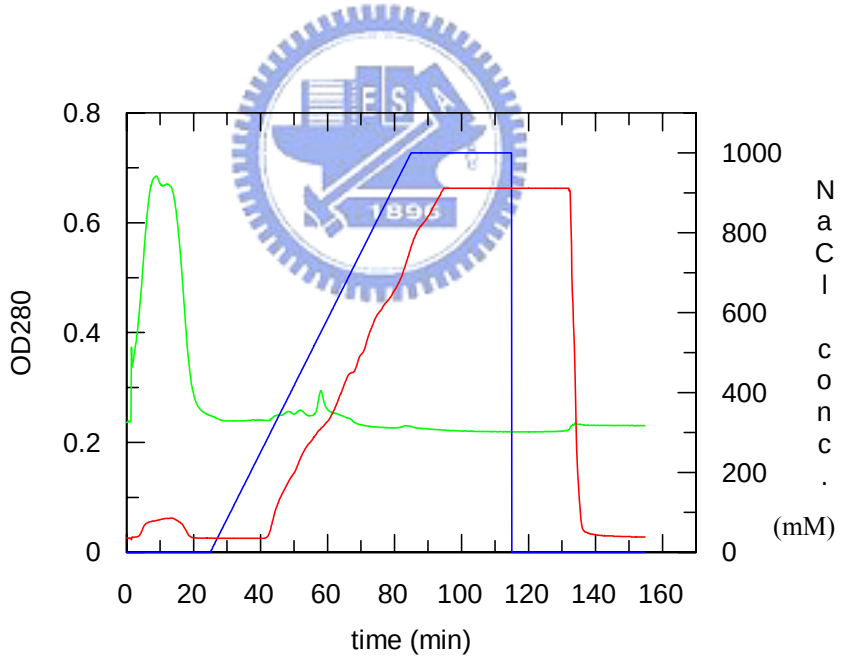


Figure 3-3-6 HiTrap SP column chromatography of intra-cellular chitosanases.

The blue line is concentration of NaCl in program; and the red line is actual concentration of NaCl in colunm. The green line in perform in UV absorption at 280 nm.

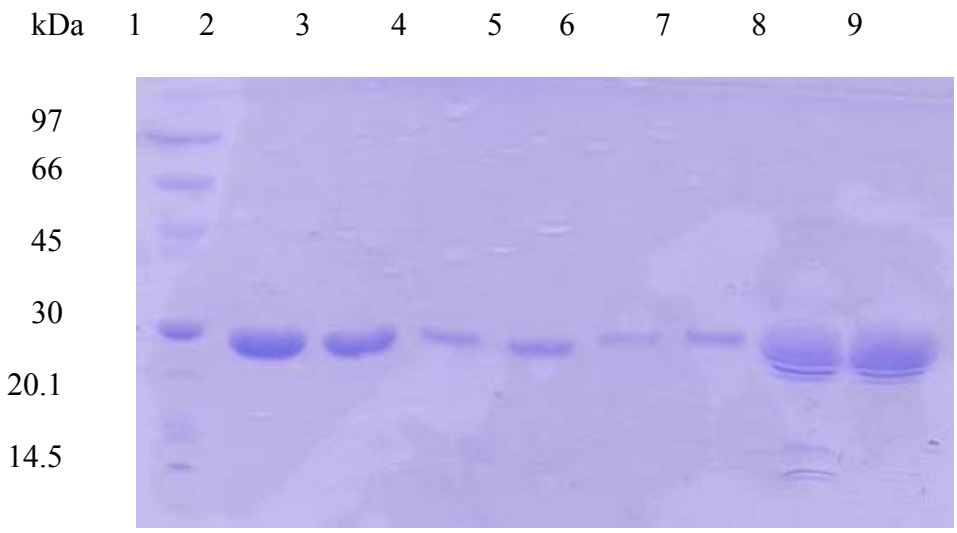


Figure 3-3-7 SDS PAGE of purified chitosanases from intra-cellular protein. Lane1, Protein marker; Lane 2, 3 wild type; Lane 4, D160N; Lane 5, E169Q ; Lane 6, D160E; Lane 7, E169Q ; Lane 8, D160C; Lane 9, E169C.

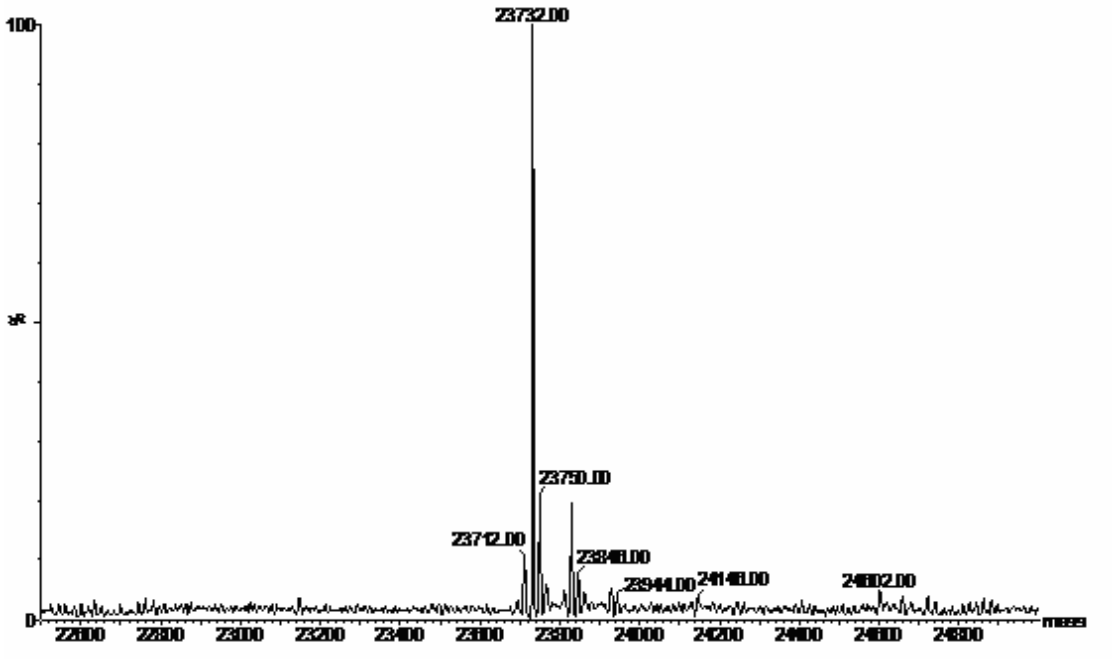


Figure 3-3-8 Mass spectrum of intra-cellular chitosanase. The molecular weight of D160E is analyzed by mass to be 23732.

Part Ⅱ Characterization of enzyme

3-4 Analysis of activity and structure

3-4-1 Comparison of catalytic activity

In order to identify the essential residues of this enzyme, 13 mutants were constructed on the basis of the 10-conserved residues and few of others. The preliminary tests on the activity of all mutants by DNS method were employed. Activity assay showed that D59N, D76N, D78N, D80N, D112N, D114N, E126Q, D194N and D229N retained significant activity (>60 %) as compared with that of the wild-type enzyme. The results were listed in table 3-4-1. Also, all of these active mutants release $(GlcN)_3$, $(GlcN)_4$, and $(GlcN)_5$ as major products. The pattern of product distribution is virtually identical to that of native chitosanase (data not shown). We conclude that these positions are unlikely to function as the essential group of *Aspergillus* chitosanase. In contrast, the activities of mutant D160N and E169Q were significantly reduced (less than 0.1 %). Obviously, Asp^{160} and Glu^{169} are likely to be the candidates mediating the catalytic activity of chitosanase if the activity loss is not due to the collapse of protein structure. 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.

Table 3-4-1 Comparison all mutants' activity with wild type (served as 100%).

Mutation sites	Wild type	D59N	D76N	D78N	D80N	D112N	D114N
Chitosanase activity	100%	96%	65%	62%	77%	102%	109%
Mutation sites	E126Q	D160N	E169Q	D191/ 194N	D229N	D160E	E169D
Chitosanase activity	108%	~0%	~0%	108%	80%	88%	40%

3-4-2 Structural analysis

Since D160N and E169Q lost nearly all enzymatic activity, the structure information of these mutants need to be evaluated before any conclusion was drew. Several spectroscopic methods, such as CD, fluorescence, and UV, were used for examining the secondary structure of these enzymes.

CD spectra of D160N, E169Q and D191/194N (active mutant) were measured and compared with that of wild type CNS. The overlaid spectra were shown in figure 3-3-1. Explicitly, the CD spectra indicate that no significant perturbation on structures for all mutants as compared with that of wild type enzymes.

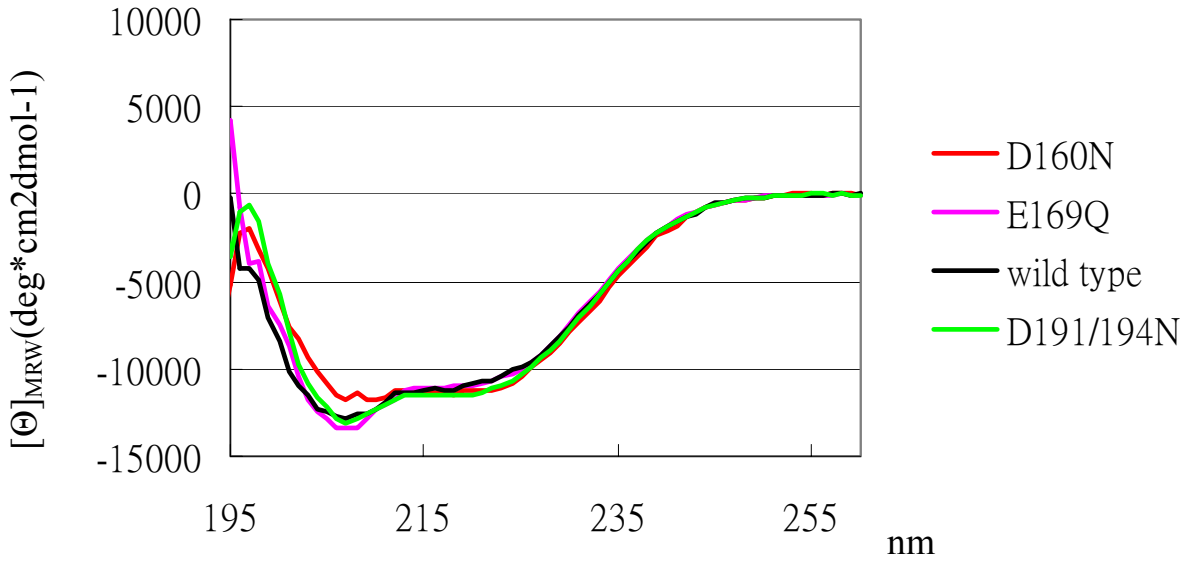


Figure 3-4-1 CD spectrum of wild type and mutants. Secondary structure of enzymes, D160N and E169Q (without activity), are compared with enzymes, wild type and D191/194N (with activity).

Note: The sophisticated experiments for this section are described in chapter 7-1 and 7-2.

3-5 Investigation of anomeric configuration by NMR

^{1}H-NMR spectroscopy has been used to investigate the stereoselectivities of various glycohydrolases such as cellulases, xylanase, β-xylosidase and many others. This study may reveal the information about the active site topology. Two different types of catalytic features of glycohydrolases have been discussed in chapter 1: retention and inversion of the anomeric configuration.

The first NMR study of Family 75 chitosanase mechanism was investigated by Dr. Cheng (Cheng, 2003) in our lab. The result showed that Family 75 chitosanase catalyzes the hydrolysis of chitosan with the inversion anomeric configuration. However, we are interested in the anomeric configuration of D160E recombinant chitosanase, because D160E not only contained the mutant of essential group Asp^{160} but also retained the chitosanase activity. To understand the catalysis of D160E mutant (purified from intra-cellular soluble protein), we performed a time course NMR investigation on enzymatic hydrolysis of chitosan (shown as figure 3-5-1). A series of partial ^{1}H-NMR spectra (4.4 ~ 5.6 ppm) recorded at 8, 16, 27, 35, 45, and 55 minutes after the addition of chitosanase. The ^{1}H-NMR spectrum of chitooligosaccharide mixture (data not shown) revealed two doublets centered at δ = 4.89 ppm (J = 8.5), and δ = 5.43 ppm (J = 3.15 Hz), corresponding to the *β* and α of the anomeric protons at C1-proton of the non-reducing end sugar, respectively. When enzyme was added to chitosan, the α anomeric proton (centered at 5.43 ppm) increased instantly, whereas the corresponding *β* anomeric proton (centered at 4.89 ppm) slowly increased with time. At 8 minutes, the ratio of α-form to *β*-form is 77:23. At 16, 27, 35, 45, 55 minutes, the α and β anomeric signals constantly decreased from 77:23, 62:38, 55:45, 53:47 to 52:48. This

NMR investigation showed that D160E recombinant chitosanase is an inverting enzyme, identical to its wild type enzyme. The transglycosylation activity of D106E mutant was also investigated by incubating 1% chitosan solution containing 10% ethanol with enzyme. Since no ethyl glycosides can be detected by mass spectrometry, we concluded that the D160E mutant has no significant transglycosylation activity.

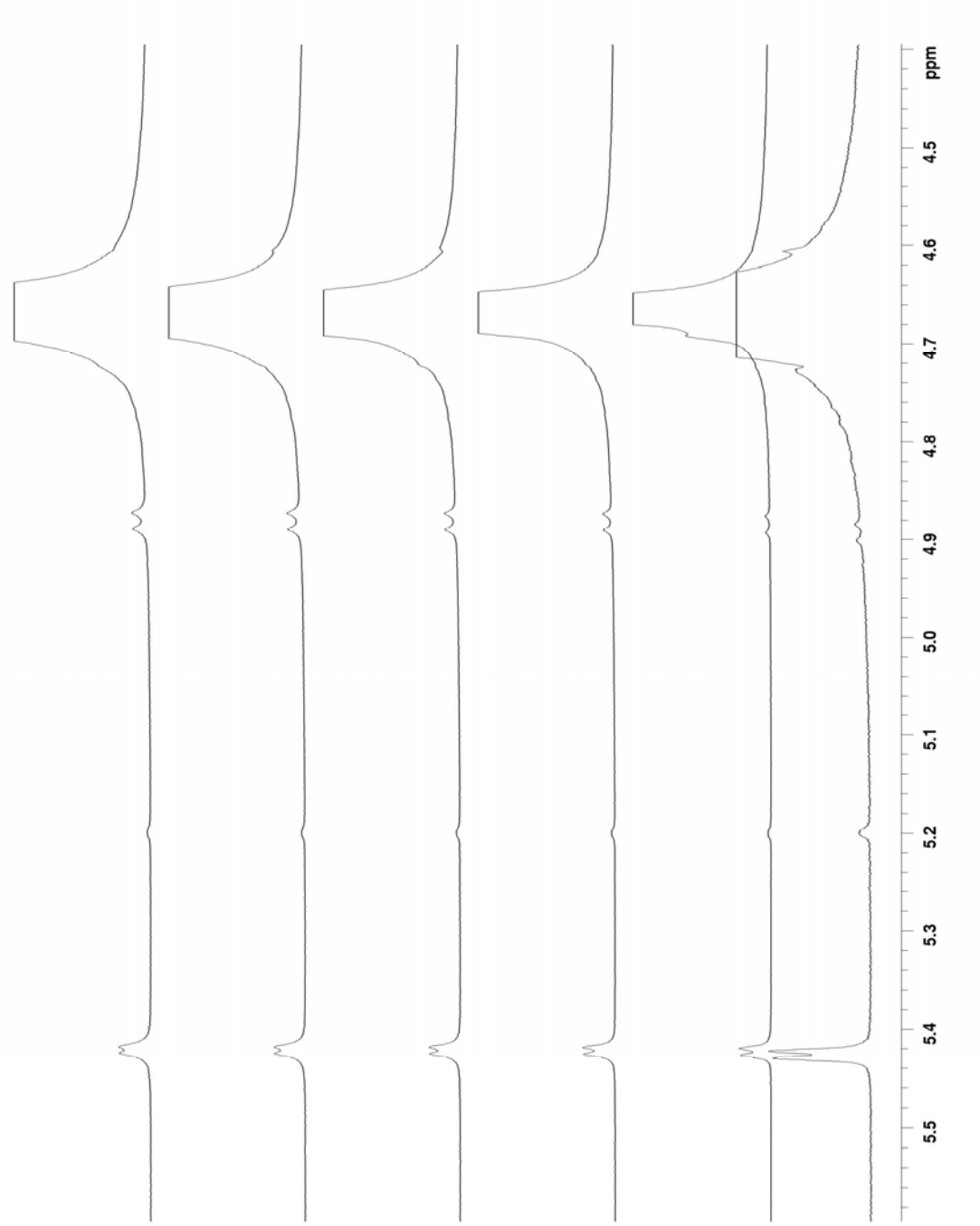


Figure 3-5-1 ^{1}H-NMR spectra during hydrolysis of chitosan by chitosanase at 35 ℃.

The ^{1}H-NMR analysis of the stereoselectivity of the D160E enzymatic hydrolysis of chitosan at 35 ℃. The initial substrate is shown in spectrum A. Spectra were obtained over 8, 16, 27, 35, 45 and 55 min in the presence of chitosanase. The signals at δ= 4.89 ppm (*J*=8.5 Hz), and δ= 5.43 ppm (*J*=3.15 Hz) correspond to α- and β-anomers of C1-H of the reducing end sugar. The ratios of α and β anomeric signals are 77:23, 77:23, 62:38, 55:45, 53: 47 and 52:48 in order.

3-6 Identification of hydrolyte pattern by LC/MS/MS

3-6-1 Identification of subclass

Three types of subclass chitosanase were described in chapter 1. The products obtained from the catalysis of wild type recombinant chitosanase (purified from intra-cellular soluble protein) with lower DDA (degree of deacetylation) chitosan as substrate were analyzed by LC/MS. Five major oligosaccharides with m/z 341, 383, 502, 544 and 705, corresponding to a chitobiose, a monoacetylated chitobiose, a chitotriose, a monoacetylated chitotriose, a monoacetylated chitotetraose, respectively, were found (figure 3-6-1.) The sequences of these acetylated chitooligosaccharides are further determined to be GlcN-GlcNAc, GlcN-GlcN-GlcNAc and GlcN-GlcN-GlcN-GlcNAc by chemical methylation on the reducing end of the oligosaccharides following ESI/MS/MS analysis. As shown in figure 3-6-2(a), the tandem mass analysis of the peak of m/z 383, the methylated chitobiose as shown in 3-6-3(a), exhibit the fragment with m/z 236 which can only be derived from GlcN-GlcNAc-OCH_3. Similarly, the mass spectrum as shown in figure 3-8-2(b) and 3-8-2(c), the tandem mass analysis of monoacetylated chitotriose (m/z 544) and a monoacetylated chitotetraose (m/z 705) exhibit the fragment derived from GlcN-GlcN-GlcNAc-OCH_3 (figure 3-8-3(b)) and GlcN-GlcN-GlcN-GlcNAc-OCH_3 (figure 3-6-3(c)), respectively. Although other oligosaccharides may be present, they were not significant. According to these sequences, *Aspergillus* chitosanase is classified to subclass Ⅰ which can hydrolyze the linkages of GlcN-GlcN and GlcNAc-GlcN.

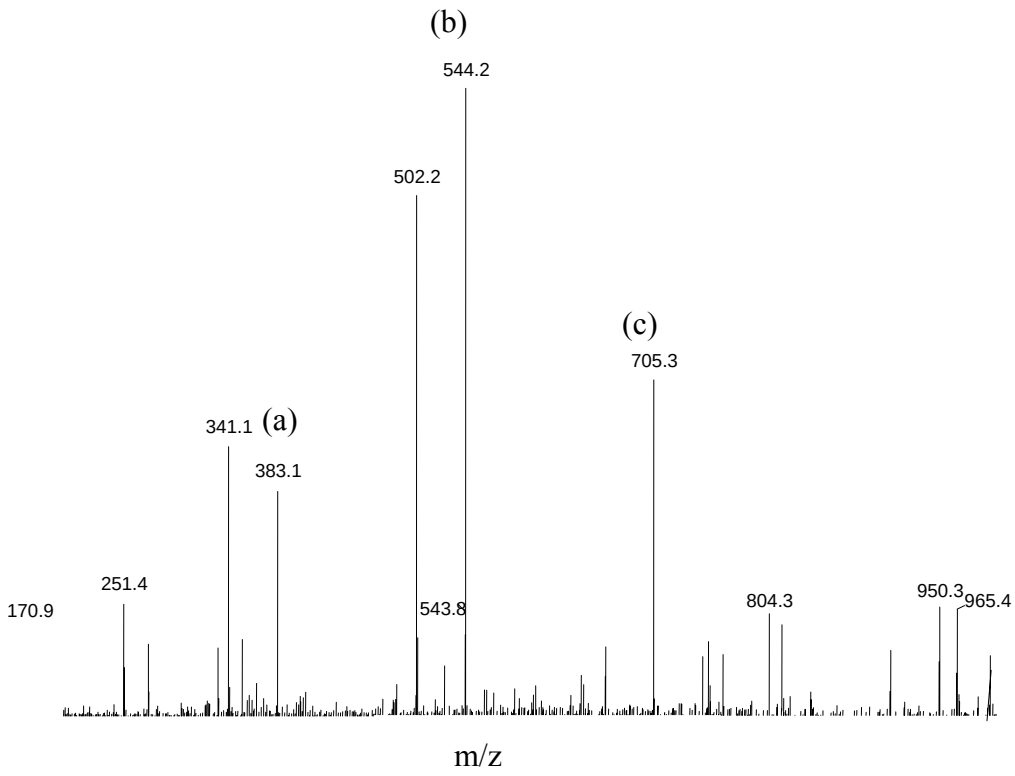


Figure 3-6-1 Mass spectrum of the enzymatic hydrolytes catalyzed by wild type recombinant chitosanase.

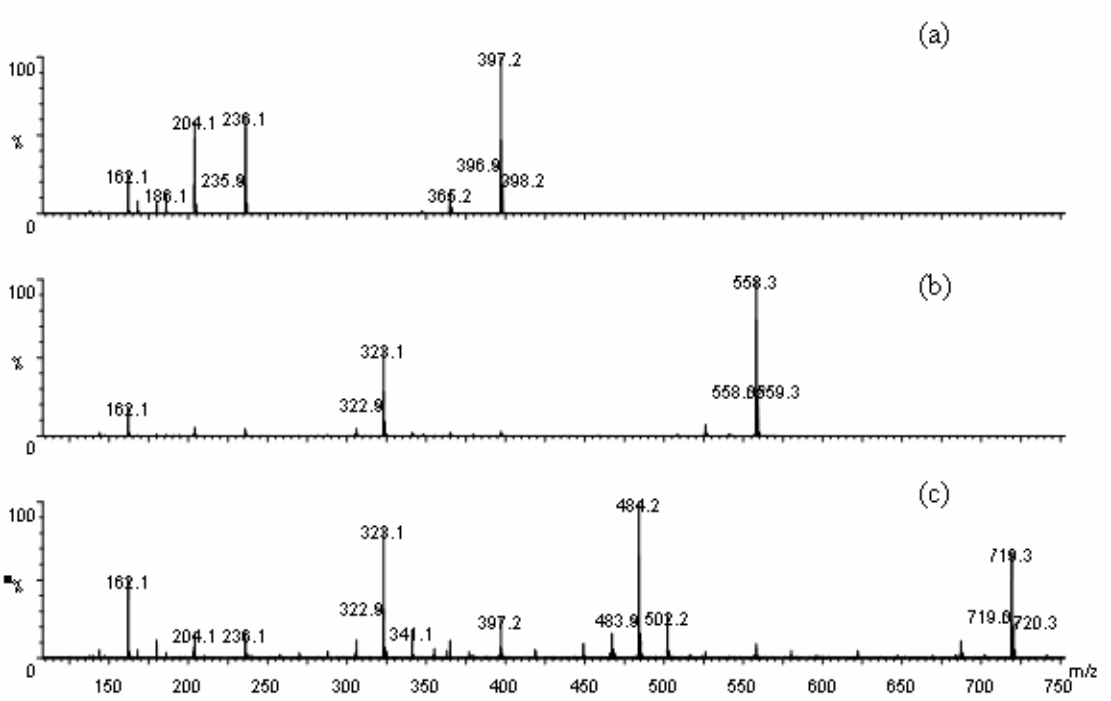
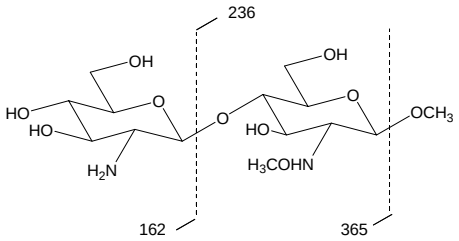
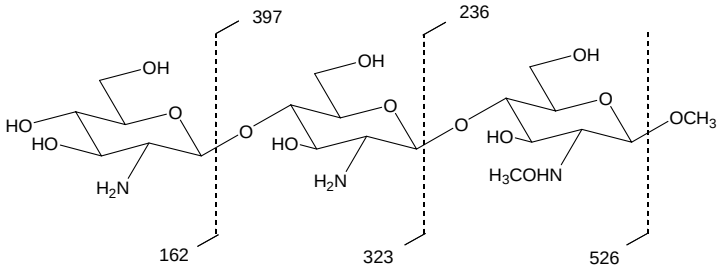


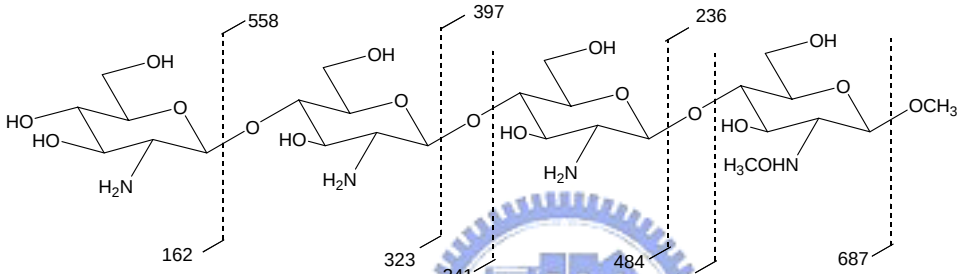
Figure 3-6-2 ESI/MS/MS analysis of (a) (b) (c) peaks in figure 3-8-1. The (a)(b)(c) peaks in fig. 3-6-1 are shown corresponding to (a)(b)(c) in this figure.



(a)



(b)



(c)

Figure 3-6-3 Illustration of all peaks in figure 3-6-2. The sub-figure (a)(b)(c) are corresponding to figure 3-6-1 and 3-6-2.

3-6-2 Identification of subsite

As descibed in chapter 1, endo-chitosanases present two types of substrate binding characters : [(-3), (-2), (-1), (+1), (+2), (+3)] and [(-2), (-1), (+1), (+2), (+3), (+4)]. When chitosan hexamer was hydrolyzed by wild type recombinant chitosanase (purified from intra-cellular soluble protein), the third glycosidic linkage is split and produced $(GlcN)_3$, shown as figure 3-6-4. This product distribution $(GlcN)_3$>>$(GlcN)_2$=$(GlcN)_4$ is resembling to that obtained by goose egg white lysozyme and *Streptomyces* sp. N174 chitosanase (Fukamizo et al., 1995; Fukamizo, 2000). Besides, the rate of degradation decreased with a decrease of substrate-binding cleft of the chitosanase can accommodate at lease six GlcN residues. These evidences suggested that the subsite structure of *Aspergillus* chitosanase is in the category of [(-3), (-2), (-1), (+1), (+2), (+3)].

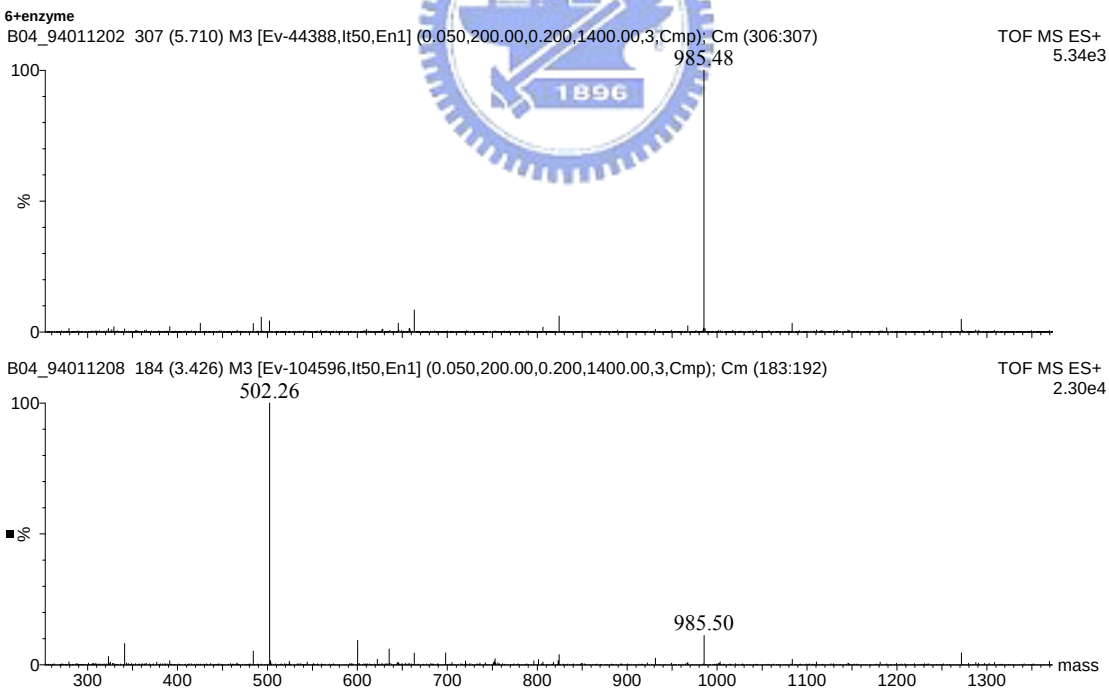


Figure 3-6-4 Mass spectrum of chitosan hexamer and its hydrolyte catalyzed by wild type chitosanase. (a)Chitosan hexamer only; (b)Hydrolysates of chitosan hexamer by wild type chitosanase.

3-7 Investigation of the binding properties of chitosanases by SPR

The activated Au chip was labeled with wild type and D160N mutant (purified from intra-cellular soluble protein) separately at flow-cell 1 (FC1) and flow-cell 2 (FC-2) of SPR system. The analyte was injected to flow through FC1 and FC2. The senorgrams of SPR with 10 mM trimer and 10 mM tetramer as analytes were shown as figure 3-7-1 and 3-7-2, respectively. The k_a, k_d and K_D values for trimer and tetramer were calculated and listed in Table 3-7-1. Binding curves of wild type and D160N towards trimer and tetramer were very similar indicating the binding powers of the two enzymes are comparable. Kinetic data suggest that trimer is bound less tightly than tetramer. Also, it seems that D160N mutant possesses stronger binding affinity than wild type enzyme. We conclude that the active site of D160N remains nearly intact. The activity loss of D160N is thus most likely due to the absence of essential group not the structural perturbation.

Table 3-7-1 Kinetic data from SPR investigation

	k_a (1/M*s)	k_d (1/s)	K_D
D160N-10 mM 3mer	72.4	5.47e-3	76 μM
wild-10 mM 3mer	53.4	7.24e-3	136.6 μM
D160N-10 mM 4mer	77.9e3	1.71e-3	22 nM
Wild-10 mM 4mer	1.24e4	1.62e-3	130 nM

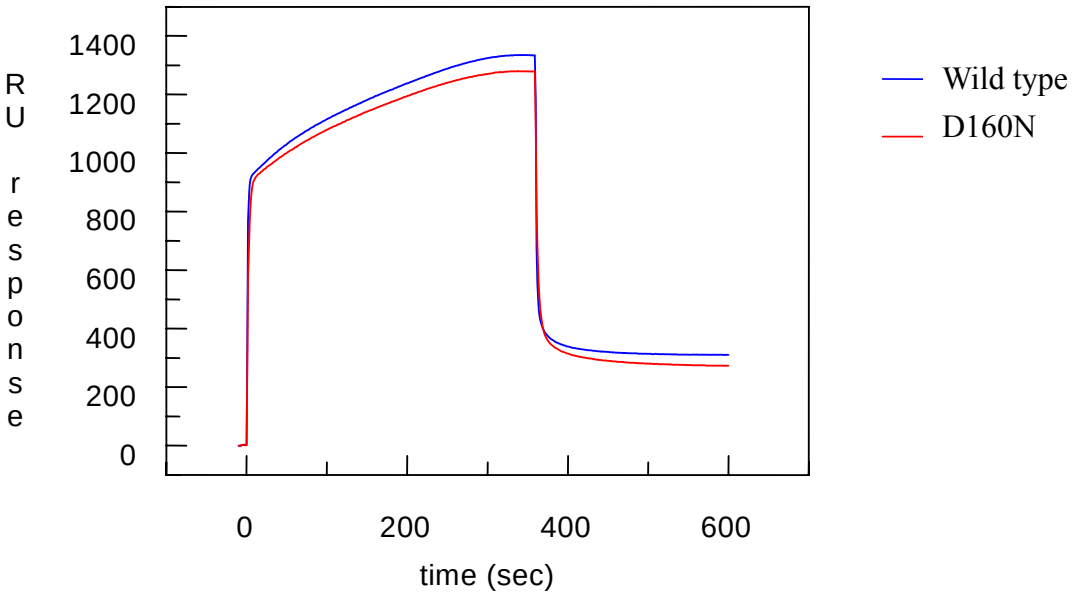


Figure 3-7-1 SPR spectrum of wild type or D160N immobilized on Au chip and 10 mM trimer as analyte. The blue line is the RU response of wild type; the red one is D160N.

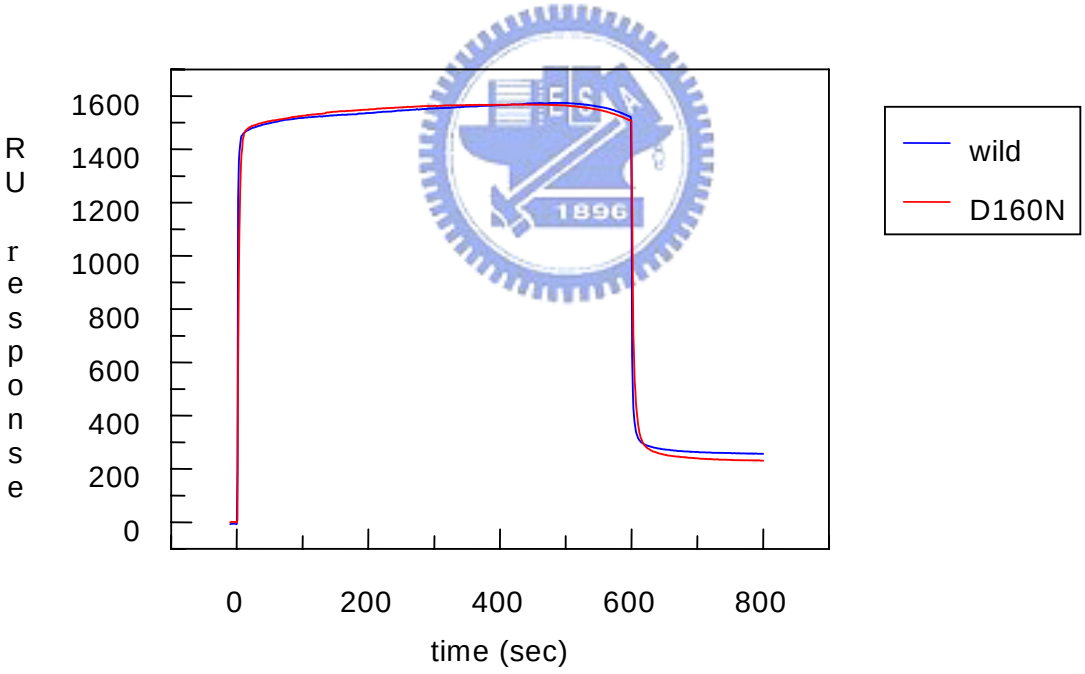


Figure 3-7-2 SPR spectra of wild type and D160N immobilized on Au chip and 10 mM chitosan tetramer as analyte. The blue line is the RU response of wild type; the red one is D160N.

Note: The opposite designed experiment for SPR is described in chapter 7-3.

3-8 Prediction of *Aspergillus* chitosanase topology

3-8-1 Experimental prediction

The native *Aspergillus* chitosanase is atypically stable with superior 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 23462 Dalton, 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 (described in 3-9). 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 23^{+} charges was observed in the ESI mass spectrum (as shown in figure 3-8-1). This phenomenon is explicable through the three-dimensional structure of this enzyme by its disulfide bonds and impacted intensity (Russell, 1994). These data indicate that the protein structure of *Aspergillus* chitosanase is highly compact and hardy. These intrinsic properties might allow the refolding process to be effective.

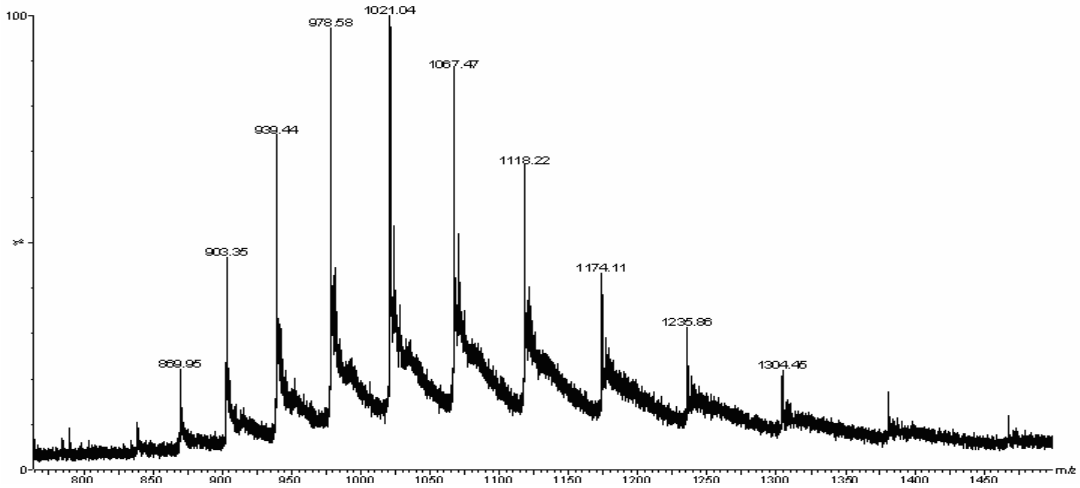


Figure 3-8-1 Mass spectrum of native chitosanase with maximal signal at 23 charge and m/z 1021.

3-8-2 Theoretical prediction

As reported by Withers and his colleagues, the distance between the two catalytic residues is closely related to the catalytic mechanism (Wang *et al.*, 1994). In the case of retaining enzymes, the average distance between two catalytic residues is about 4-5 Å, while the distance is about 10-11 Å in inverting enzymes (Davies *et al.*, 1995). Since *Aspergillus* chitosanase had been identified into inversion anomeric configuration (Cheng, 2003), the distance between two catalytic residues, Asp^{160} and Glu^{169}, will be 10-11Å. Regardless of whether the enzyme is inverting or retaining, the overall topologies of the active sites of chitosanases fall into only three general classes, (Davies *et al.*, 1995). These three topologies (figure 3-8-2) can, in principle, be built on the same fold, with the same catalytic residues.

Pocket or crater

This topology (Fig. 3-8-2a) is optimal for the recognition of a saccharide non-reducing extremity and is encountered in monosaccharidases and in exopolysaccharidases.

Cleft or groove

The 'open' structure (Fig. 3-8-2b) allows a random binding of several sugar units in polymeric substrates and is commonly found in endo-acting polysaccharidases.

Tunnel

This topology (Fig. 3-8-2c) arises from the previous one when the protein evolves long loops that cover part of the cleft. Found so far only in cellobiohydrolases, the resulting tunnel enables a polysaccharide chain to be

threaded through it.

Because the *Aspergillus* chitosanase is endo type, its structure is believed to be the cleft or groove topology. The structure of the active site is open so that can cleave substrate into various size in length of oligosaccharides.

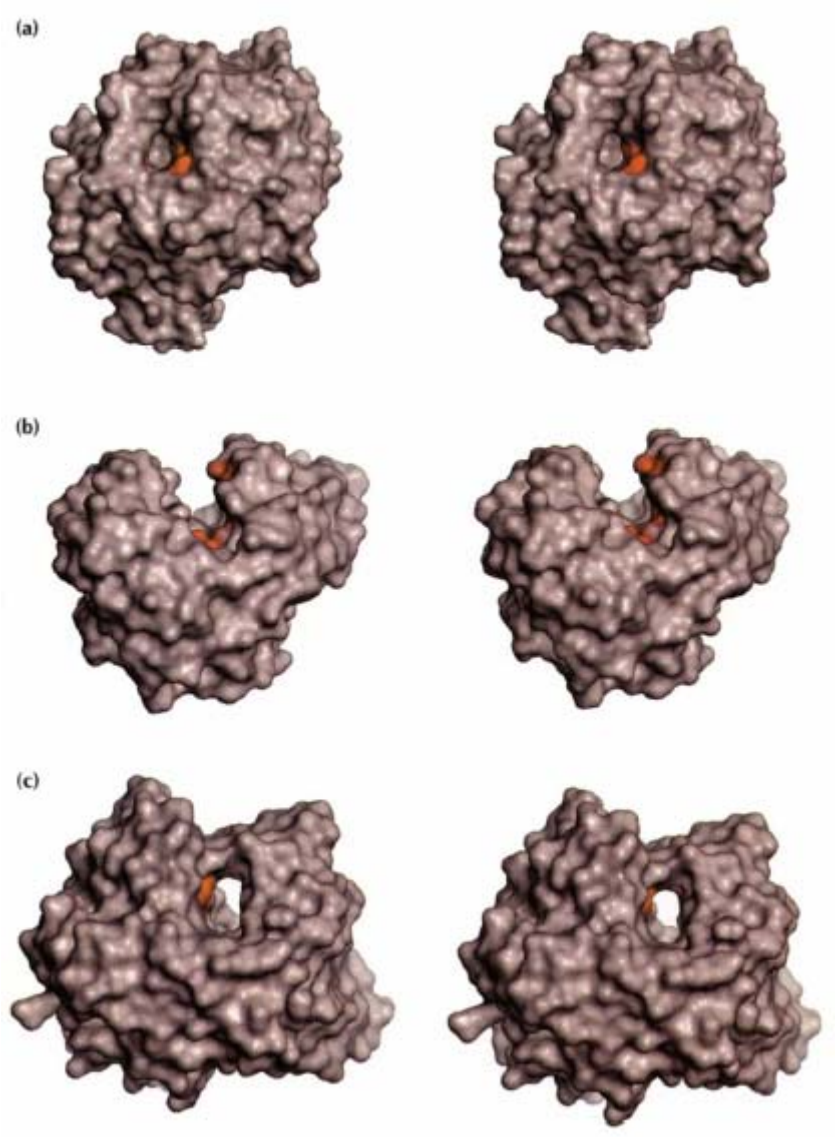


Figure 3-8-2 Three forms of active site found in glycosyl hydrolases.

(a) Pocket or crater; (b) Cleft or groove; (c) Tunnel

Note: The computational predictions are described in chapter 7-4.

3-8-3 Prediction of the roles of Asp^{160} and Glu^{169}

The remaining question is the specific function, general acid and general base, of D160 and E169. Though no conclusive evidence for answering this question at present, the statistical data can provide clue for reaching a possible prediction. Many studies have shown that the glycosyl hydrolase family, when two distinct carboxylic-acid residues collaborate for catalysis, Asp 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 (Chir *et al.*, 2002), GH-13, GH-33, GH-68, GH-71, and GH-77 and inverting enzymes from GH-8 (Collins *et al.*, 2002), GH-9, GH-25, GH-46 (Boucher et al., 1995; Fukamizo *et al.*, 1995) 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 essential groups in a glycohydrolase. Comparison of the relative acidity of Asp and Glu in a similar environment, such as a catalytic core, can provide information useful for understanding the natural preference. The pKa of Asp is less than that of Glu according to an empirical relationship (Forsyth *et al.*, 2002) or theoretical calculations (Juffer *et al.*, 1998); 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. The Asp^{160} and Glu^{169} are likely to function as the general base and the general acid, respectively, in the catalytic center of *Aspergillus* chitosanase, but this prediction still requires proof through extensive investigation such as with active-site affinity labeling or from its crystal structure.

3-9 Chemical label

As described in chapter 2, chemical label are used for various purposes. Here in, three purposes are attended including estimation of disulfide bond, recovery of chitosanase activity, and label of inhibitor.

3-9-1 Estimation of disulfide bond

In our case, *Aspergillus* chitosanase has 6 cysteines in amino acid sequence. Therefore, we have to estimate how many free thiol and disulfide bond are there in *Aspergillus* chitosanase.

The result of UV spectrum (shown in figure 3-9-1) is indicated that there is no free thiol to interact with DTNB, indicating the formation of disulfide bond(s) or the free thiol residue(s) may not be accessible.

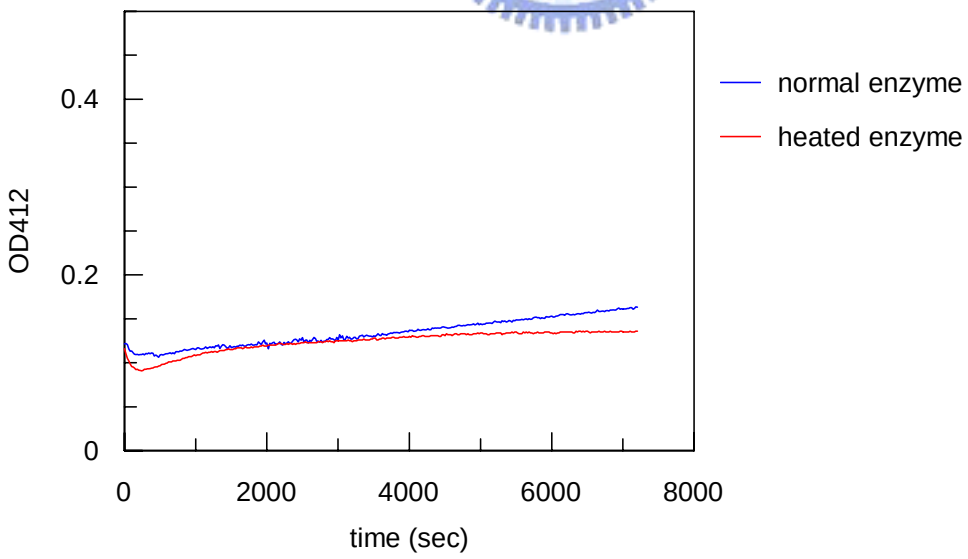


Figure 3-9-1 UV spectrum of *Aspergillus* chitosanase with DTNB. Normal and heated form chitosanase are separately incubated with 3 mM DTNB at 37 ℃ and detected 412 nm by UV spectrometer.

3-9-2 Specific site of chitosanase labeled by inhibitor

The inhibitor which was designed and synthesized by our laboratory was a derivative of chitobiose (chitin dimer) modified with a bromine. In our design, the inhibitor is assumed to be attacked by the catalytic amino acid in enzyme shown in figure 3-9-2, bromine as a leaving group and then stuck at the enzyme. Finally, the labeled enzyme will be digested by protease and analyzed by peptide mapping to find out which amino acid is labeled by the inhibitor.

As the results shown in figure 3-9-3, peak 23789 and peak 24252 differ in 463 Da which exactly equal to the inhibitor molecular weight except the leaving group. Although the enzyme molecular weight has error, the spectrum still can give the clue that only one molecule the inhibitor can attact and stick onto enzyme. The result suggested that the inhibitor label specifically at one site, but not randomly at many sites in enzyme. The peptide mapping to localize the residue with label is in progress.

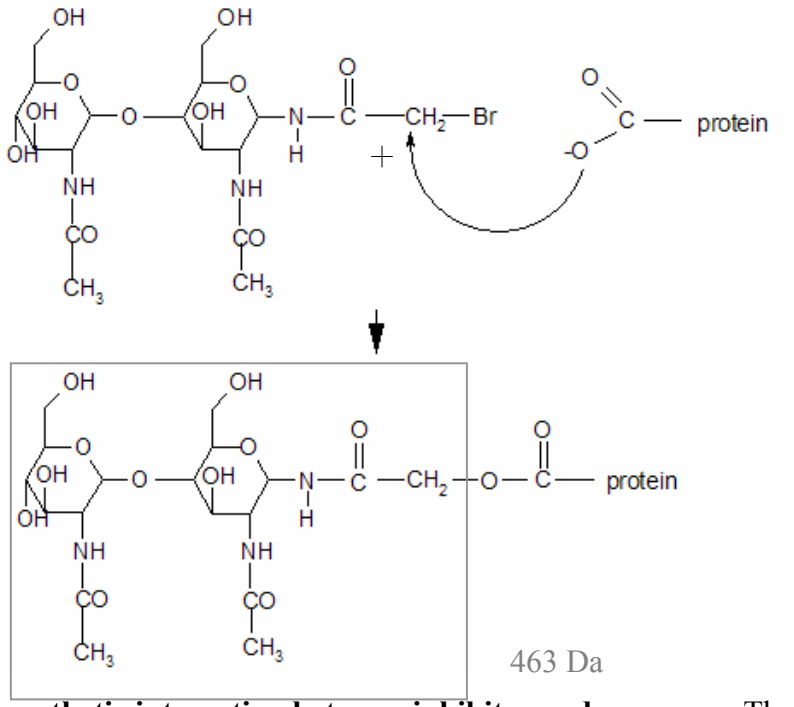


Figure 3-9-2 The hypothetic interaction between inhibitor and enzyme. The hypothetic mechanism is that the carboxyl group of enzyme will attack and stick to inhibitor. The molecular weight of enzyme will be plus 463 Da.

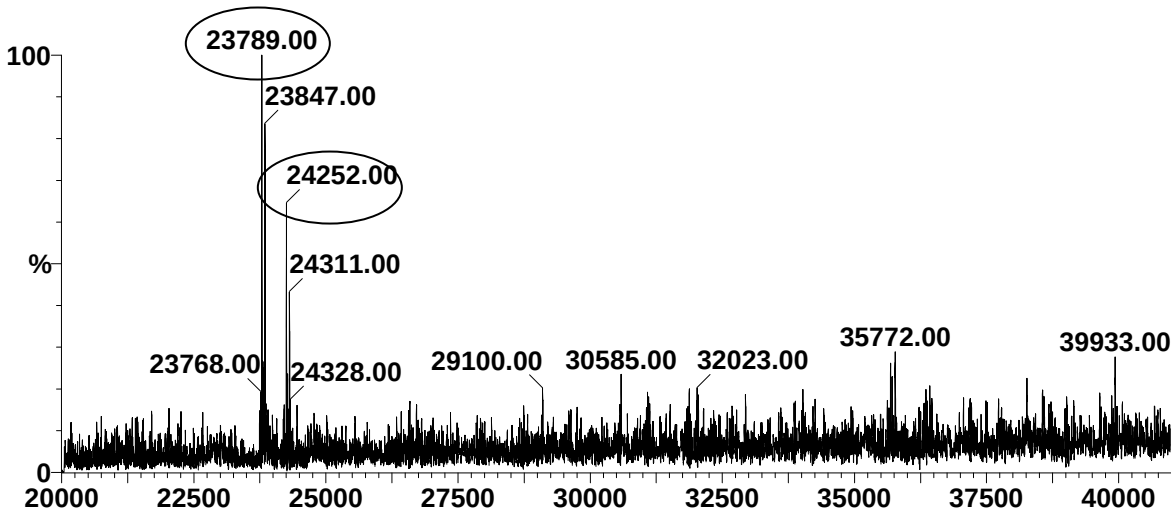


Figure 3-9-3 Mass spectrum of enzyme labeled with inhibitor. The peak-23789 is the original molecular weight of *Aspergillus* chitosanase; the prak-24252 is the molecular weight of *Aspergillus* chitosanase (23789) plus inhibitor (463).

Chapter 4 Conclusion

Chitosan is the principal fiber polymer of the cell wall in addition to chitin in certain strains of fungi. Owing to the increasing number of immunosuppressed patients and an increase in the severity of immunosuppressive therapies, continuously inhaling conidia by humans has become more and more harmful. *Aspergillus fumigatus* is now the most prevalent airborne fungal pathogen, and causes severe and usually fatal invasive infections, for example aspergillosis (IA), in immunocompromised hosts in developed countries (Latgé, 2001). Chitin is the major structural component of its cell wall and this organism is known to secrete extracellular chitin-related proteins with enzymatic activities of chitinase, chitosanase and transglycosylation (Xia *et al.*, 2001). Therefore, either to understand the pathogenesis associated with *A. fumigatus* infection or to create a new target for drug design, the chitinolytic system of this organism is intriguing. In addition, extracellular stress-related plant chitosanase can lyse fungal spores of some plant pathogens (Grenier *et al.*, 1990). As chitinous polymer is absent in mammals, the chitolytic enzyme has thus been proposed as prospective targets for novel antifungal agents. *Aspergillus* chitosanase, which is remarkably resistant to physical and chemical manipulation, may be a good candidate as a new alternative agent against mycosis.

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 (data not shown). This phenomenon is explicable through the three-dimensional structure of this enzyme by its disulfide bonds and impacted intensity. 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; Tsai *et al.*, 2004, Tang *et al.*,

2003). 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 (Chir *et al.*, 2002), GH-13, GH-33, GH-68, GH-71, and GH-77 and inverting enzymes from GH-8 (Collins *et al.*, 2002), GH-9, GH-25, GH-46 (Boucher et al., 1995; Fukamizo *et al.*, 1995) 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 (Forsyth *et al.*, 2002) or theoretical calculations (Juffer *et al.*, 1998); 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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Appendix

A few of preliminary results and miccerlaneon were summarized in the following paragraphes. More sophisticated studies need to be performed.

A. Comparison of all mutants

Ⅰ Further comparison of hydrolysis activity of all mutants

Preliminary activity assay showed that D59N, D76N, D78N, D80N, D112N, D114N, E126Q, D194N and D229N retained significant catalytic activity (>60 %) compared with that of the wild-type enzyme. Hence, these nine positions are unlikely to function as the essential group of *Aspergillus* chitosanase. In contrast, the activities of mutant D160N and E169Q were significantly reduced (less than 0.1 %). Obviously, Asp^{160} and Glu^{169} are likely to be the essential groups of chitosanase for the catalytic activity if the activity loss is not due to structural collapse. Therefore, further activity assay may focus on the comparison of these two mutants with others which have activities.

The precise activity evaluation proceeds from 2 parts. Chitosan and enzymes in catalytic reactions are mixed which proceeds for 100 hours in the ratio of 1:1 (shown in figure A1) and 1: 0.01 (shown in figure A2), separately. Then, we combine the two results by pick up the data of the no activity lines (D160N and E169Q) from figure A1 and the data of the activity lines (besides D160N and E169Q) from figure A2. Here, the absorption data from the ratio of 1: 0.01 (i.e. figure A2) have to multiply 100 in order to restore the ratio. The processed data is shown in figure A3.

As the results of these three figures, especially figure A3, D160N and E169Q are severely lost chitosanase activity. Therefore, we propose that Asp^{160} and Glu^{169}

are essential amino acids.

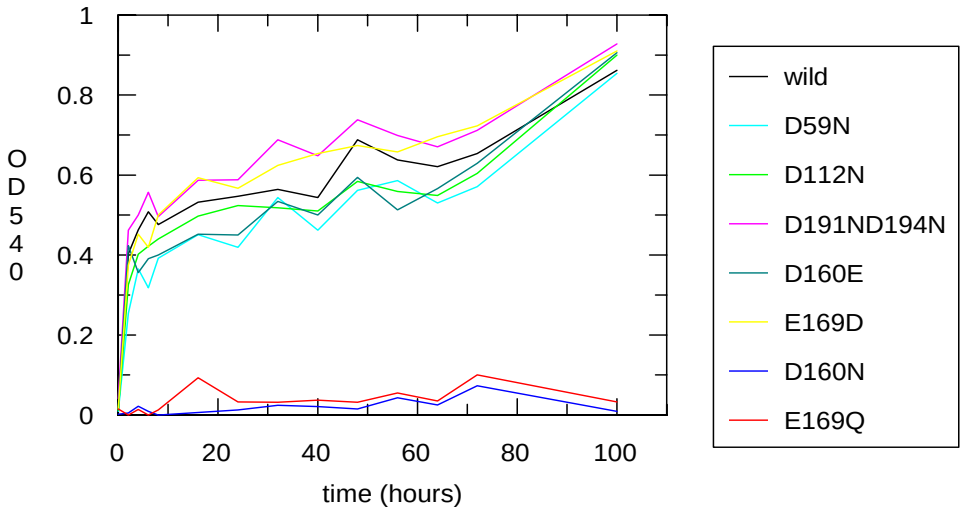
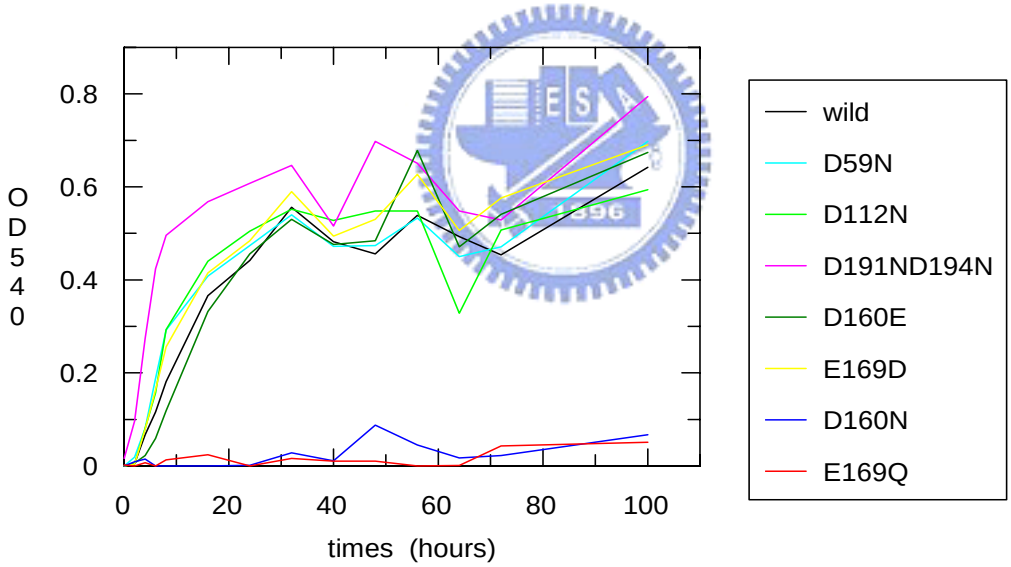


Figure A1 Activity test by DNS method. (chitosan : enzyme = 1 : 1)



citosan : enzyme = 100 : 1

Figure A2 Activity test by DNS method. (chitosan : enzyme = 1 : 0.01)

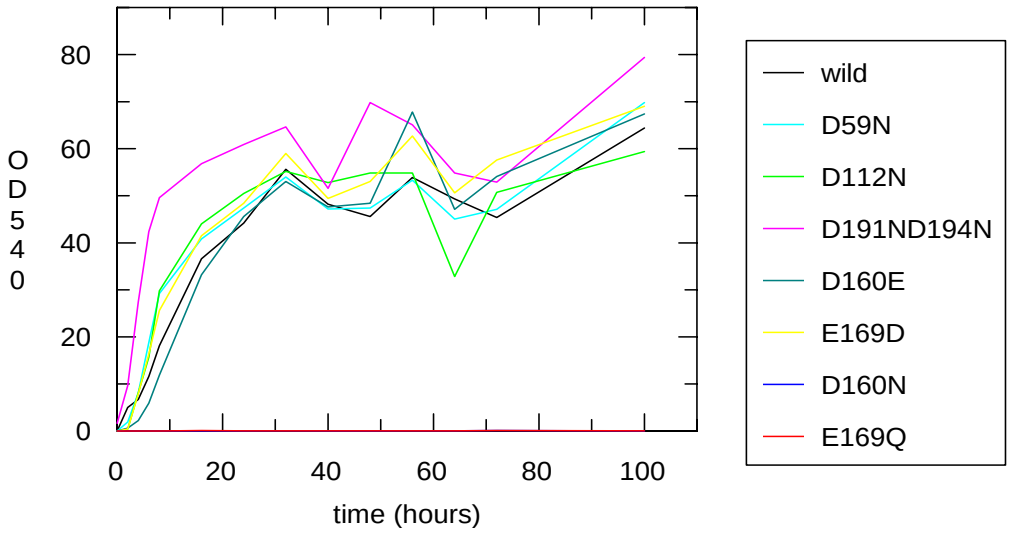


Figure A3 Combinational figure from figure 7-1-1 and 7-1-2.

Ⅱ Comparison of secondary structure

To identify that the influence of mutants, D160N and E169Q, do destroy the catalytic center but not disturb the protein structure, we have to check the secondary structure of these enzymes. Therefore, CD, fluorescence, and UV spectrum are employed to examine whether all types of enzymes have the same features.

Additionally, we discover that the recombinant protein purified from inclusion body will change its conformation with time. Because the recombinant protein express as inclusion body, we set up a strategy in order to obtain the correct folding enzyme which has activity. After solving the inclusion body in 5M urea, the recombinant enzyme structure will be destroyed. However, wild type and some of mutants will regain their activity with time, if we just put it in 4 ℃ without dialysis. Thus, we assume that the recombinant protein will self refold its structure and restore its activity with time if the enzyme solution is put

statically at 4 ℃. Therefore, CD and fluorescence are employed to trace the structure change of wild type enzyme and to prove the hypothesis.

CD spectrum

CD spectra for these recombinant proteins are obtained. These data are presented in mean residues molar ellipticity ($[\Theta]_{MRW}$) shown as A4.

CD spectra of D160N and E169Q are similar to those of recombinant enzyme with significant chitosanase activity including wild type and D191/194N. Explicitly, these CD spectra indicate that none of these mutations seriously modified the tertiary structure.

Separately, to prove the hypothesis of self folding at 4 ℃ with time, the wild type enzyme is laid aside at 4 ℃and then examined its secondary structure. After 1 day, 14 days, and 28 days stocked urea treatment, chitosanase was via SP column and screened. Their CD spectra are compared with native chitosanase shown as figure A5. As the spectrum, we confirm that the wild type enzyme tends to recover gradually its conformation to the native chitosanase. The secondary structure of recombinant protein almost approaches to that of native protein after 28 days.

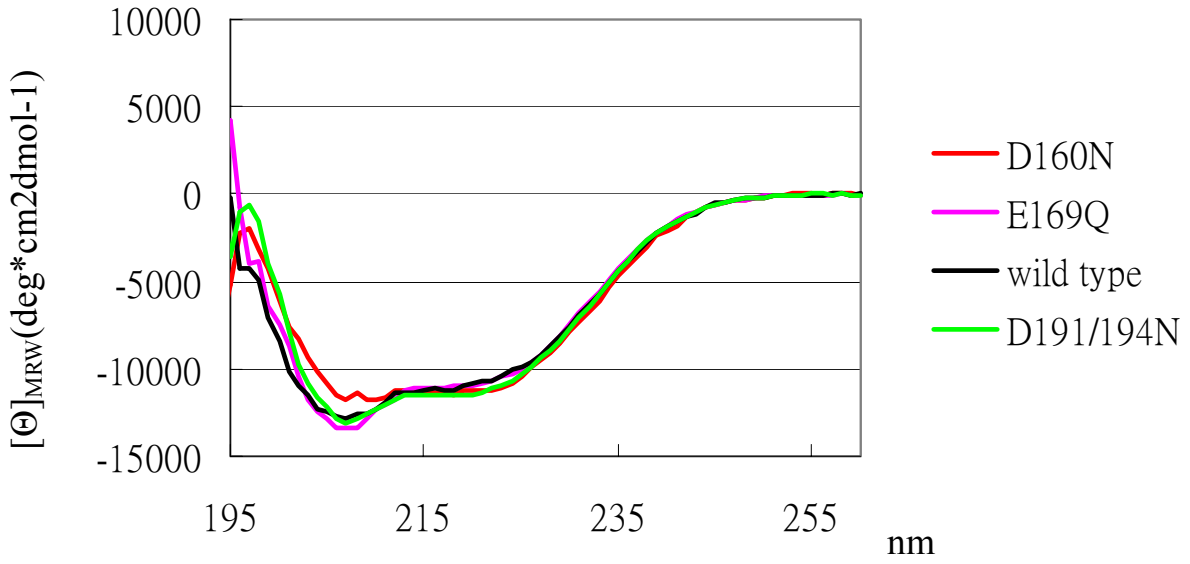


Figure 7-1-4 CD spectrum of wild type and mutants.

Secondary structure of enzymes, D160N and E169Q (without activity), are compared with enzymes, wild type and D191/194N (with activity).

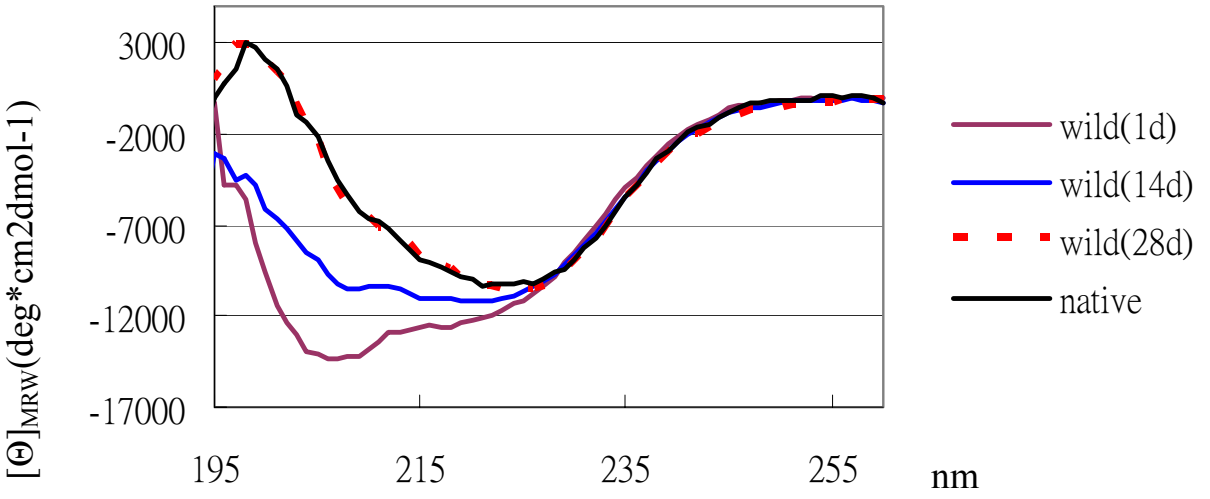


Figure A5 CD spectrum of inclusion body refolding with time.

The degree of folding with time are shown and compared. Wild type recombinant chitosanase is put in 4 ℃ for few days after purification.

Fluorescence spectra

Besides CD spectrometer, we try to get the information of two structures by fluorescence spectrometer. As well known the 3D fluorescence spectra of enzyme will change when the conformation of enzyme has changed. The spectrum of nature and denature recombinant chitosanase which is heated at 95 ℃ for 1 hour is shown in figure 3-2-6(a) and 3-2-6(b). The center of contour shifts to right hand side if the enzyme is denatured. In addition, the highest spot of both figures are excitation at 284 nm but emission shifting from 333 nm to 337 nm with less folding.

Therefore, we use fluorescence spectrum to confirm the hypothesis of self-folding in urea at 4 ℃ with time (shown as figure A6). The detections of fluorescence are all excite at 284 nm because its emission is highest. Because of the declination of enzyme concentration with time, the height of peaks are shorter and shorter. But obviously, the peaks move to short-wavelength and reveal that the enzyme folding at 4 ℃ is better and better with time. Therefore, the result show that recombinant chitosanase at 4 ℃ will reduce enzyme solubility which leads the decreasing of enzyme concentration and improve enzyme folding.

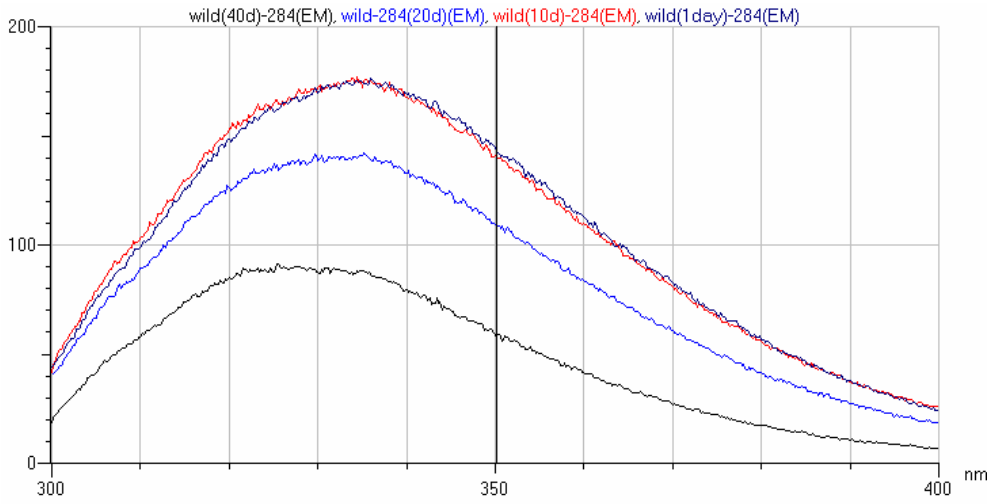


Figure A6 Overlap fluorescence spectra of wild type chitosanase at successive days after rescution

The concentration is lower and lower with time but the peaks move to short-wavelength.

B. Further comparison of hydrolysis activity of important mutants

The hydrolysis activity evaluation is estimated colorimetrically by DNS method and the strategy is described in A. Two reactions, one is that the ratio of chitosan and enzyme is 1:1 shown in figure B1; the other is that the ratio of chitosan and enzyme is 1: 0.01 shown in figure B2. Additionally, the data combined the former (enzyme without activity, D160N, E169Q and E169D) and the latter (enzyme with activity, wild type, D160E; the absorption data have to multiply 100) are shown as figure B3.

The results of these three figures, especially the last one, show that only D160E retains chitosanase activity which approximate to wild type. Therefore, we firmly believe that Asp^{160} and Glu^{169} are essential amino acids. However, the unexpected question is why E169Q losses activity.

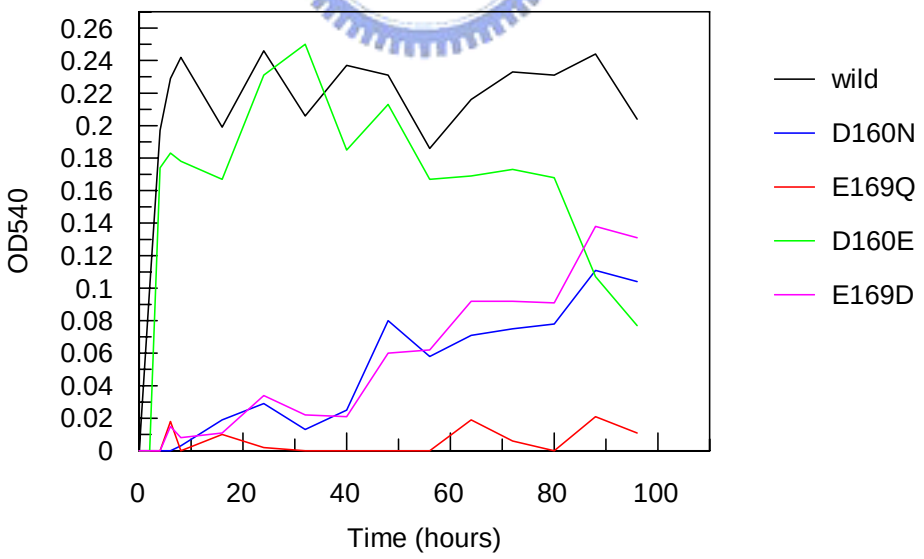


Figure B1 Activity test by DNS method

Chitosan : enzyme = 1 : 1

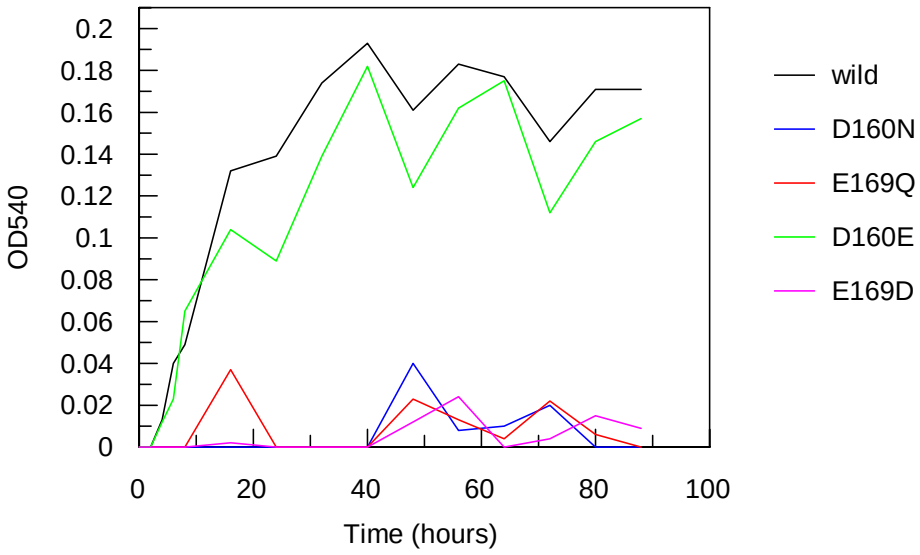


Figure B2 Activity test by DNS method

Chitosan : enzyme = 1 : 0.01

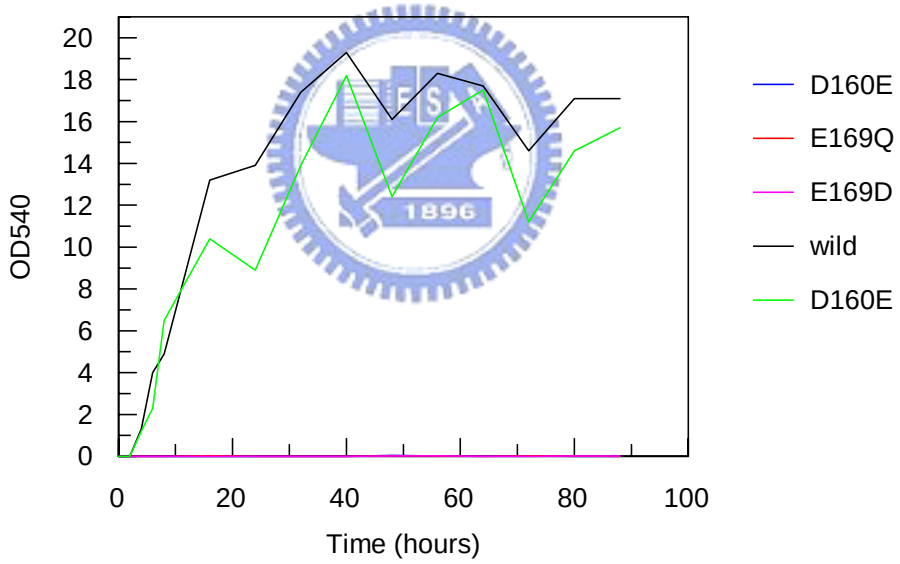


Figure B3 Combinational figure from figure B1 and B2

C. Kinetic analysis by SPR

Immobilization chitosan tetramer as ligand

Ⅰ Method

1. The activated chip is then coupling with chitosan tetramer in coupling buffer (0.1 M, pH 8.0, $NaHCO_3$ containing 0.5 M NaCl) for 1~2 hours at RT or 4 hours at 4℃.
2. The excess ligand is washed away by coupling buffer.
3. The chip is immersed in 1M , pH 8, ethenolamine for 1 hour, in order to block any remaining active groups.
4. The chip is washed by at least 3 cycles of alternating pH by 0.1 M, pH4, acetate buffer containing 0.5 M NaCl and 0.1 M, pH 8, Tris-HCl buffer containing 0.5 M NaCl.
5. The chitosan tetramer chip is then stocked in 20 mM phosphate buffer.

Ⅱ Results

RU response data

The activated Au chip is coupled with chitosan tetramer and its residual activated site are blocked the non-occupied Au sites by BSA. Recombinant protein of wild type, D160N, E169Q, D160E, and E169D are analyzed by SPR machine in turn. All enzymes used as analyte are purified from intracellular soluble protein and diluted to 1 μg/mL. The concentration of enzyme is measured by Bicinchonic Acid (BCA) Assay. After analyzing one enzyme sample, the chitosan tetramer labeled Au chip will be washed by 0.1 % SDS to remove the binding enzyme flowed before. Each datum is processed to merge

together shown as figure C1.

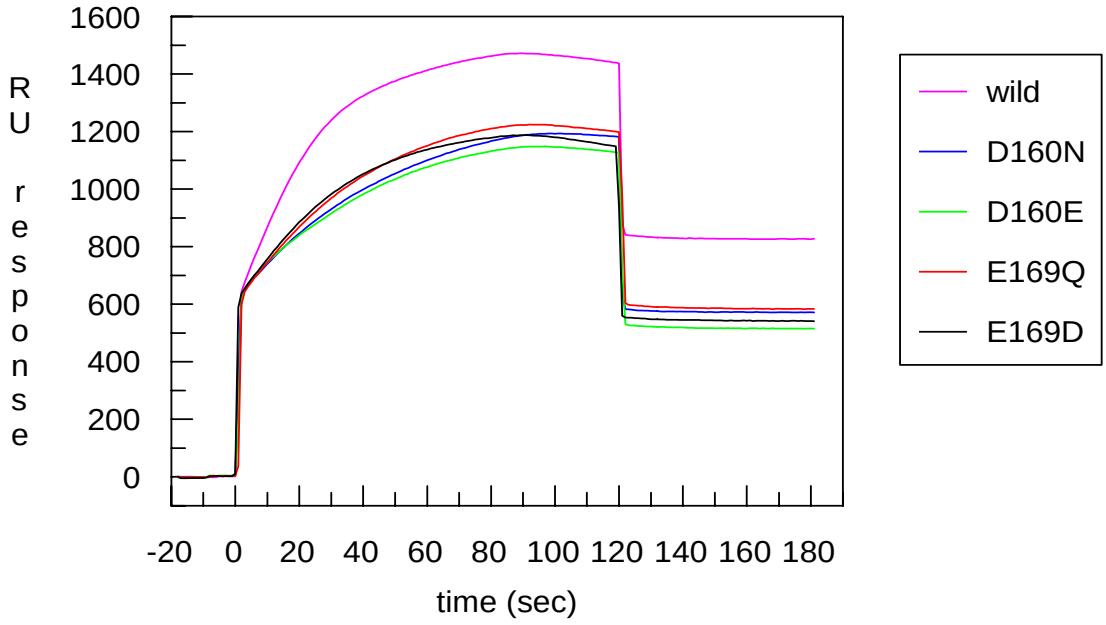


Figure C1 SPR spectrum of chitosan tetramer immobilized Au chip as ligand

The pink, blue, green, red, and black line are RU response of wild type, D160N, D160E, E169Q, and E169D tower chitosan tetramer, respectively.

Kinetic data

Response of all enzyme tower chitosan tetramer are calculated by SPR system and obtained the kinetic date of ka and kd, association constant and dissociation constant respectively, shown in table C.

	wild	D160N	E169Q	D160E	E169D
ka(1/mS)	6.84 e4	2.48 e4	7.95 e4	5.57 e4	7.4 e4
kd(1/s)	1.61 e-4	2.66 e-4	1.3 e-4	1.08 e-4	1.79 e-4
K_D	$2.4e^{-8}$	$1.1e^{-8}$	$1.6e^{-9}$	$1.9e^{-9}$	$2.4e^{-9}$

Table C Kinetic data from SPR.

D. Computational prediction

CLUSTALW alignment

Ten conserved residues, Asp^{59}, Asp^{76}, Asp^{78}, Asp^{80}, Asp^{112}, Asp^{114}, Asp^{160}, Glu^{169}, Asp^{194}, and Glu^{229}, of 5 amino acids sequences in GH-75 are described in 3-1.

Sanger's alignment

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, Asp^{160} and Glu^{169}, and the "DCDID" motif, which will be described in Appendix E-Ⅰ.

MuSiCME

Asp^{160} and Glu^{169} were recognized as catalytic sites *via* theoretical computation with MuSiCME (http://genome.life.nctu.edu.tw/MUSIC; Tsai *et al.*, 2004, Tang *et al.*, 2003).

Secondary structure –Prof and Jnet

Two 2^{nd} structure prediction tool were used (Prof and Jnet), and both generated similar results (helixes at N-terminals and C-terminals, sheets in the middle). The alignment of Prof data is shown as figure D1.

Tertiary structure – HMMSTRE

Althought we send many *Aspergillus* chitosanase to crystallize, the crystal is difficult to obtain. Thus, HMMSTRE (from http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php) is utilized here to predict the 3D structure (shown in figure D2) in order to understand the position of

essential amino acids, Asp^{160} and Glu^{169}. Asp^{160} and Glu^{169} are labeled in figure and the estimated C_α- C_α and C_β- C_β distance are 5.6 Å and 7.8Å, respectively. (Data provided from NTHU)

Additionally, the same structure is shown in figure D3 in different point of view. The highly conserved region DIDCD (describe in Appendix E-Ⅰ) is labeled.

However, there are several unreasonable phenomenons in this 3D structure predicted by HMMSTRE. First, the structure shown in figure D2 seems not compact enough to present those atypical stable features described in 3-8-1. Second, the structure dose not match the inductive inference described in 3-8-2. Third, the prediction distant of Asp^{160} and Glu^{169} is too short for inverting enzyme. Finally, the "DCDID" motif, which was proposed to be in the substrate binding domain, locates at outer face of protein and exposes to outside. For these irrational reasons, we infer that the structure predicted by HMMSTRE is unreliable.

```
tr|Q8NK76 Chitosanas  ----------------------------------------MRLSEILAVA-LVTGATA
tr|Q875I9 Endo-chito  ----------------------------------------MRLSEILTVA-LVTGATA
tr|Q9P961 Chitosanas  ----------------------------------------MHFAGIVAIA-LATGATA
tr|Q6PY43 Chitosanas  ----------------------------------------MRSNALLGLAAFGTVASA
tr|Q9HFL8 Putative c  ----------------------------------------MRSTSLFAVVTLGAVASA
tr|Q9C172 Chitosanas  ----------------------------------------MMRSSAVLTLTTLSSIACS
```

```
tr|Q00867 Chitosanas  ----------------------------------------MPSLRNTLLASLLAASVSG
tr|Q8NK77 Chitosanas  ----------------------------------------MPSLRNTLLASLLAASVSA
tr|Q6T5L4 Chitosanas  -MFFEIAKTLTLALAITAPVIAKKVH----------PSA--FAASKAIDVAAISSASEKV
tr|Q75R32 Chitosanas  MPIKSFASRLALSLAICGTAMGQKVNGADYNKPDGGPPAKFFQASSSIPVAAIQAAAAKA
                                                                              :

tr|Q8NK76 Chitosanas  YDLPDNLKQIYEKH-K-GKCSKVYQKGFTNGGHSDGKSFEYCGDIE---GAIFMHSSAKG
tr|Q875I9 Endo-chito  YNLPNNLKQIYDKH-K-GKCSKVLAKGFTNGDASQGKSFSYCGDIP---GAIFI-SSSKG
tr|Q9P961 Chitosanas  YDLPENLKQIYEKH-KSGKCSKELQGGYDNGHSHDGKSFSYCGDIP---NAIYLHSSKNG
tr|Q6PY43 Chitosanas  FSLPANLKTIYDNH-KSGTCAKKLSGTFS-GG---A---SYCGDLA---GAIFLKGSS--
tr|Q9HFL8 Putative c  YQLPANLKKIYDQH-KAGTCSNKLSGTFS-GG---A---TYCGDLP---NAIFLKGSN--
tr|Q9C172 Chitosanas  YELPAKLKSLYDKH-QSGPCSNKLSGTFK-GG---A---SYCGDIP---NAIFLKG-N--
tr|Q00867 Chitosanas  RDVPANVKTFKDSIIKQGSCKSTLATGFFSSDGDSG-TYSYCGDHVKDYNVIYLQGKN--
tr|Q8NK77 Chitosanas  RDVPANVKSFKDSIIKQGSCKSTLATGVYSSDGDSG-TYSYCGDHVKDYNVIYLQGKN--
tr|Q6T5L4 Chitosanas  KQVPAH--ATYPMSIKDFKDKSTIHSDW--ASFAEGAAFVFRAD----------------
tr|Q75R32 Chitosanas  SKVPSH--ATYP--IGQGSTKSTIHSDW--AGFSEGAAFSFIAD----------------
                       .:* :                .       .    . : .*

tr|Q8NK76 Chitosanas  GQYTNMDVDCDGANNSA---GKCSNDPSGQGVTAFKDEVKKFGIP--DLDANLHPYIVFG
tr|Q875I9 Endo-chito  --YTNMDIDCDGANNSA---GKCANDPSGQGETAFKSDVKKFGIS--DLDANIHPYVVFG
tr|Q9P961 Chitosanas  GQYADMDIDCDGANRHA---GKCSNDHSGQGETRWKDEVQKLGID--DLDANIHPYVVFG
tr|Q6PY43 Chitosanas  GNYDNLDIDCDGANNSA---GACANDPSGQSETAFKDTVRTFGIS--DLDANIHPYVVFG
tr|Q9HFL8 Putative c  GNYDNMDIDCDGANNSA---GGCANDPTGQGQTAFKDTVKTYGIP--DLDANLHPYVVFG
tr|Q9C172 Chitosanas  GTYDNMDIDCDGVNRSA---GACANDQTGQDQTAFMDTVKTYGIP--DLDANVHPYVVFG
tr|Q00867 Chitosanas  GKLVNMDIDCDGVQGSPADDGRCGSSGDTQSITSFQWVLESYGTSQKDLDANIHPYIVFG
tr|Q8NK77 Chitosanas  GKLVNMDIDCDGIQGSPADDGRCGSSGDTQSITSFQWVLESYGTSQKDLDANIHPYIVFG
tr|Q6T5L4 Chitosanas  -----MDTDCDGVNY------KCDGNVDGGPLTNW-----------GALSAFEVPYIVIP
tr|Q75R32 Chitosanas  -----MDVDCDGLNH------GCKGNPDGQKETNW-----------GALSAYEVPFIVIP
                           :* **** :        * ..      * :             *.*   *::*:
                            D D D

                                                          Asp160      Glu169
tr|Q8NK76 Chitosanas  NEEHS---PQFKPQKYGMEPLSVMAVVCNGKLHYGIWGDTNGG----TSTGEASLSMAEL
tr|Q875I9 Endo-chito  NEDHS---PKFKPQSHGMQPLSVMAVVCNGQLHYGIWGDTNGG----VSTGEASISLADL
tr|Q9P961 Chitosanas  NENDDGDDPEFDPRKHGMEPLSVMAVVCNKKLFYGIWGDTNGH----TATGEASLSMAEL
tr|Q6PY43 Chitosanas  NS---GSSPSFNPQSSGMQPLSVMAVVCNNQVFYGIWGDTNGG----TSTGEASLALGKL
tr|Q9HFL8 Putative c  NE---GASPSFNPQSKGMKPLSVMAVVCNNQVFYGVWGDTNGF----TSTGEASLALGKL
tr|Q9C172 Chitosanas  NA---EADPSFVPQDHGIEPLSVMAVVCNDQVHYGVWGDVNGG----VLTGEASLSMAKL
tr|Q00867 Chitosanas  NEGTKKGWKTFDPEKHGIKPLSVMAVVCGNKMFYGIWGDENGDDGDQPMVGEASISLATA
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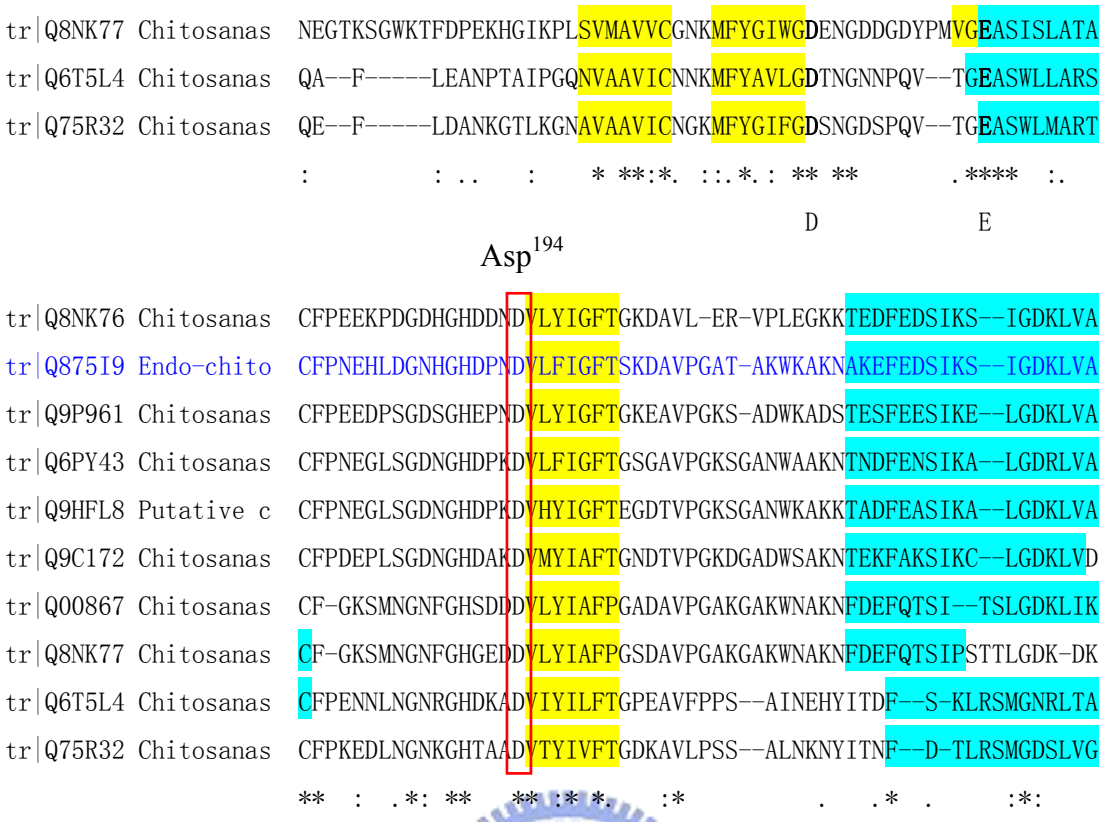

Figure D1 Secondary structure prediction by Prof

The blue box is Prof predicted helix; the yellow box is Prof predicted sheet. The amino acids in rectangle are the result after alignment.

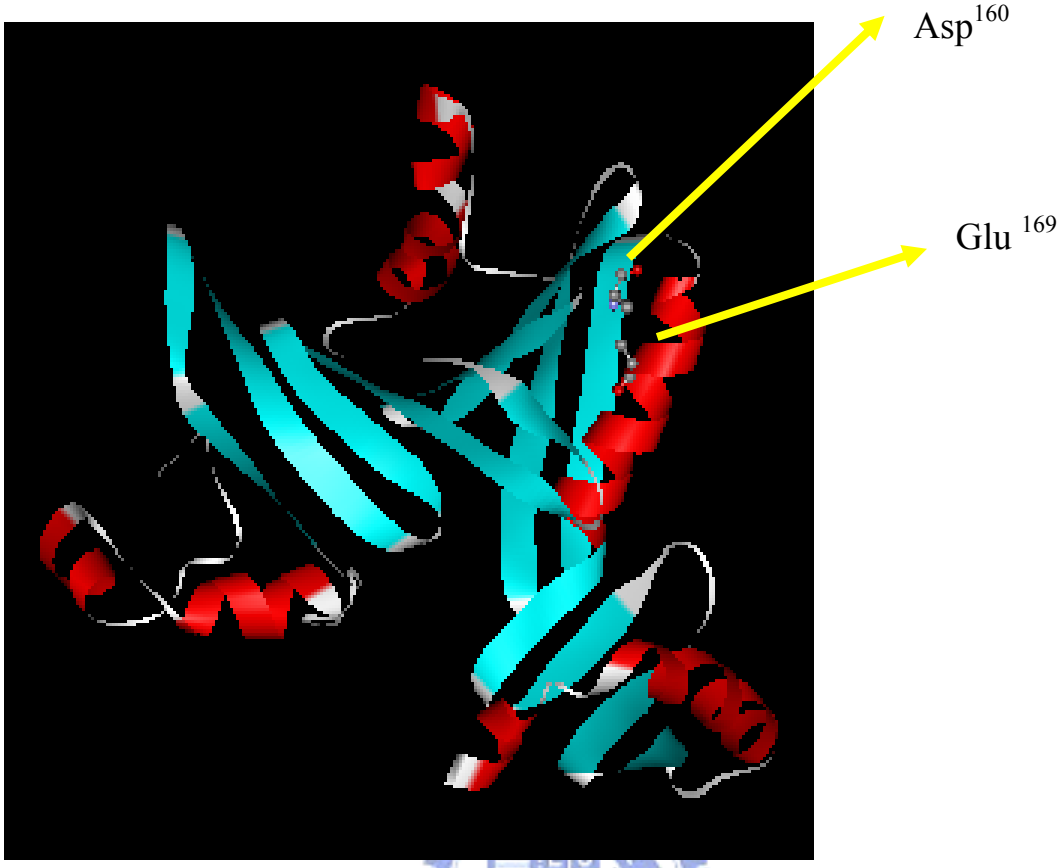

Figure D2 Computational prediction of *Aspergillus* chitosanase 3D structure

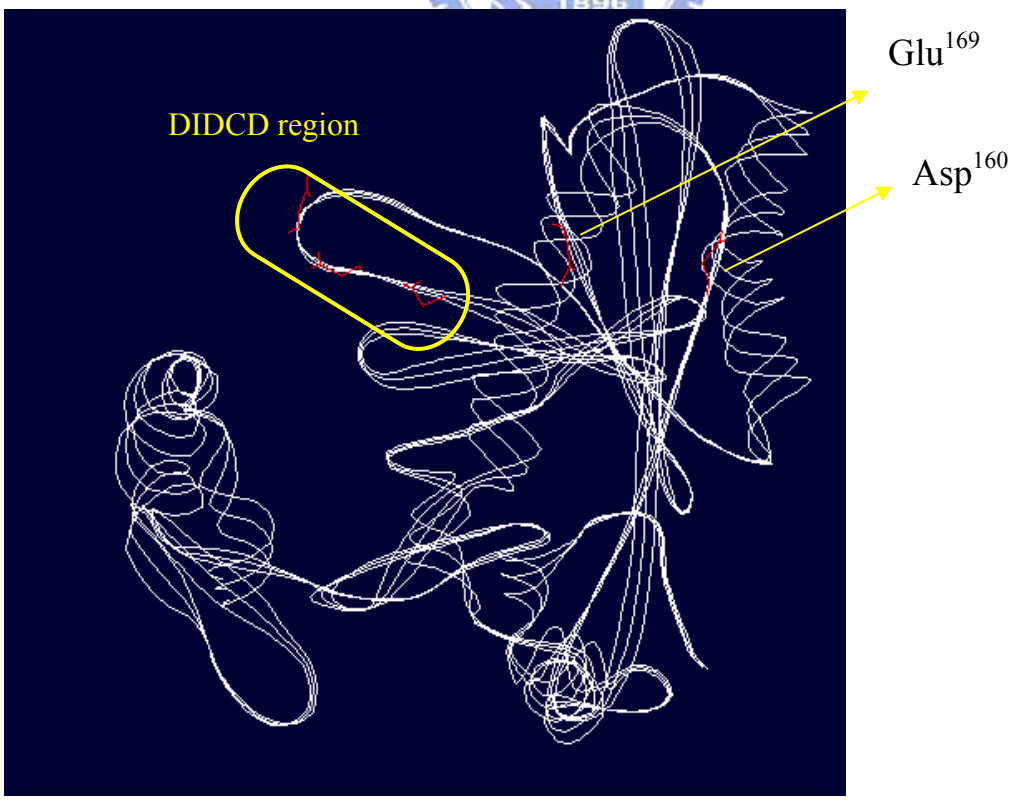

Figure D3 Computational prediction of *Aspergillus* chitosanase 3D structure

E. Other experiments

Ⅰ DIDCD region

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 (Asp^{76}, Ile^{77}, Asp^{78}, Cys^{79}, Asp^{80}), which is proposed to be in the substrate binding domain. The activity test results of mutants D76N, D78N, and D80N are shown in table 3-4-1. The average activity compared with wild type is about 65%.

Besides, the catalytic domains of family 18 chitinase have a $(\beta\alpha)_8$ (TIM barrel) fold (Perrakis *et al.*, 1994) and are characterized by several conserved sequence motifs (Terwisscha *et al.*, 1996). The most prominent of these motifs is the DXDXE motif that spans strand 4 of the TIM barrel and includes the glutamate that acts as the catalytic acid. The active site grooves of these chitinases are lined with araomatic amino acids that contribute to substrate binding (Aalten *et al.*, 2000).

Therefore, we consider that the DIDCD region in *Aspergillus* chitosanase will play an important role in catalytic and substrate binding domain. This hypothesis can explain why the D76N, D78N, and D80N mutants only have 65% remained activity. So, we analyze the hydrolytes of these mutants and wild type enzymes tower chitosan (shown in figure E1) and try to find out the difference. As shown in figure E1, the degree of acetylation of mutant's hydrolytes seems higher than that of wild type's. We presume that the acting specificity of mutants are lost and consider firmlier that DIDCD motif has

important role of substrate binding.

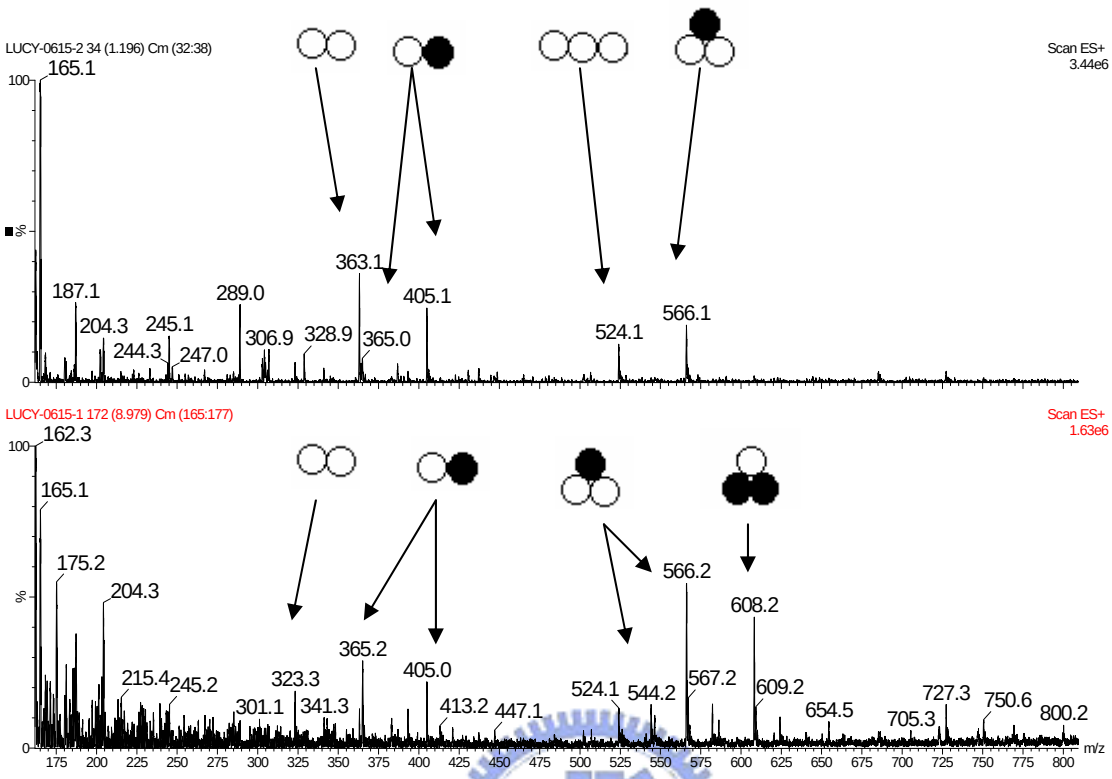

Figure E1 Mass spectrum of hydrolytes

The upper one spectrum is hydrolytes of wild type chitosanase; the lower one is hydrolytes of mutants (D76N, D78N, and D80N are identical). Because of adding H_2O (18 Da), Na^+ (22 Da), or both (40 Da), the same component of hydrolyte has different molecular weight. The black circle is N-acetylglucosamine; the white one is glucosamine.

Ⅱ Transglycosylation

In order to investigate the transglycosylation activity of mutants, the hydrolytes

of transglycosylation reaction of chitosan and alcohol using the D160E mutant are been analyzed by mass.

The hydrolytes pattern of D160E (shown in figure E2) catalysis reaction with 10% ethanol is similar with that without ethanol and the two reactions' patterns are identical to those of wild type. The results shoe that the hydrolytic character of D160E is the same with wild type and has no transglycosylation activity.

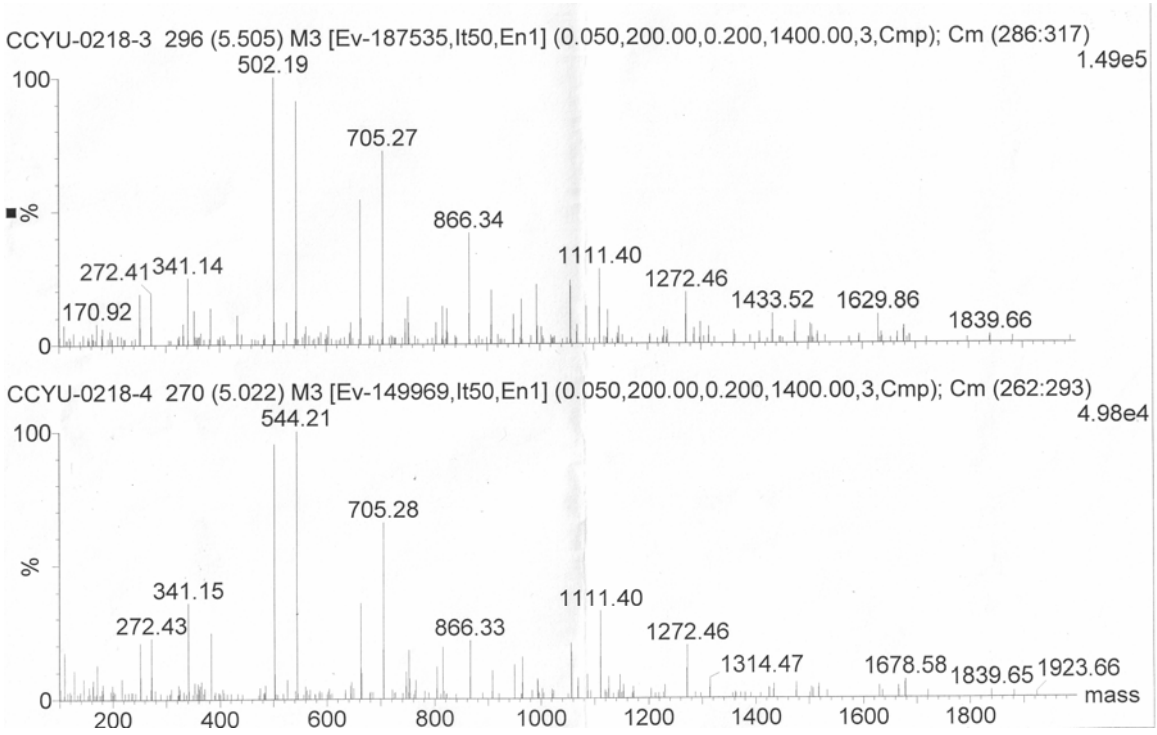

Figure E2 Mass spectrum of hydrolytes of D160E with or without ethanol

The upper spectrum is the chitosan hydrolytes of D160E with 10% ethanol; the lower one is the chitosan hydrolytes of D160E without ethanol.

Ⅲ Recovery of chitosanase activity

D160C and E169C mutants, which replace their original amino acid with cysteine are designed for advance modification of the putative activity site. This modification can be obtained by the interaction between this extra thiol residue and some other thiol compound through disulfide bound. DTNB is employed in this study and will link with free thiol of cysteine in enzyme (shown as figure E3). The activities of the labeled enzymes (D160C and E169C) have not yet been confirmed.

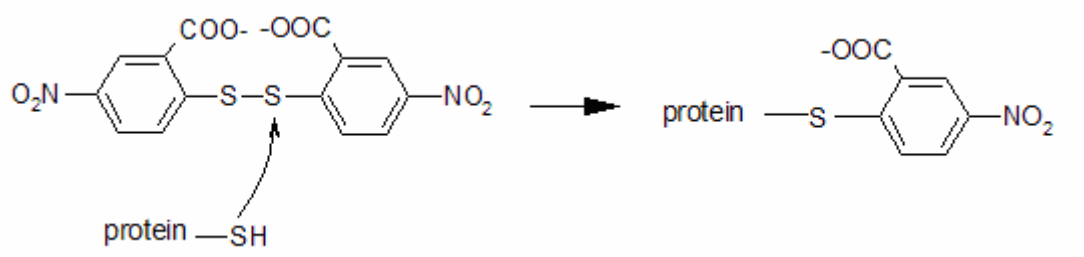

Figure E3 DTNB interact with cysteine in protein.