行政院國家科學委員會補助專題研究計畫成果報告

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※ **Reconstruct the product specificity of chitinase** ※
※ **by genetic protein engineering (1)--Gene cloning** ※
※ **and catalytic ptoperty** ※

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計畫類別：個別型計畫

計畫編號：NSC 89-2113-M-009-020-

執行期間：89 **年** 8 **月** 1 **日至** 90 **年** 7 **月** 31 **日**

計畫主持人：李耀坤

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□**赴國外出差或研習心得報告一份**
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執行單位：國立交通大學應用化學系

中 華 民 國 90 **年** 10 **月** 25 **日**

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中文摘要

一種可分解幾丁質的菌種已被篩選得到並經菌種鑑定為枯草菌屬(命名為 NCTU2)**。該菌種經誘導作用後可得一胞外幾丁質水解酵素。經疏水性層析管柱和凝膠層析管柱之純化程序可得高純度之酵素，純化倍率為** 15.6 **倍，回收率** 58%**。此純化酵素為單分子蛋白，分子量為** 37 kDa**，**pI **值** 6.3**。此酵素可於** 60 **℃、**pH6.6 **下穩定至少三小時以上，其最佳活性在** 45~60**℃間。此酵素可水解幾丁四糖和幾丁六糖，而得幾丁二糖為唯一產物，顯示此酵素為外切型幾丁質水解酵素。其前十五個胺基酸序列為** "ANNLGSKLLVGYWHN"**，經由基因庫比對得知其與** *Bacillus cereus* **之** *ChiA* **相同。經由** PCR **基因放大作用後，可得** *Bacillus* NCTU2 **之完整幾丁質水解酵素基因，此基因已被定序完成，其中有七處胺基酸與** *Bacillus cereus* **者不同。我們已著手進行該酵素之應用與學理之研究。**

Abstract

A chitin-degrading *Bacillus* strain (designated as NCTU2) was screened from soil and identified. An extra-cellular chitinase was induced and purified. The purification process to gain 58% yield and 15.6-fold purification involved two chromatographic steps including a hydrophobic interaction column and a gel-filtration column. The purified enzyme was characterized to be a monomeric protein with the M*r* value of 37 kDa and p*I* 6.3. It is thermally stable at 60 C, pH 6.6 for more than 3 h. The optimal activity is in the range of 45 to 60 ℃ at neutral pH. The purified enzyme hydrolyzed chitin tetraose, and chitin hexaose to form *N,N'*-diacetyl chitobiose (chitin biose) as the sole product. The disaccharide was the predominant product through out the enzymatic time-course reaction. Indicate that the purified chitinase is an exo-chitinase. The first 15 *N*-terminal amino acid sequence is determined to be "ANNLGSKLLVGYWHN". By searching this sequence in Genbank, the *ChiA* of *Bacillus cereus* was found to be identical. PCR amplification was then employed for gene cloning on the NCTU2 strain. Complete gene sequence revealed that only 7 amino acids are found to be different between the two enzymes. Although genetic information of this enzyme is known, the correspondent chitinase from *B. cereus* has not yet been purified and studied.

Introduction

Chitin, an insoluble linear *b*-(1,4)-linked N-acetylglucosamine (GIcNAc) polysaccharide [1,2], is a major structure component of fungal cell wall and the exoskeletons of invertebrates, including insects and crustaceans[3,4]. It is one of the most abundant natural occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology, and biotechnology [5-7]. This linear polymer can be hydrolyzed by base or acid or alternatively by enzymes such as lysozyme, some glucanases and chitinase. Chitinases (EC 3.2.1.14), an essential enzyme catalyzing the conversion of chitin to its monomeric or oligomeric component, have been found in a wide range of organisms including bacteria [8,-10], plants [11], fungi [12], insects [13], and crustaceans [14]. Plants produce chitinases as a defense against fungal pathogens [15-17]. Because chitin is not found in vertebrates, it has been suggested that inhibition of chitinases may be used for the treatment of fungal infections and human parasitosis. In addition to the potential applications of chitinase itself, the chitin oligosaccharides [$(GlcNAc)_n$] have been found to function as antibacterial agents, elicitors, lysozyme inducer, and immuno-enhancers [18]. Due to these biological interests, the preparation of *N*-acetylchitooligosaccharides is becoming one of the new targets in carbohydrates industry. To prepare chitin oligosaccharides with specific degree of polymerization is particularly valuable. To pursue this goal a chitinase from *Bacillus sp. NCTU2* was purified and characterized, and the correspondent gene was cloned and studied.

Materials and methods

Chemicals. Buffers, N-acetylglucosamine, purified chitin, carboxy methyl-chitin, ammonium sulfate, standard molecular weight markers, and isoelectric point (p*I*) standard proteins(*pI* 4.45–9.6) were purchased from Sigma-Aldrich Inc. (St. Louis,MO). Phenyl sepharose, Sephadex G-75, HiTrap SP, and HiTrap Q resin was purchased from Pharmacia LKB(Uppsala, Sweden).

Preparation of colloidal chitin. Colloidal chitin was prepared from commercial chitin by the method of Roberts and Selitrennikoff [19] with few modifications described as follows: 5 grams of chitin powder was added slowly into 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 liters of ice-cold 95% ethanol with rapid stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 5000 *g* for 20 min at 4°C. The precipitate was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0), then dried by lyophilization.

Microorganism screening and identification. A bacterial strain from soil was isolated by using colloidal chitin as a sole carbon source. This strain was further identified as a *Bacillus sp*. by Centraalbureau Voor Schimmelcultures (CBS) in The Netherland.

A single colony was inoculated into 5 ml of

LB medium containing 1% colloidal chitin and incubated at 37°C for 24 h on a rotary shaker. 5 ml of the overnight culture was inoculated into a 2-L Erlenmeyer flask containing 500 ml of the M9 medium (containing the following salts per liter: Na_2HPO_4, 0.65 g, KH_2PO_4, 1.5 g, NaCl, 0.25 g, NH_4Cl, 0.5 g, $MgSO_4$, 0.12 g, $CaCl_2$, 0.005 g) supplemented with 1% colloidal chitin and further incubated for 72 h. The culture broth was centrifuged at 4°C for 20 min at 15,000 g. The supernatant was collected for further purification of the enzyme.

Purification of chitinase. The supernatant obtained from last manipulation was then concentrated by ~50-fold with rota-evaporator at 35 °C. The resulting sample solution (12 ml) was then loaded onto a hydrophobic interaction column (high performance phenyl sepharose, Pharmacia Co.) with 1M $(NH_4)_2SO_4$ as initial salt concentration. Fractions with chitinase activity were pooled (18 ml) and subjected to concentration by 10-fold. The resulting solution was loaded on a G-75 gel filtration column (2.5x30 cm) with phosphate (20mM, pH7.0) as eluent. The active fractions were collected , pooled, and used for further study.

Enzyme activity assay. Chitinase activity was analyzed by estimating the reducing end of sugars. The assay was performed by mixing 0.3 ml of 1 % colloidal chitin, pH 6.6 and 0.3 ml of suitably diluted enzyme. After incubation for 10, 20, and 30 min, individually, at 37 ℃, hydrolysis reactions were terminated and analyzed by adding 0.6 ml of dinitrosalicylic acid reagent [20]. The mixture was boiled for 15 min and chilled and centrifuged to remove insoluble chitin. The resulting adduct of reducing sugars were measured spectrophotometrically at 540 nm. The extinction coefficient of the resulting adduct (at 540 nm) was determined to be 788 $M^{-1}cm^{-1}$ when N-acetyl-D-glucosamine was the reducing sugar. One unit of chitinase activity is defined as the amount of enzyme required to release 1 mole of detectable reducing sugars at 37 ^oC in 1 minute.

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

Amino acid analysis. The N-terminal amino acid sequence of purified chitinase was determined with a Milligen 6600.

Products analysis. The products of the enzymatic hydrolysis were analyzed by HPLC. After enzymatic incubation, 2 volumes of acetonitrile were added to the reaction solution. Trace amount of insoluble material was removed by centrifugation. The resulting solution was then subjected to analysis. HPLC (Waters C626) coupled with a light scattering detector (PL-ESL1000, UK) was used to determine the oligosaccharides in all samples. An IB-SIL NH2 column (5 , 4.6x250 mm, Phenomenex, CA,

USA) was employed. The mobile phase was a water/acetonitrile (2:3)mixed solvents with a flow rate of 0.8 ml/min.

PCR cloning. On the basis of the ChiA from *B.cereus*, two primers were designed for PCR cloning shown as follows: 5'-GGAGTGTTCATATGTTAAACAAGTTCAAAT-3', and 5'-ATTAAAGCTTTACTTTGTTATTTTTGCAAGGAAAG-3' (The underline showed the location of start coden and the correspondent stop coden). Genomic DNA was isolated based on the protocol reported (ref.). The PCR amplification condition for each cycle was set as follows: 95 (1 min), 55 (1.5 min), and 72 (1 min), with total 30 cycles. The correspondent Chi gene from NCTU2 was then constructed into pCR cloning vector (Stratagene Co.), and sequenced with dye-terminator cycle sequencing on ABI 310.

Results and discussion

Microorganism screening and enzyme induction. Some microorganisms screened by using colloidal chitin as a sole carbon source were isolated from soil. A chitin-degrading bacterium, which formed the largest clear zone on 1% colloidal chitin plate, was isolated, identified and designated as *Bacillus sp. NCTU2*. This strain grew well at temperature 28-37 ^{o}C and pH 6.6. The induction process in terms of chitinase activity was insensitive to some physical and chemical parameters since no significant activity change was detected with regarding to pH (5.5-7.5), temperature (28-37 C), and the added nutrients such as glucose, N-acetylglucosamine, and trypton. Although the presence of colloidal chitin was essential the induced chitinase activity was unaffected when chitin was added to greater than 0.5% and the culture was harvested after 72 h). This might indicate that the induction of chitinase is controlled at a limited level in the medium.

Purification of chitinase from *Bacillus sp. NCTU2*. In classical protein purification, the culture filtrate of fermentation is normally treated with ammonium sulfate to precipitate crude proteins. Unfortunately, this is not applicable in this study. Only scarce amount of protein can be salted out by ammonium sulfate (85% saturation). Precipitation of crude proteins by pre-chilled organic solvent such as ethanol or acetone caused large activity loss. Interestingly, a vacuum-equipped rota-evaporator could be conveniently employed for concentration of bulk culture filtrate without significant loss of enzyme activity. The 50-fold concentrated sample solution was first passed through a phenyl sepharose column, figure 1. Significant chitinase activity was found in the region containing extremely low salt fractions suggesting that the protein surface of chitinase is fairly hydrophobic. This explains why the chitinase activity was always found in the unbound fractions in regarding less a cationic or anionic exchange column was employed. Four bands could be seen after the

phenyl sepharose column separation, figure 3. Impurities could not be removed further by either HiTrap Q (anionic exchange) or HiTrap SP (cationic exchange) column, see figure 3, lane 2. With the application of G-75 gel filtration chromatography, figure 2, the chitinase could be purified to nearly homogeneous. Based on the analysis of gel filtration, the molecular weight of the purified chitinase was estimated to be 26 kDa, which is much smaller than that estimated from SDS-PAGE analysis (37 kDa). This reveals that the native form of the chitinase is in a form with tight structure packing. This is consistent with the observation regarding to high stability of the enzyme in very low ionic strength. The yield and purity for each purification step were summarized in Table 1. The total yield is 58% with 15.6-fold purification. The pI of the enzyme is measured to be 6.3.

Optimal conditions of chitinase. The enzyme hydrolyzed colloidal chitin at a range of pH. The optimal pH was near 7.0. At least 60% of the optimal enzyme activity was retained in pH 5.5-9.0 (Fig. 4). The optimal temperature was around 60°C, above which chitinase activity decreased dramatically (Fig. 5) indicating the denaturation of enzyme structure occurred at temperature greater than 60°C.

Thermal and pH stability. The purified chitinase was stable for more than 3 months when stored at 4 °C and no significant activity loss was observed when it was kept at 37 °C for a week. Figure 6 showed the thermal stability of the enzyme in higher temperature (37 ~80 °C). The experiments were performed by keeping the suitable amount of enzyme at various temperatures in sodium phosphate buffer (50 mM, pH7.0). An aliquot of enzyme was removed for assaying the residual activity in 30-min intervals. The enzyme was stable at 60 °C for at least 180 min without any activity loss. However, the activity decreased rapidly at 70°C and above. The pH stability was also investigated by assaying the remaining activity of the enzyme after incubating in various pH buffers (4-10) for 120 min. Results showed that the purified chitinase was very stable in the pH range of 6-8 at 60 °C (figure 7).

Substrate specificity and effect of metal ions. In addition to colloidal chitin, the purified chitinase also hydrolyzed chitosan with various degree of acetylation. The activity of the enzyme showed a positive trend with regarding to the degree of acetylation of chitosan. For instance, the relative activities (as compared with colloidal chitin) were 0.85, and 0.10 when 56% and 10% acetylated chitosan were used as substrate, respectively. However, no significant activity was found when chitin powder, O-carboxymethyl, and N-carboxymethyl chitin were served as substrate. The effects of various metal ions on activity levels were also examined. Among the metal ions tested, Hg^{2+} and Cu^{2+} were strong inhibitors. The presence of 10 mM of the two ions resulted in a 95% of the activity loss. However, interestingly,

enzyme activity was enhanced by ~100% in the presence of 10 mM Ca^{2+}.

Enzymatic hydrolysis. The purified enzyme hydrolyzed chitin tetraose, and hexaose to form its correspondent dimer (chitin biose) as the sole product, while the monomer, dimmer, and trimer were found with chitin pentaose and triose as substrate. Catalytic hydrolysis of colloidal chitin by the purified enzyme yielded *N,N'*-diacetyl chitobiose as the predominant product through out the time-course reaction. These results strongly suggested that the purified chitinase is an exo-chitinase. The enzyme has no activity on chitin biose, while it posses a weak activity on chitin triose. Oligosaccharides with DP (degree of polymerization) value greater than (and equal to) 4 are believed to be good substrates. This chitinase was also shown to be suitable for preparing a gram-scale of *N,N'*-diacetyl chitobiose in a batch reaction. 20 ml of 5% colloidal chitin was incubated with 30 units of the purified chitinase at 45 C for 3 days. The end product of the enzymic reaction was analyzed by HPLC and confirmed to be a disaccharide (figure 8).

Amino acid analysis and PCR cloning. The N-terminated amino acid sequence of chitinase was determined as "ANNLGSKLLVGYWHN", which provides an useful information for further gene cloning of this enzyme. By searching the homology sequence in Genbank, a *ChiA* of *Bacillus cereus* was found to be highly consistent with the fragment sequenced. PCR amplification was then employed for gene cloning of the chitinase from *NCTU2* strain. A 1.1 kb of DNA fragment was amplified by PCR with genomic DNA of *NCTU2* strain as template. The amplified fragment was inserted into pCR cloning vector. Complete gene sequence revealed that only 29 nucleotides, resulting in 7 amino acids, were found to be different in both genes (figure 9). The signal peptide of the enzyme is identified as the first 27 amino acids, MLNKFKFFCCILVMFLLL PLSPFQAQA, of the gene.

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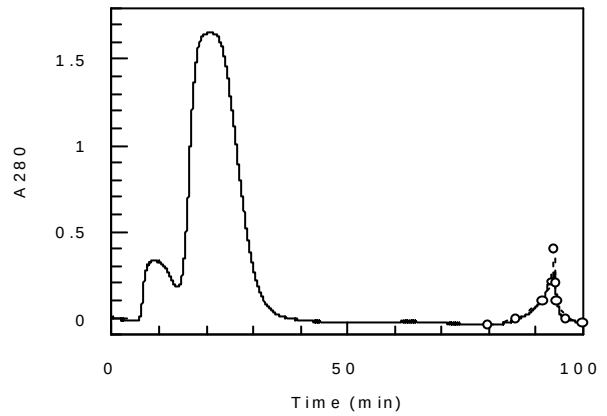

Figure 1. Chromatogram of hydrophobic interaction column

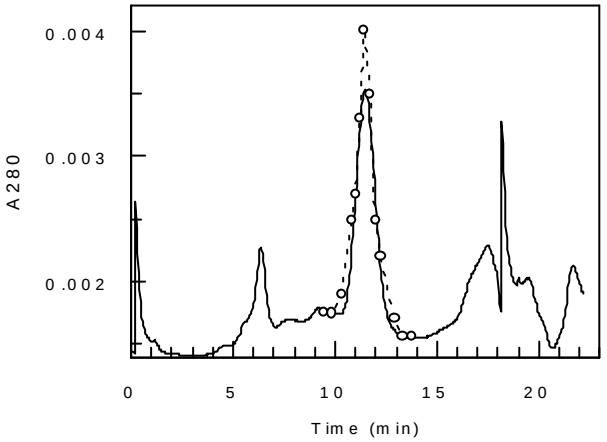

Figure 2. Chromatogram of G-75 gel filtration column

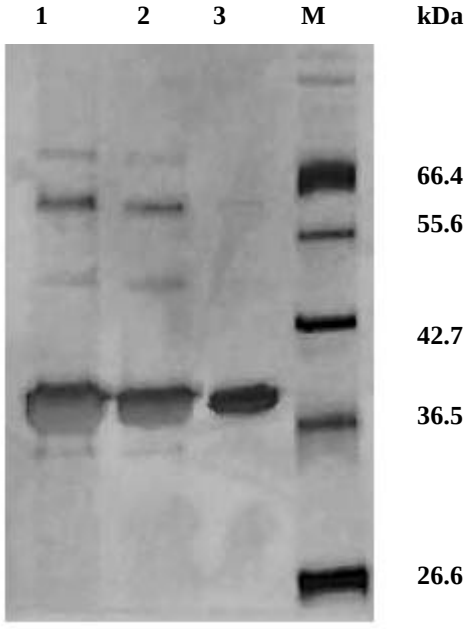

Figure 3. SDS-page analysis of protein sample obtained from various steps of purification. Lane 1: HIC eluent; Lane 2: HiTrap SP combining with HiTrap Q; Lane 3: Sample after G-75 column chromatography; M: Protein marker.

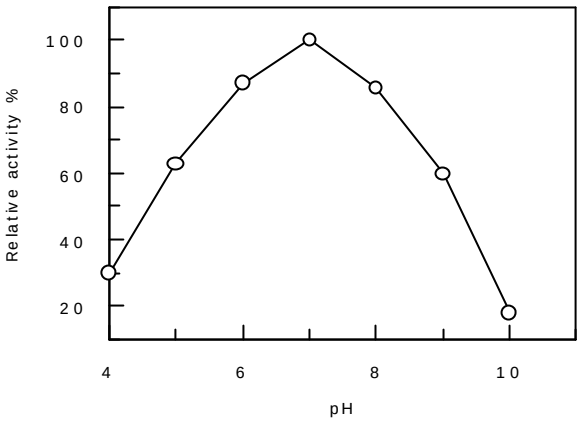

Figure 4. Optimal pH activity

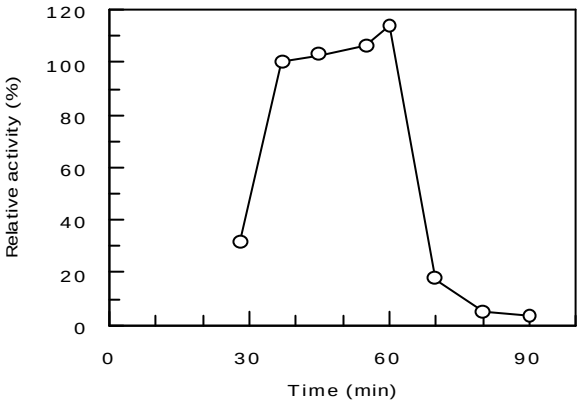

Figure 5. Optimal temperature activity

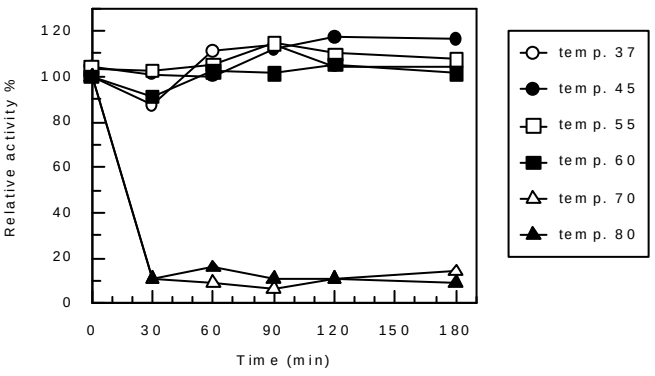

Figure 6. Thermal stability

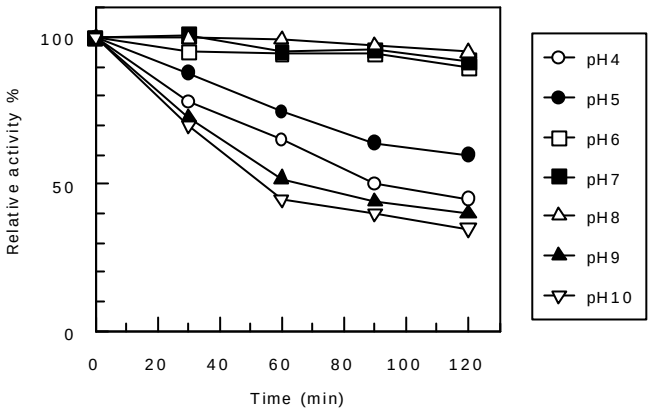

Figure 7. pH stability

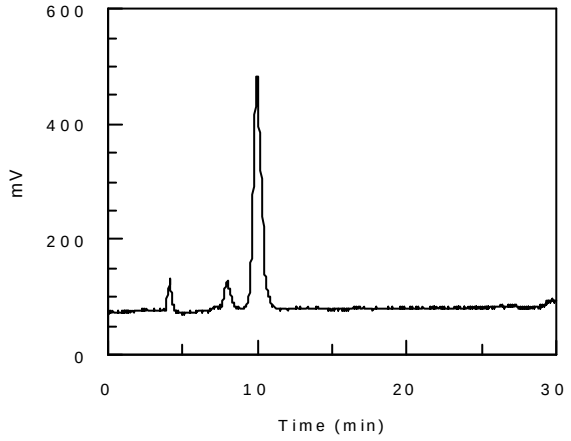

Figure 8. HPLC analysis of the end product of enzymatic reaction

ATG TTA AAC AAG TTC AAA TTT TTT
TGT TGT ATT TTA GTA ATG TTC TTA
CTT CTA CCG CTA TCC CCT TTC CAA
GCA CAA GCA GCA AAC AAT TTA GGT
TCA AAA TTA CTC GTT GGA TAT TGG
CAT AAC TTT GAT AAC GGT ACT GGC
ATT ATT AAA TTA AAA GAC GTT TCA
CCA AAA TGG GAT GTA ATC AAT GTA
TCT TTT GGT GAA ACT GGT GGT GAT
CGT TCC ACT GTT GAA TTT TCT CCT
GTG TAT GGT ACA GAT GCA GAC TTC
AAA TCA GAT ATT TCT TAT TTA AAA
AGT AAA GGA AAG AAA GTA GTT CTT
TCA ATA GGT GGA CAA AAT GGA GTC
GTT TTA CTT CCT GAC AAT GCC GCT
AAG GAT CGT TTT ATT AAT TCC ATA
CAG TCT CTA ATC GAT AAA TAC GGT
TTT GAT GGA ATA GAT ATT GAC CTT
GAA TCA GGT ATT TAC TTA AAC GGA
AAT GAT ACT AAT TTC AAA AAT CCA
ACT ACT CCC CAA ATC GTA AAT CTT
ATA TCA GCT ATT CGA ACA ATC TCA
GAT CAT TAT GGT CCA GAT TTT CTA
TTA AGC ATG GCT CCT GAA ACA GCT
TAT GTT CAA GGC GGT TAT AGC GCA
TAT GGA AGC ATA TGG GGT GCA TAT
TTA CCA ATT ATT TAC GGA GTG AAA
GAT AAA CTA ACA TAC ATT CAT GTT
CAA CAC TAC AAC GCT GGT AGC GGG
ATT GGA ATG GAC GGT AAT AAC TAC
AAT CAA GGT ACT GCA GAC TAC GAG
GTC GCT ATG GCA GAT ATG CTC TTA
CAT GGT TTT CCT GTA GGT GGT AAT
GCA AAT AAC ATT TTC CCA GCT CTT
CGT TCA GAT CAA GTC AAG ATT GGG
CTT CCA GCA GCA CCA GCG GCA GCT
CCA AGT GGT GGA TAC ATT TCG CCA
ACT GAA ATG AAA AAA GCT TTA AAT
TAT ATC ATT AAA GGA GTT CCG TTC
GGA GGA AAG TAT AAA CTT TCT AAC
CAG AGT GGC TAT CCT GCA TTC CGC
GGC CTA ATG TCT TGG TCT ATT AAT
TGG GAT GCA AAA AAC AAC TTC GAA
TTC TCA AAT AAC TAT AGA ACA TAT
TTT GAT GGT CTT TCC TTG CAA AAA
TAA

Figure 9. Gene sequence of chitinase from *Bacillus NCTU2* strain. The sequences (underlined) correspond to the first 27 N-terminal amino acids was identified as the signal peptide of the enzyme.