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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計畫編號：NSC　90-2313-B-009-002-

執行期間：90 年　8 月　1 日至　91 年　7 月　31 日

計畫主持人：李耀坤

本成果報告包括以下應繳交之附件：

- □赴國外出差或研習心得報告一份
- □赴大陸地區出差或研習心得報告一份
- □出席國際學術會議心得報告及發表之論文各一份
- □國際合作研究計畫國外研究報告書一份

執行單位：國立交通大學應用化學系

中　華　民　國　91　年　10　月　28　日

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一、中文摘要

聚醣胺可進一步分解而製成寡醣胺或再製成其他衍生物，以提高其利用價值，例如含六醣及七醣分子之寡醣胺已被証具有抗菌、增強免疫力及抗腫瘤等生理活性，因此這類寡醣在醫藥和機能性食品方面的開發應用極具潛力。雖然，以β-1,4 D-葡萄醣胺鍵結而成的寡醣胺可由聚醣胺經化學水解反應而得，但其主要水解產物為單醣和雙醣，較長鏈之寡醣產物含量很低。酵素法是較可行的方法。一般而言，此類酵素可分為內切型與外切型兩種，前者之產物為一群寡醣體之混合物，而後者可得較單一之成份，但通常醣鏈數較短。由於 *Bacillus circulans* 聚醣胺水解酵素之立體結構為已知，我們因此選擇此酵素為技術開發之對象之一。該聚醣胺水解酵素為 29-kDa 之內切型胞外酵素。我們已取得該基因，由於該酵素在大腸菌中表達狀況不佳故已積極將其建構於枯草菌之表達系統中。另一聚醣胺水解酵素為本研究室從徵菌中誘導純化而得，經証實為一活性很強的酵素，可有效製備 DP3 至 DP11 之寡醣混合體，該酵素之相應基因已被篩檢完成。完整基因含有一段十七個胺基酸之訊號胜腱基因和兩段 Intron。我們已著手基因之表達研究。

關鍵詞: 枯草桿菌、聚醣胺、寡醣胺、聚醣胺水解酵素、基因選殖

Abstract

Chitosan, a polysaccharide composed of the -14-linkage of glucosamine, has been found to posses several biological functions such as antibiotic activity, lowing cholesterol level in blood, enhancing immune system and many others applications in cosmetics and health care. However, the functions of oligosaccharides derived from this natural occurring polymer are not very well studied, presumably owing to the availability of these oligosaccharides, pure oligomer in particular. Though further study and application of chitooligosaccharides [$(GlcN)_n$] need to be explored, the result shown that the hexa-oligomer possesses prominent anti-tumor activity is encouraging. For many years, carbohydrate synthesis has been an important research topic. Unfortunately, owing to the complexity of the stereochemistry and the multi-hydroxyl groups of carbohydrate, to synthesize oligosaccharides with specific degree of polymerization (DP) has not yet been achieved. In this project, .we have started to approach the goal by two different enzymes. The chitosanase gene from *Bacillus Circulans* is now available. Since the expression of this enzyme in *E. coli* is not working properly, we have tried to construct this gene in *Bacillus subtilis* expression system. The other enzyme obtained from *Aspergillus* Y2K, which was screened from soil by our lab, has been shown to be a powerful endo-chitosanase. With the application of this enzyme, the chitooligosaccharides with DP3~DP11 can be produced in large scale. The correspondent gene has cloned. The entire gene consists of 866 bp. The first 51 bp correspondent to 17 amino acids is the sequence of signal peptide. Two introns can be found in the gene with 67bp and 82 bp each. The mature protein consists of 221 amino acids with the calculated molecular weight of 23446 Da.

Keywords: *Bacillus circulans, polyglucosamine,* chitosan, chitosanase, cloning, over-expression,

緣由與目的

For many years, pharmaceutical industry and academic researcher have paid their great attention in developing glycol drug. Yet, unlike peptide and nucleotide drug, only very few of glycol drug were available for medical applications. Few reasons can be account for this situation. However, the complexity of carbohydrate chemistry and the supply of oligosaccharides, in most of

cases, are the main task. The goal of this project was designed to directly approach in preparation of chitosan oligosaccharides with specific degree of polymerization.

Chitosan is a -1, 4-linked polyglucosamine with vary degrees of N-acetylated residues. Its hydrolytes, glucosaminyl-oligosaccharides, have received attention because of their interesting biological properties, including their inhibitory effects on the growth of fungi/bacteria and their ability to induce disease-resistance-response genes in higher plant [1,2]. Exceptionally, chitohexaose shows anti-tumor activity against Sarcoma 180 solid tumor in ddY mice [3]. These properties suggest further potential for chitooligomers and its degrading enzyme--chitosanase.

Chitosan oligomers have been obtained by sonic irradiation, hydrodynamic shearing and chemical hydrolysis of chitosan [2,4]. However, yields of the oligosaccharides from DP2 to DP5 were low because of low efficiency and random cleavage. Enzymatic degradation of chitosan seems to be the better way to prepare its oligosaccharides. In an attempt to find chitosanases that are capable of depolymerization of chitosan for the large-scale preparation of oligomers, we have screened various types of microorganisms and found a group of fungi that produce extra cellular chitosanolytic enzyme-- chitosanase (EC 3.2.1.99). One of the screened fungi with powerful chitosan-degrading activity was identified and designated as *Aspergillus sp. Y2K*. We have shown that the chitosanase purified from this strain was potentially useful for large-scale preparation of chitosan oligosaccharides (5). With the application of our enzyme, kilogram-scale of chitosan oligosaccharides can be easily achieved. Owing to its application potential, we have cloned its correspondent gene and will be discussed in this report. With this gene, we may be able to step further to generate mutant enzyme(s) for specific oligosaccharide preparation.

結果與討論

The *Aspergillus sp.* Y2K strain (5) was used as a gene source of the cloning. The cultivations in Chitosan-M9 medium as described previously (5) for 5 and 4 days were used for preparation chromosomal DNA and total RNA, respectively. The *Escherichia coli* strains TOP10 was used for plasmid transformation.

Isolation of Chromosomal DNA and RNA from *Aspergillus. sp*

The mycelium were collected by filtration and disrupted to a fine power with a mortar and pestle in the present of liquid nitrogen. Chromosomal DNA was then extracted by the traditional method (Molecular Cloning) and RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction.

Construction of cDNA library

A total of 5 μg of RNA isolated from mycelium was used for the construction of cDNA library. Using the set of primer, SmartT and SmartG, for RT-PCR by ThermoScript RT-PCR System (Invitrogen), the annealing temperature of PCR was set on 65°C. The fresh cDNA fragments were cloned into pCR 2.1 TOPO-vector with TOPO TA Cloning kit (Invitrogen) and transformed to competent *Escherichia coli* TOP10 under conditions recommended by the manufacturer.

Amplification of a chitosanase-specific cDNA fragment

The N-terminal amino acid sequence of chitosanase of *Aspergillus*Y2K was determined as YNLPNNLKQIYDKHK (5). According to the N-terminal sequence, two degenerated primers, AFN-1and AFN-2, were designed to pair with SmartT for PCR. The cDNA library was served as template and a chitosanase-specific DNA fragment was amplified and sequenced.

Inverse PCR to obtain genomic DNA containing chitosanase (CTN) gene

For inverse PCR amplification, five micrograms of chromosomal DNA was completely digested by *Eco* RI and self-ligated with 200 U T4 DNA ligase (Roche) at 16°C overnight. The aliquots of the ligation mixture were added in a 50-«l reaction mixture containing 2U *Taq* DNA

polymerase (Viogene), 0.5 mM of the back-to-back primers (I45 and I112) and 1.5 mM $MgCl_2$ for inverse PCR amplification. Reaction mixture in 0.5-ml micro-centrifuge tube was treated sequentially for 30 seconds at 94°C, 30 seconds at 53°C, and 1 minute at 72°C in a programmable PCR machine for 35 cycles. At last step, the reaction mixture was incubated for 10 min at 72°C to complete any unfinished single-stranded DNA. Fresh inverse PCR products were ligated to pCR 2.1-TOPO vector and transformed to competent *E. coli* TOP10 (Invitrogen) as described above.

Table 1 Primers designed for this study

No	Smbol	Oligonucleotides sequence
1	SmartT	5'>AAA CAG TGG TAA CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT T(AGC)(AGCT)<3'
2	SmartG	5'>AAA CAG TGG TAA CAA CGC AGA GTA CGC GGG<3'
3	AF-N1	5'>TA(CT) AA(CT) (CT)T(AG) CCA CC(CT) AA(CT) (CT)T(AG) AA(AG) C<3'
4	AF-N2	5'>C(AGCT)A A(CT)A A(CT)(CT) T(AGCT)A A(AG)C A(AG)A T(ACT)T A(CT)G A(CT)G A<3'
5	I45	5'>CTT GTG TTT GTC GTA GAT CTG TTT CAA GT<3'
6	I112	5'>GGA AAA TGT TCC AAG GTA CTG GCA AAA G <3

DNA sequence and the encoding amino acid sequence

The chitosanase gene (CTN) of *Aspergillus* Y2K was sequenced and shown in Figure 1. The entire gene consists of 866 bp. The first 51 bp (double-underlined) correspondent to 17 amino acids is the sequence of signal peptide. Two introns can be found in the gene with 67bp and 82 bp each, which are shown in bold type. TATA box-like sequences and a CT-rich region are also indicated.

GAATTCCAAAATCAAAAGAGCAACCAACCTGTCGATCAAAACATTAGGTAAAGTTTTGGCCTCATGGTTACATCTCAGG
ATGTGTACTGATGCAAGGATTTGAGGTAGTTCTGAAACTGCAGGGTCTTCAGATGAGACATGCATAGTCCTTGCAGCAT
CAGTGGGTCTATCACGGCGGGGAGATTCGAATCCCTCCTATGGCCTGATCTTACAGTTCCACTGGGCTGGACCCTTATG
GCTACCCTTCGTATCCGTAGATCCAAATCCTTGTGTCAACTTCCCCGGATTTTCCGGATTCGCCGGCTTGTGTGCGAGG
GGGATTCACTTGGTCATCCGACTTGATCAAGCCGGAGCCCGACATCCACGCCGAACTATCAGACTGCCCTGGCGCTTCA
TTTCGAGTATATAAAGAGTACGATATCTGTCTACGACGTAGCCGAACCAACAAGCCCAGGACCTCTTAGATATTAAGGC
AATTGCTCTTCCTGACAATCAATCCGGCACGATGCGTCTCTCTGAAATTCTTACTGTTGCTCTGGTCACTGGGGCCACT
GCTTATAATTTGCCCAACAACTTGAAACAGATCTACGACAAACACAAGG**TATACTGCACACATCTCAAGGTTTGTATTA**
TAGCCAGCCAGCTAATATCTCTTTTTGCGCCGTCAGGGAAAATGTTCCAAGGTACTGGCAAAAGGGTTCACCAATGGTG
ATGCTAGCCAAGGCAAGTCTTTCAGTTACTGCGGCGACATCCCGGGTGCCATTTTCATCTCCTCCTCCAAGGGGTACAC
CAATATGGACATTGACTGCGACGGCGCCAACAACTCCGCCGGCAAGTGCGCCAACGACCCGTCCGGCCAGGGCGAGACT
GCCTTCAAGTCCGACGTGAAGAAGTTTGGCATCTCCGACCTGGACGCCAACATCCACCCCTATGTGGTGTTTGGAAACG
AGGACCACTCTCCCAAGTTCAAGCCCCAGTCACATGGCATGCAGCCATTGAGTGTTATGGCTGTCGTGTGCAATGGCCA
ACTG**GTATGTCTCTCTCATCCATCCAGCAGCCTGCAAGTCGATGTCTGTGTCCGTTTCCGTAGCTAACGCCGAATCCAT**
CACACAGCATTACGGAATCTGGGGTGACACCAACGGTGGCGTTTCTACCGGCGAAGCCTCCATTTCTTTGGCCGACCTT
TGCTTCCCCAACGAGCATCTCGATGGCAACCATGGTCACGATCCCAATGATGTCCTCTTCATTGGCTTCACTAGCAAGG
ACGCCGTGCCTGGAGCGACTGCCAAGTGGAAGGCAAAGAATGCGAAAGAATTCGAGGACAGTATCAAGTCGATTGGTGA
CAAGCTGGTTGCTGGTTTGAAAGCATAGCGAACTAGAAC

Figure 1. Nucleotide sequence of chromosomal DNA of *Aspergillus* sp. CTN. Start (ATG) and stop (TAG) coden are boxed.

The gene sequence and the deduced amino acid sequence were shown in Figure 2. The mature protein consists of 221 amino acids with the calculated isoelectric point and molecular weight of 6.4

and 23446 Da, respectively, which are consistent with those from purified enzyme. Based on amino acid multi-alignment, this enzyme is classified as a member of family 75 glycohydrolases. In addition to the cloning study of CTN from *Aspergillus* Y2K, the chitosanase gene from *Bacillus circulans* is also available. Unfortunately, this enzyme cannot be expressed properly in *E. coli* system. A great deal of effort has been spent on constructing this gene in a *Bacillus subtilis* expression system. After overcoming the transformation problem, we will be able to express this particular chitosanase.

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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'
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Figure 2. Nucleotide sequence and deduced amino acid sequence of *CTN* cDNA from *Aspergillus* sp. Y2K. The first N-terminal 17-amino-acid sequence amino acid is determinated as a signal pepetide is underlined and the N-terminal 15-amino-acid sequence determined from the purified enzyme by Edmann degradation is double-underlined. The asterisk indicates the stop codon.

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