行政院國家科學委員會專題研究計畫 期中進度報告

挑戰聚醣胺水解酵素之自然設計(1/3)

計畫類別：個別型計畫
計畫編號：NSC91-2113-M-009-012-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立交通大學應用化學系

計畫主持人：李耀坤

報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 92 年 6 月 2 日

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計畫參與人員：

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中 華 民 國 92 年 5 月 27 日

中文摘要

關鍵詞: 聚醣胺水解酵素、寡醣、化學修飾、噬菌-抗體外表技術、定點突變、液態層析質譜

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

Abstract

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

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

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

Introduction

Chitosan is a β-1, 4-linked polyglucosamine with vary degrees of *N*-acetylated residues. Its hydrolytes, glucosaminyl-oligosaccharides, have received high attention because of their interesting biological properties, including the inhibitory effect on the growth of fungi/bacteria and the 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 biological properties reveal the application potential of chitooligomers and its degrading enzyme--chitosanase.

Chitooligomers can be obtained by sonic irradiation, hydrodynamic shearing and chemical hydrolysis of chitosan [2, 4]. However, yields of the oligosaccharides from DP2 to DP5 were very low because of low efficiency and random cleavage. Enzymatic degradation of chitosan seems to be the better way to prepare its oligosaccharides. In attempt to find enzymes that are capable of depolymerization of chitosan in large-scale, various types of microorganisms including bacteria and fungi were screened and isolated. An *Aspergillus* strain, designated as *Y2K*, was found to possess powerful chitosan-degrading activity. 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 prepared. For approaching the ultimate goal of preparing chitooligosaccharide with specific DP values and converting the intrinsic catalytic properties of chitosanase, the *Bacillus circulan* chitosanase was extensively inspected in terms of its catalytic function and x-ray structure. It turns out that the *Bacillus* chitosanase is a good candidate for further structural engineering, with which the mutated enzyme can be used for this particular study.

Phage display has proven to be a powerful technology for selecting and engineering novel protein functions (6, 7). Antibodies are well recognized as indispensable tools for recognizing, and tracking target molecules. However, traditional methods for preparing antibodies are cumbersome and labor intensive. The phage display method enables to quickly evaluate a huge range of potentially useful antibodies and then produce large quantities of the selected ones. The success of phage display depends not only on the diversity of the library at the DNA level, but also on the efficiency with which the encoded proteins are displayed on the phage surface. Phage

libraries each typically contain a billion different antibodies, a number comparable to that in human immune systems. To select the phage with the desired antibody from a library, the phage is allowed to bind to the target molecule, which is attached to a solid surface. The phage with antibodies that recognize the target molecule bind tightly and the remaining unbound phage are simply washed away. The DNA contained within the desired phage then can be used to produce more of the selected antibody for use in research or medical diagnostics. The development of high-affinity ligands capable of selectively recognizing a variety of small motifs in different oligosaccharides would be of significant interest as experimental and diagnostic tools. There are currently no antibodies that can discriminate among similar sugar linkages. We are trying to obtain high-affinity chitooligosaccharides from phage display random polypeptide library.

Method and Materials

Construction of *Bacillus cns.* The chitosanase gene (*cns*) was amplified and constructed in pET22b (+) vector at the *NdeI* site and the *NcoI* site to obtain the expression vectors designated as pNde/cns and pNco/cns, respectively. The pNCMO2 vector, serving as PCR template, containing a *Bacillus cereus* chitosanase gene is a generous gift from Professor Ando in Japan. The following two oligonucleotides, cns-Nterm and cns-Cterm, serve as primer for the *cns* gene amplification:

Primers	***NcoI***	***NdeI***
cns-Nterm: 5'-GCT C	CC ATG G	CT TTC CAT ATG GCT TCT CCT
cns-Cterm: 5'- GAC AAT GTA ATT GTT CCC TAC		

Enzyme purification. All purification steps were performed at ambient temperature (approximately 25 ^{o}C). A 1-liter culture of *E. coli* BL21(DE3) with pNde/cns or pNco/cns was grown at 37 ^{o}C to a mid-log phase in LB-medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl for each liter, pH 7.0) containing 100 μg/ml ampicillin. The culture was induced by adding IPTG to a final concentration of 0.5 mM and continued growing for 6 more hours. After centrifugation, cell pellet was resuspended in 15 ml phosphate buffer (20 mM, pH 7.0) containing a suitable amount of PMSF and sonicated for a total of 10 minutes with four intervals of 2 minutes each. The lysate was centrifuged at 10,000 x g for 30 minutes to obtain the cell-free extract which was subjected to ammonium sulfate fractionation. The precipitate between 0 ~ 80 % ammonium sulfate saturation was collected and desalted using HiTrap desalting column (Pharmacia Co.) The filtrate was then applied onto HiTrap SP (5 ml) column pre-equilibrated with phosphate buffer (20 mM, pH 7.0). The column was eluted with a 100 ml linear gradient of NaCl (0 ~ 300 mM) at the flow rate of 1 ml/min. The purified enzyme was collected and stored at 4 ^{o}C for further studies.

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.

Site-directed mutagenesis. Site directed mutagenesis was carried out by Quick change method with the following two pairs of oligonucleotides as primers.

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

Chemical modification. A suitable amount of enzyme was incubated with 10 mM thiol agents at pH8.0 for 2 h. The stoichiometric ratio of labeling reaction was analyzed by ESI/LC/MS. The resulting protein was stored at 4 C for further activity assay.

Phage Library. Phage library was amplified and the phagemid was cultured with TG-1 bacteria in 2xTY medium. The phage/phagemid infects F+-*E.coli* via sex pili. The helper phage M13K07 bacterial filamentous phage (Pharmacia) was used to rescue the phagemid library. From the culture of 500 ml 2xTY containing amphicillin and glucose, 470 ml was used as secondary library stock. The 30 ml was infected with M13K07 bacterial phage and cultured with 470 ml of 2xTY medium and incubated at 30℃ overnight. By spinning the phage was separated from the supernatant and added with 1/5 volume of PEG/NaCl. The phage was separated out by centrifugation after one hour. The phage pellet was resuspended in 5 ml PBS. The phage supernatant was tiered against TG1 and stored at 4℃ in PBS.

Chitosan coupling on sepharose 4B. Sepharose 4B in a glass filter tube was washed for 15 min with 50 ml of 1mM HCl. 0.5g chitooligosaccharides (DP2-7) in 0.1M $NaHCO_{3,\ pH}$ 8.0 was immobilized on sepharose 4B column and rotated continuously for 2 hrs at room temperature. The unbound chitooligosaccharides were washed out with $NaHCO_3$ 0.1M, pH 8.0 for 5 times. 0.1M Tris-HCl buffer, pH 8.0 was added to the resulting sepharose 4B gel. After one hour, it was washed three times with acetate buffer 0.1M, pH 4.0. The final chitooligosaccharides-immobilized gel was stored in Tris-HCl buffer 0.1M, pH 8.0 containing 0.5M NaCl, at 4 C.

Antibody Library Panning. Phage was prepared by infecting the original library stock with M13K07 helper phage as described above. The phage library then used for selection. The chitooligosaccharides-immobilized Sepharose 4B was loaded onto a plastic column and washed with PBS. The PBS washing procedure was carried out for ten times. Next, phage units washed with PBS containing 0.1% Tween-20 for another ten times. Bound phage were eluted with 100 mM triethylamine, neutralized

with one-third volume of 1 M Tris, pH 7.4, and used to infect exponentially growing TG1 cells for 30 min at 37℃. Cells were pelleted, resuspended in 2xTY and plated on a Petri dish containing TYE (15 g of Bacto-Agar, 8 g of NaCl, 10 g of Tryptone, and 5 g of yeast extract in 1 L) with ampicillin and 1% glucose. After growing the bacteria overnight at 30℃, 2xTY containing 15% glycerol was added to the plate, the cells were loosened with a glass spreader, and 100 μl of the mixture was inoculated into 100 mL of 2xTY containing ampicillin and 1% glucose. Cells were grown at 37 ℃, infected with the helper phage in the ratio of 1:20, pelleted, resuspended in 50 ml of 2xTY containing ampicillin and kanamycin, and incubated with shaking overnight at 30℃. Phage particles were precipitated from the supernatant with one-fifth volume PEG/NaCl and resuspended in 2 ml of PBS, and 1 ml of the suspension was used for further selection. Essentially four rounds of selection were performed as described above.

Results and discussion

Construction and overexpression of *B. circulan* chitosanase. DNA fragment containing *cns* was first amplified by cns-Nterm and cns-Cterm primers with the pNCMO2 as template. The target gene was further inserted into the NcoI site or NdeI site for expression. The cns and the deduced amino acid are shown in Figure 1. In fact, two expression systems, *E. coli and Bacillus megaterium*, were constructed for producing the recombinant chitosanase. Although *E. coli* system is much common for protein expression, many proteins are failed to be expressed in active form. *Bacillus* expression systems including *Bacillus brevis*, *Bacillus subtilis* and *Bacillus megaterium*, were first tried. Owing to the low copy number of the vector and low transformation efficiency, the construction of

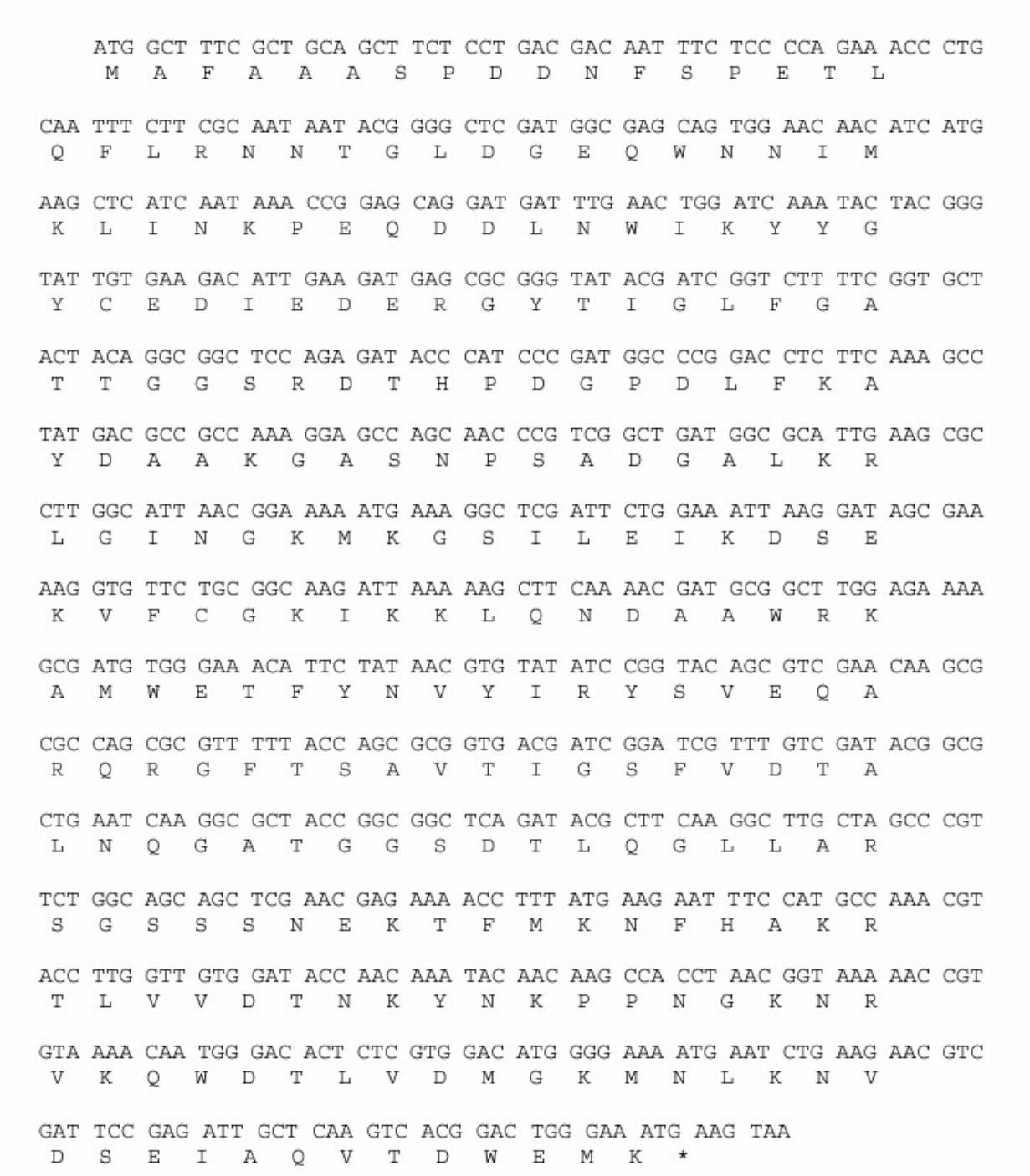


Figure 1. The sequence and deduced amino acid sequence constructed in pET22b (+) expression. Calculated molecular weight is 39441 Da.

chitosanase in *Bacillus* systems was not straightforward. We have been successful in expression of the chitosanase with *Bacillus megaterium* system. However, the protein expression level was not satisfied. Figure 2a showed the SDS–PAGE analysis of the recombinant wild type chitosanase expressed in *Bacillus megaterium* system. Fortunately, this chitosanase can be expressed in *E. coli*. The target gene was inserted in either the *Nde* I or *the Nco* I (with pelB leader sequence) site of pET22b (+). Both constructs are functioning for protein expression. Table 1 summarized purification yield and purity of wild type chitosanase at various stages. Figure 2b presented the purity of all purified mutants.

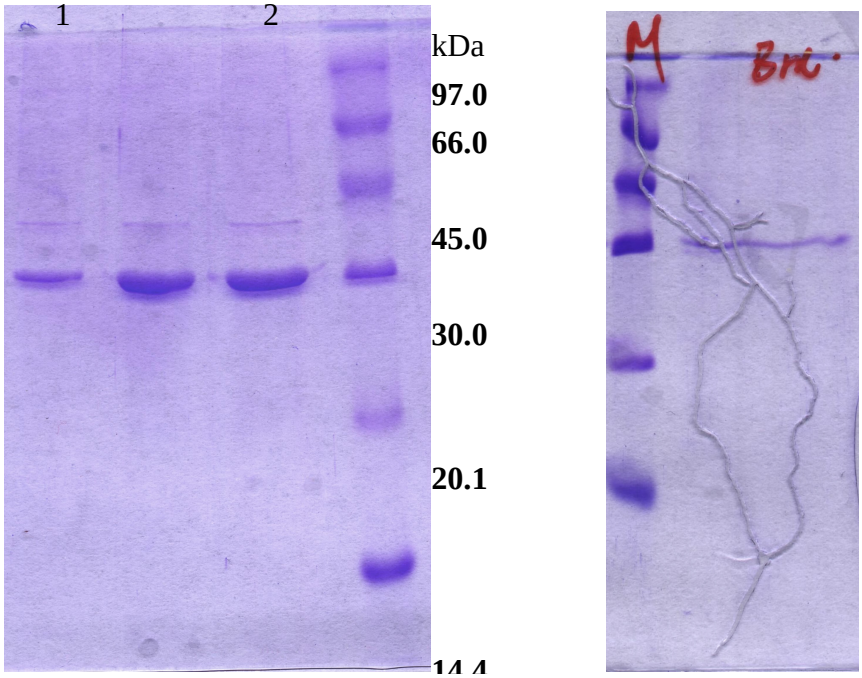


Figure 2a. The SDS-PAGE analysis of WT chitosanase expressed with B. megaterium system (b) Chitosanase and mutants purified by HiTrap SP column. lane 1: E37C; land 2: D55C; lane 3: WT chitosanase; M: marker.

Table 1. Purification of wild type chitosanase

Step	Total Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Protein yield	Enzyme Recovery Yield (%)
Crude extract	411	4073	9.9	1	100
0-80% $(NH_4)_2SO_4$	287	3863	13.5	1.36	94.8
HiTrapSP pH=7.0	3.2	959	300.0	30.03	23.5

1 unit of chitosanase activity: releasing 1 μmole sugar with reducing end in 1 min.

Mutants and chemical modifications. In order to approach our research goal, two conserved amino acids (E37 and D55), which are believed to be the essential groups of this enzyme, were replaced by cysteine residue separately or simultaneously. All mutants, E37C, D55C and E37C/D55C, can be purified with the protocol similar to that of wild type (WT) enzyme. The molecular masses for all mutants and WT chitosanase were confirmed by ESI/LC/MS. Interestingly, the protein expressed by pET22 system with pelB leader showed a nonspecific signal peptide scission. As can be seen in Figure 3, several mass peaks can be observed from the measurement of the purified WT chitosanase. For example, the peaks with values of 29305, 29234, and 29037 Da are corresponding to the proteins (39441 Da) with a Met, Met-Ala, and Met-Ala-Phe shortage of the expecting protein, respectively. Similar results were also observed in the case of E37C and D55C mutants. However, after the treating with 2, 2-dithiodiacetic acid, 3, 3-dithiodipropionic acid, or 4, 4-dithiobutanoic acid, two major protein components with only one expected label can be observed for the cases of both mutants. Figure 4a-c showed the mass analysis of thiolated D55C.

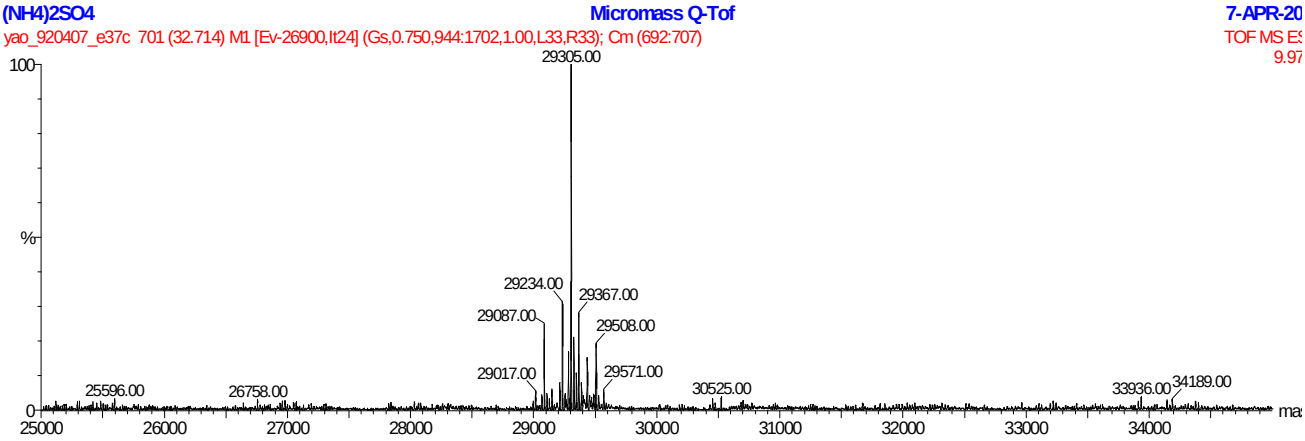
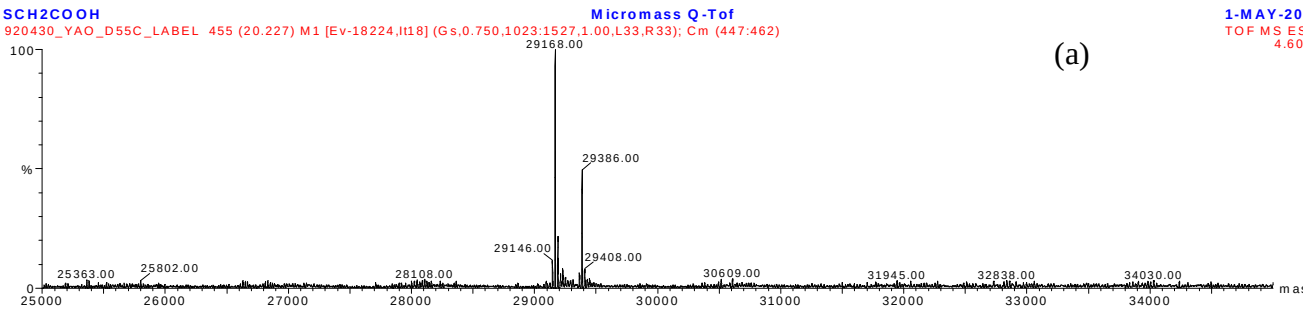


Figure 3. The wild type chitosanase expressed by the pET 22b system with the pelB leader.



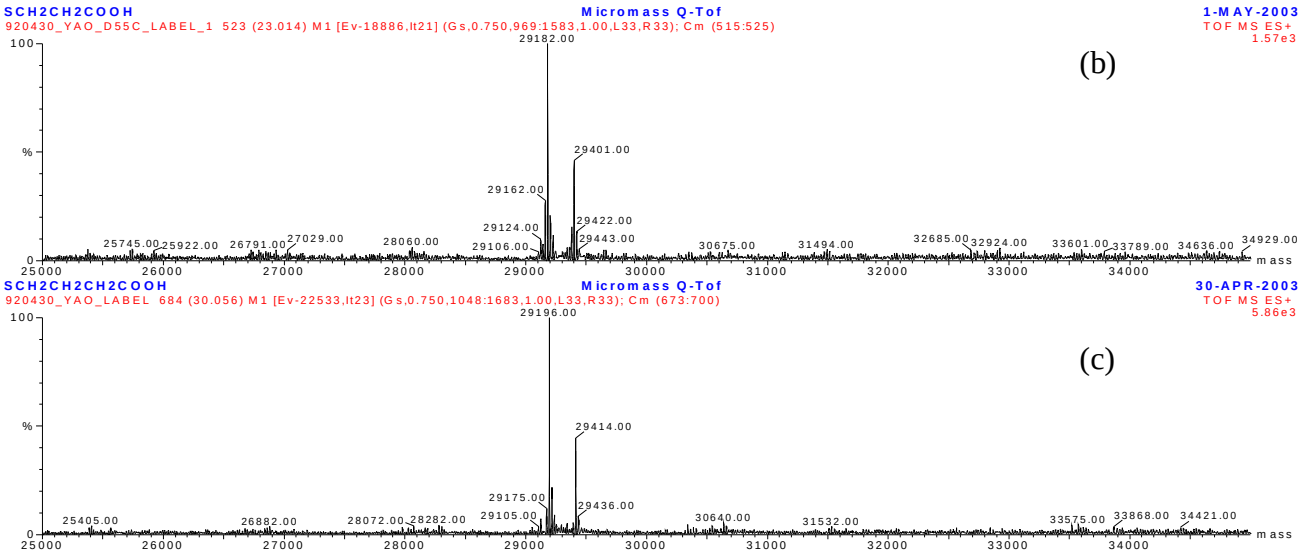


Figure 4. Chemical modification of D55C (major protein peaks: 29077 and 29255) by (a) 2, 2-dithiodiacetic acid (91 Da will be added), (b) 3, 3-dithiodipropionic acid (105 Da will be added), (c) 4, 4-dithiobutanoic acid (119 Da will be added).

Activities and product specificity of the modified enzymes. All mutants with and without labeling of the thiol groups have been studied in terms of their reactivity and product specificity. In comparison with the activity of WT chitosanase, the catalytic activities of D55C and E37C were estimated to be at least 100-fold decrease, while no significant activity of the double mutant, E37C/D55C, can be detected. By inspecting the x-ray structure of this chitosanase, the distance between the carboxyl oxygen of D55 and E37 side chains to the active anomeric center on substrate are estimated to be ~6 A and ~3 A, respectively. This 6 A distance allows water molecule to accommodate and be polarized by the carboxylic site chain of D55, whereas, 3 A is within the hydrogen-bond range. It is reasonable that D55 is believed to serve as the general base for the catalysis, while E37 is functioning as the general acid. Unlike the catalytic mechanism of β-glucosidase (8, 9) and many other glycohydrolases (10), chitosanase precedes the catalytic reaction with the inversion of the anomeric configuration. Chemical modification by 2.2-dithiodiacetic acid, 3,3-dithiodipropionic acid, or 4,4-dithiobutanoic acid were designed to increase the site chain of the general base so that the catalytic mechanism may be converted into the retaining type. Two common methods, known as glycosyl-transferring reaction and *in situ* NMR analysis, are often employed for investigating the catalytic stereo specificity of glycohydrolases. The former method is to analyze the anomeric configuration of the product obtained through glycosyltransferase activity of enzymes. For example, β-xylosidase from *Trichderma koningii* can effectively transfer xylosyl moiety from substrate (e.g. *p*-nitrophenyl-β-xyloside) to many alcohols such as methanol, ethanol, and benzylalcohol to form β-xylosides. This β-xylosidase is therefore believed to be a retaining enzyme (11). In this study, all modified enzymes did not show transglycosylation activity. In order to unequivocally elucidate the catalytic features of the labeled enzymes, the *in situ* NMR analyses are currently performed. The products specificity of all labeled and unlabeled mutants were very similar. As can be seen from the ESI/MS analysis (Figure 5), the major products are the chitobiose, chitotriose and chitotetraose. The catalytic activity of the labeled enzyme is somewhat stronger than that of its corresponding unlabeled enzyme.

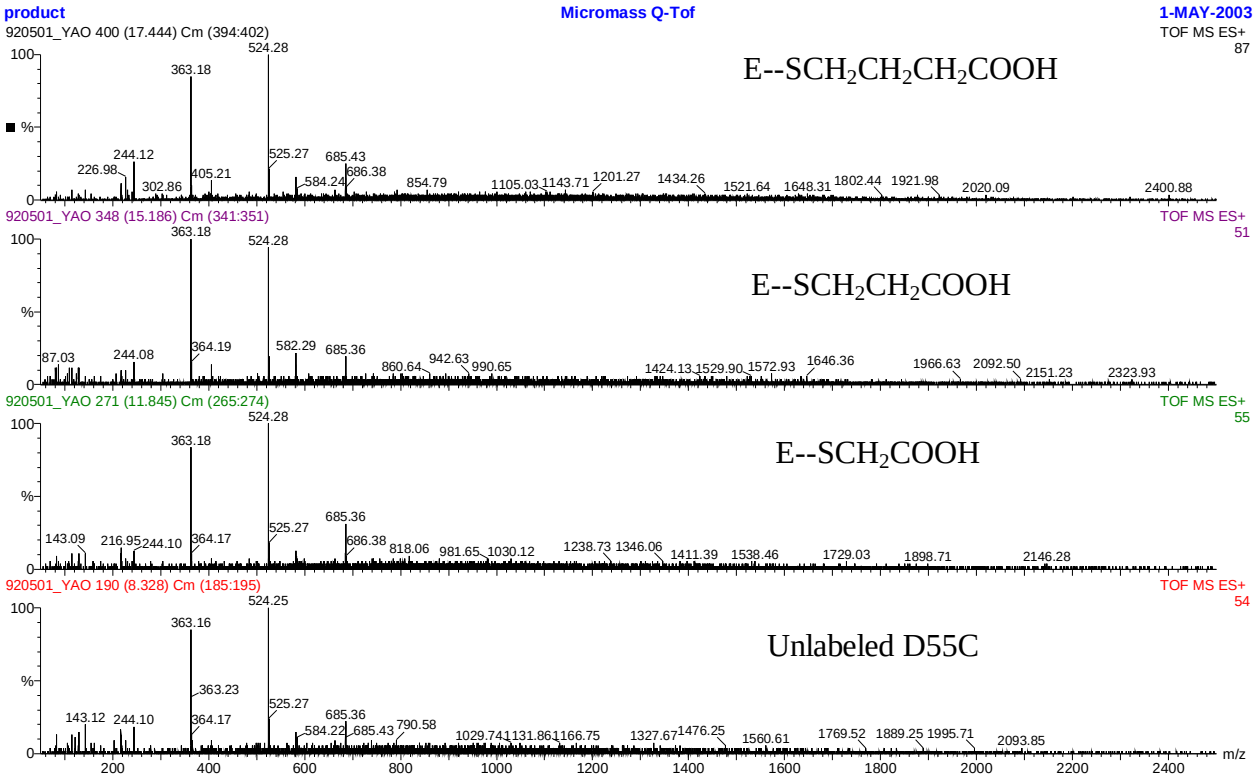


Figure 5. Products analysis by ESI/MS. Chitobiose (DP2+Na: 363), chitotriose (DP3+Na: 524) and Chitotetraose (DP4+Na: 685) were found to be the major products for the unlabeled and labeled D55C chitosanases.

Monoclonal antibody screening by phage display. The standard protocol of phage display and oligosaccharides immobilization on sepharose 4B gel was successfully developed. We have performed at least 5 runs of screening and obtained 50,000 plaques which can bind on the gel immobilized with chitooligosaccharides strongly. The gene of antibody will be sequenced and protein will be expressed in the following year's project.

Reference

1. Izume, M., and Ohtakara, A. *Agric. Biol. Chem.* 1987, 51, 1189-1191.
2. Izume, M., Nagae, S., Kawagishi, H., and Ohtakara, A. *Biosci. Biotechnol. Biochem.* 1992, *56*, 1327-1328.
3. Nanjo, F., Sakai, K., Ishikawa, M., Isobe, K., and Usui, T. *Agric. Biol. Chem.*, 1989, *53*, 2189-2195.
4. Usui, T., Matsui, H., and Isobe, K. *Carbohydr. Res.* 1990, *203*, 65-77.
5. Cheng, C-Y, and Li, Y-K. *Biotechnol Appl Biochem*. 2000, 32, 197-203.
6. Sidhu, S. S. *Curr Opin Biotechnol* 2000, 1l, 610–616.
7. Smith, G. P., Petrenko, V. A. *Chem Rev* 1997, 97,391–410.
8. Li, Y-K., Chir, J., Tanaka, S. and Chen, F-Y. *Biochemistry* 2002, 41, 2751-9.
9. Li, Y-K., Chir, J. and Chen, F-Y. *Biochem. J.* 2001, 355, 835-840.
10. Li, Y-K., Yao, H-J., and Pan, I-H. *J. Biochem*. 2000, 127, 315-320.
11. Li, Y-K., Yao, H-J., and Cho, Y-T. *Biotechnol. Appl. Biochem*. 2000, 31,

119-125.