

Structural simulation and protein engineering to convert an endo-chitosanase to an exo-chitosanase

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To obtain an enzyme for the production of chito-disaccharides (GlcN₂) by converting endo-chitosanase to exo-chitosanase, we chose an endo-chitosanase from *Bacillus circulans* MH-K1 (Csn) as the candidate for protein engineering. Using molecular modeling, two peptides with five amino acids (PCLGG) and six amino acids (SRTCKP) were designed and inserted after the positions of D¹¹⁵ and T²²² of Csn, respectively. The inserted fragments are expected to form loops that might protrude from opposite walls of the substrate-binding cleft, thus forming a ‘roof’ over the catalytic site that might alter the product specificity. The chimeric chitosanase (Chim-Csn) and wild-type chitosanase (WT-Csn) were both over-expressed in *Escherichia coli* and purified nearly to homogeneity. The products formed from chitosan were analyzed by ESI-MS (electrospray ionization-mass spectrometry). A mixture of GlcN₂, GlcN₃ and GlcN₄ was obtained with WT-Csn, whereas Chim-Csn formed, with a smaller catalytic rate (3% of WT-Csn activity), GlcN₂ as the dominant product. Measurements of viscosity showed that, with similar amounts of enzyme activity, Chim-Csn catalyzed the hydrolysis of chitosan with a smaller rate of viscosity decrease than WT-Csn. The results indicate that, on inserting two surface loops, the endo-type chitosanase was converted into an exo-type chitosanase, which to our knowledge is the first chitosanase that releases GlcN₂ from chitosan as the dominant product.

Keywords: chitosan/exo-chitosanase/mass spectrometry/structural modeling

Introduction

Chitosanases (EC 3.2.1.132), catalyzing the hydrolysis of the β -1,4 glycosidic linkage of chitosan, are classified into glycoside hydrolases (GH) in five main families – GH-5, 8, 46, 75 and 80 (<http://afmb.cnrs-mrs.fr/CAZY/>), based on the homology of the amino-acid sequences (Henrissat and Bairoch, 1996). All have been shown to be the endo-type enzyme. In general, glycoside hydrolases can hydrolyze their

substrates in an endo-type or exo-type manner: the exo-type glycoside hydrolases hydrolyze their substrates from one end of the substrate in an orderly manner to produce the corresponding monosaccharide and/or disaccharide, whereas the endo-type glycoside hydrolases randomly degrade polysaccharides at any accessible glycosidic bond to give a mixture of oligosaccharides. Many microbial chitosanase have been found to function as the endo-type enzyme, which exhibit powerful catalysis on chitosan to release chito-oligosaccharides with various degrees of polymerization (DP) (Cheng and Li, 2000; Cheng *et al.*, 2006), but few chitosan-degrading enzymes possess the exo-type catalytic feature. Perhaps, at present, only exo- β -D-glucosaminidase (GH-2) (Cote *et al.*, 2006), which is not classified as a member of the chitosanase families listed above, can cleave chitosan in an exo-type manner to generate glucosamine (GlcN). There is, so far, no successful method to produce a pure chito-oligosaccharide, such as GlcN₂, directly with an enzyme; laborious separation is commonly employed, but allows an isolation of GlcN_n ($n = 2-6$) in pure form on only a small scale.

Although exo- β -D-glucosaminidase, which is considered an exo-chitosanase, was first discovered and characterized in 1990 (Nanjo *et al.*, 1990), the structure of this enzyme is not yet available, so it is difficult structurally to reconstruct a mutant with a desired function. One can, however, learn from nature about the structural designs of glycoside hydrolases in other GH families. In many glycoside hydrolases, such as GH-6, 8, 9, 17, 18, 28 and 55, the exo- and endo-type enzymes are present in the same family. As enzymes in the same family not only possess similarity of their amino-acid sequence, they are also commonly classified into the same clan, indicating that the endo- and exo-type enzymes might be endowed with similar amino-acid sequences and protein folding but with minor structural variation at the catalytic site. For example, both endo-1,4-glucanase and cellobiohydrolase belong to GH-6 and are endo- and exo-type glucanases, respectively. Of their amino-acid sequences, one quarter are identical, and their protein foldings are similar with only few and minor differences (Fig. 1A and B). As shown in the exo-type glucanase (Fig. 1B), two loops were observed to protrude from opposite walls of the substrate-binding cleft to form a roof over the catalytic center. This feature probably results in an exo-type catalytic function of this glucanase. Further work has demonstrated the conversion of the exo-1,4-glucanase into an endo-1,4-glucanase on removing the two loops (Meinke *et al.*, 1995).

A chitosanase from *Bacillus circulans* MH-K1 (Csn) (GH-46) hydrolyzed GlcN₆ in an endo-splitting manner, and released GlcN₂, GlcN₃ and GlcN₄ in almost equivalent proportions (Fukamizo *et al.*, 2005). The enzyme, composed of 259 amino acids (Ando *et al.*, 1992), consists of two globular domains that generate the substrate-binding cleft (Fukamizo *et al.*, 2005). Inspection of protein structures of Csn (PDB

Abbreviations: GlcN, 2-amino-2-deoxy-D-glucopyranose; GlcN_n, β -1,4-linked oligomer of GlcN with a polymerization degree n .

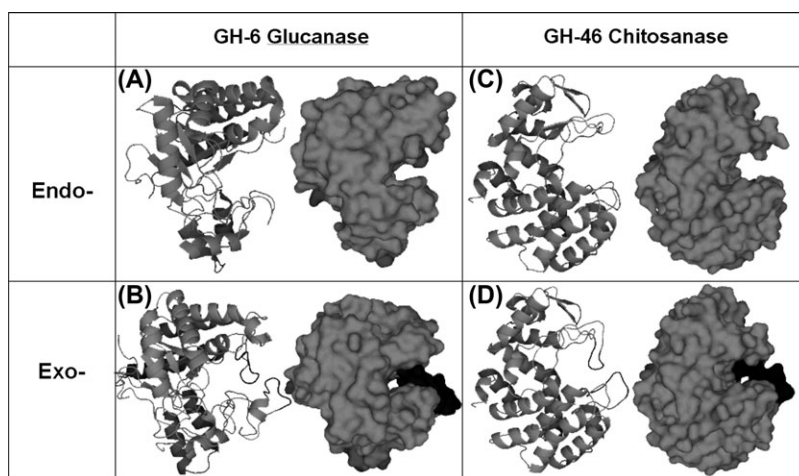


Fig. 1. Comparison of three-dimensional structures of endo- and exo-type glycoside hydrolases. (A) endo-1,4-glucanase E2 of *Thermobifida fusca* YX (PDB ID: 2BOD); (B) cellobiohydrolase, i.e. exo-glucanase of *Humicola insolens* (PDB ID: 1BVV); (C) endo-chitosanase of *B. circulans* MH-K1 (PDB ID: 1QGI); (D) *in silico*, artificial exo-chitosanase derived from endo-chitosanase of *B. circulans* MH-K1.

ID: 1QGI) and the endo-glucanase (PDB ID: 2BOD) in GH-6, Fig. 1C and A, respectively, reveals that both enzymes possess a similar structure that encloses a deep cleft for substrate binding and for catalysis. Our idea to convert an endo-chitosanase to an exo-chitosanase was derived from the above-mentioned information. We designed, and proposed to add, two loops at the appropriate positions of Csn by structure modeling, as shown in Fig. 1D, to form a prospective exo-type chitosanase.

Materials and methods

Chitosan and chito-oligosaccharides

Chitosan with 85% degree of deacetylation was obtained from local suppliers in Taiwan. Chito-oligosaccharides (GlcN₂, GlcN₃, GlcN₄, GlcN₅ and GlcN₆) were purchased (Seikagaku, Tokyo, Japan). All other chemicals (Sigma-Aldrich) were molecular biology grade.

Bacterial strains and plasmids

The vector pNCMO2 containing the *B. circulans* MH-K1 chitosanase gene was provided by Professor Ando in Japan. Two primers, N-term primer 5'-GCTCCCATGGCTTTCCATATGGCTTCTCCT-3' containing an *Nde*I restriction site (in bold) and C-term primer 5'-GACAATGTAATTGTTCCCTAC-3', were used for PCR amplification. The amplified DNA fragment (~1.0 kb) was digested with *Nde*I and *Xho*I restriction enzyme, and then ligated into pET22b (+). This new clone, pET-*csn*, containing the gene for mature Csn with an added methionine at the N-terminus, was transformed into *Escherichia coli* (*E. coli*) strain BL21 (DE3) for further protein expression.

Site-directed mutagenesis

The site-directed mutagenesis was performed according to the Quik-change™ method (Stratagene). The basic procedure involved PCR amplification with pET-*csn* as the template and two synthetic oligonucleotides containing the desired mutation as primers. Insertion of peptides PCLGG and SRTCKP was achieved by double mutations of pET-*csn*. These two primer pairs were designed as follows:

Csn-115-F: 5'-CA GGC GGC TCT AGA GAT CCG TGC CTG GGC GGT ACC CAT CCC GAT GGC C-3',
Csn-115-B: 5'-G GCC ATC GGG ATG GGT ACC GCC CAG GCA CGG ATC TCT AGA GCC GCC TG-3',
Csn-222-F': 5'-CTG AAT CAA GGC GCT ACT AGT CGT ACC TGC AAA CCG GGC TCA GAT ACG CTT C-3', and
Csn-222-B': 5'-G AAG CGT ATC TGA GCC CGG TTT GCA GGT ACG ACT AGT AGC GCC TTG ATT CAG-3'.

Each PCR product was treated with *Dpn*I restriction enzyme to cleave the methylated template DNA, transformed into *E. coli* and confirmed on DNA sequencing of the full gene.

Enzyme production and purification

All purification steps were performed at ambient temperature (~25°C). A single colony of *E. coli* strain BL21 (DE3) harboring plasmid pET-*csn* was inoculated into LB medium (5 ml) containing ampicillin (0.1 mg/ml) and cultivated at 37°C for 12 h. The overnight culture was then transferred into a conical flask (1 l) containing LB medium (500 ml) with ampicillin (0.1 mg/ml) and IPTG (1 mM), at 37°C for 19–20 h. The cell pellet was obtained on centrifugation (6000g, 10 min, 4°C), resuspended in sodium phosphate buffer (10 ml, 20 mM, pH 7.0) and sonicated for 10 min. The lysate was centrifuged at 10 000g for 20 min at 4°C to obtain the cell-free extract. The extract was then applied onto a cation-exchanged chromatographic column (HiTrap™ in 5 ml SP-Sepharose; Amersham Biosciences). The column was pre-equilibrated with phosphate buffer (pH 7.0, 20 mM) and eluted with a linear gradient of NaCl (200–400 mM) in the same buffer at a flow rate 2 ml/min. The fractions with chitosanase activity were pooled and stored at 4°C for further experiments.

Chitosanase activity assay

Chitosanase activity was analyzed by estimating the amount of the reducing ends of sugars according to the dinitrosalicylic-acid (DNS) method (Miller, 1995; Cheng et al., 2006). This standard assay, namely DNS assay, was prepared on mixing chitosan (0.3 ml, 1%, pH 6.0) and enzyme (0.3 ml) with suitable dilution in phosphate buffer

(20 mM, pH 7.0) and incubated for 1 h at 37°C to allow for enzymatic hydrolysis; DNS reagent (0.6 ml) was then added, and the resulting mixture was boiled for 15 min, chilled and centrifuged to isolate the insoluble chitosan. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm. The absorption coefficient of the resulting adducts was determined to be $788 \text{ M}^{-1} \text{ cm}^{-1}$ when D-glucosamine served as the control sample. One unit of chitosanase activity is defined as the amount of enzyme required to release 1 μmol of detectable reducing sugars at 37°C in 1 min (Ando *et al.*, 1992; Cheng and Li, 2000).

Two other methods used to monitor enzyme activity were viscometry (Ohtakara, 1988) and electrospray ionization-mass spectrometric (ESI-MS) analysis. Although the former method is cumbersome and insensitive, it has the potential to detect early variations in the reaction solution to determine the splitting pattern of exo- or endo-type. It was performed with a Brookfield DV-II viscometer (Model LVDV II+, USA). The enzymatic reactions (8 ml) were performed with Chim-Csn (15 μg) [or 0.3 μg wild-type chitosanase (WT-Csn)] at 25°C using chitosan (0.5%, w/v) as substrate and analyzed at appropriate intervals. The ESI-MS method is described later.

Protein concentration and purity determination

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. The purity of the enzyme was verified with sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a gel system (12.5%, Laemmli, 1970) and enzyme ($\sim 5 \mu\text{g}$). The protein bands were visualized on staining with Coomassie brilliant blue R-250.

Identification of the disulfide bond in Chim-Csn

The disulfide bond was investigated on labeling via chemical treatment and further analyzed colorimetrically or with ESI-MS. Purified protein was treated with or without dithiothreitol (DTT, 10 mM) for 30 min at $<25^\circ\text{C}$. It was then exposed to iodoacetamide (IAA, 50 mM, 50 mM Tris, pH 8.0) in the dark at room temperature for 2 h to alkylate any cysteine that existed or had been released from previous disulfide bonding. After chemical treatment, proteins were analyzed with ESI-MS as described in the following section. Alternatively, the purified protein was treated with 5,5-Dithiobis (2-nitrobenzoic acid, 10–50 mM) (DTNB; Ellman's reagent) (50 mM Tris, pH 8.0), and the absorbance of the sample supernatant was characterized at 412 nm against the control.

Electrospray ionization-mass spectrometry analysis

Mass spectra of enzyme were recorded with a quadrupole time-of-flight mass filter (Micromass, Manchester, UK) scanning with a ratio of mass to charge in the range 500–2500 units (m/z), with a scan 2 s/step and an interscan interval 0.1 s/step. In all ESI-MS experiments, the quadrupole scan mode was used under a capillary needle at 3 kV and the source block temperature 80°C. For the MS measurements, the desalted proteins were obtained from a column (Zip-Tip, Amersham Biosciences) or ultrafiltration spin column (Vivaspin, Vivascience). Protein samples (2–5 μg) were infused in the mass spectrometer with acetonitrile solution (10%, containing 0.1% formic acid as eluent).

A triple-quadrupole MS system (Quattro Micro; Micromass) was used for the analysis of the enzymatic product. Chitosan (0.05%, w/v) was mixed with appropriately diluted chitosanase (9.4 μg Chim-Csn, or 0.3 μg WT-Csn; total volume 5 ml). The enzymatic reaction was performed at 25°C; an aliquot (10 μL) withdrawn from the reaction mixture at a constant interval was injected into the quadrupole MS with an auto-sampler.

Structure modeling

A structural modeling was employed to generate a model of chimeric chitosanase (Chim-Csn) on inserting peptides to form two loops protruding from opposite walls of the substrate-binding cleft. Each peptide consists of one cysteine that is near enough in space to form a disulfide bond. The procedures of structure modeling are listed as follows: First, we selected a high-resolution (1.6 Å) crystal structure of chitosanase [PDB accession code 1QGI (Saito *et al.*, 1999)] from *B. circulans* MH-K1 as the protein template. Secondly, we selected two insertion points located at regions Arg¹¹⁴ to Thr¹¹⁶ and Thr²²² to Ser²²⁵ because they are at the edge of the active site cleft. The insertion of the peptides into these two regions is expected to maintain the conformation of the active site and the core structure of chitosanase. Thirdly, to estimate the length of the inserted peptide, we calculated the space around both insertion points. Peptides containing 4–8 amino-acid residues were selected to form the protruding loop. Fourthly, we designed the inserting peptides based on the conformation and composition of β -turn. Proline and glycine are commonly observed in the β -turn because of the steric hindrance imposed by the turn geometry (Creighton, 1993). Each peptide required one cysteine for disulfide bond formation. Based on these criteria, peptides composed of a cysteine, several prolines and glycines, and others can be suggested and further examined. Fifthly, the modified protein sequence was modeled for a theoretical structure using SWISS-MODEL (Guex and Peitsch, 1997; Schwede *et al.*, 2000, 2003). Sixth, SYBYL version 6.9 (Tripos, Inc.) was used for energy minimization. Parameters used in SYBYL were set as follows: initial optimization = simplex, termination = gradient, 0.05 kcal/(mol*Å), NB (non-bonded) cutoff = 8.0 Å, dielectric constant = 1.0 and maximum iterations = 500. Finally, we evaluated the structural models based on two criteria: the inserted peptide pair can form thermodynamically stable loops to form a roof over the catalytic site, and two cysteines on the peptide pair are near enough in space to form a disulfide bond.

Results and discussion

Protein engineering and structure modeling

More than 25 peptide pairs, such as [GLCG, AGLCGA], [PCLGG, ARCTKL], [PCLGG, SRCTKP] and [PCLGG, SRTCKP], were modeled based on the structure-modeling procedure. On observing these structural models, we found that proline directly influenced these two peptides to bend toward each other to form a disulfide bond. Finally, a Chim-Csn with two peptides of PCLGG and SRTCKP was designed and modeled as shown in Fig. 2.

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1  MHMSNARPSK SRTKFLLAFL CFTLMASLFG ATALFGPSKA AA1MASPDNFS
51  PETLQFLRNN TGLDGEQWNN IMKLINKEPEQ DDLNWKIYYG YCEDIEDERG
101 YTIGLFGATT GGSRD1pc1lgg1THPDG PDLFKAYDAA KGASNP SADG ALKRLGINGK
151 MKGSILEIKD SEKVFCKGKIK KLQNDAAWRK AMWETFYNVY IRYSVQARQ
201 RGFTSAVTIG SFVDTALNQG AT1sr1tkcp1GGSDTLQG LLARSGSSSN EKTFMKNFHA
251 KRTLIVVD1TNK YNKPPNGKNR VKQWDTLVDM GKMNLNKVD1S EIAQVTDWEM
301 K

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Fig. 2. Amino-acid sequence of Csn and Chim-Csn. The signal peptide of Csn is underlined. The three boxes in order are added methionine (start codon), inserted peptide 1 and peptide 2. The WT-Csn, with a deduced molecular mass 29081 u, was established on deleting the signal peptide and inserting one extra methionine at the N-terminus of the mature protein. The Chim-Csn, with a deduced molecular mass 30124 u, was constructed on inserting peptides 1 and 2, and removing the glycine (shown in shaded) right after peptide 2.

The distances between C_{β} atoms and S atoms of the two cysteines in the two inserted peptides were 5.06 and 5.99 Å, respectively, in this structure. Because of the dynamic flexibility of these two inserted peptides in an aqueous solution and the rotation of C_{β} -SH bonds, these two cysteines can form a disulfide bond between the inserted peptide pair. The root-mean-square deviation (RMSD) between WT-Csn and the Chim-Csn was 0.6 Å (excluding the two inserted peptides). This RMSD value indicates that the conformation of the active site is preserved after insertion of the peptide pair. These results indicate Chim-Csn to be a satisfactory candidate for our work.

Plasmid construction, over-expression and purification of recombinant chitosanase

The *Bacillus* chitosanase gene was first reported to be expressed in the *Bacillus brevis* system (Saito *et al.*, 1995), but we successfully employed the *E. coli* expression system in this work. A DNA fragment containing *csn* was translocated from pNCMO2 into the pET22b (+) vector for protein expression.

To construct the gene of the mature Csn, we removed the DNA fragment encoding the signal peptide while adding a codon of methionine (ATG) at the N-terminus of the mature protein. The Chim-Csn was constructed by a double mutation to insert peptide 1 and peptide 2 after the positions of D¹¹⁵ and T²²² of Csn, respectively. The amino-acid sequences of Csn and the mutant are shown in Fig. 2. Both enzymes were over-expressed in *E. coli* and further purified with cation-exchange chromatography. The deduced molecular masses of WT-Csn and Chim-Csn are 29079 and 30122 u, respectively. ESI-MS analysis showed the molecular masses of WT-Csn and Chim-Csn to be 28948 and 29991 u, respectively (Fig. 3A and B). The difference 1043 u between the two recombinant proteins confirmed the insertion of the two peptides, whereas the lack of 131 u, relative to the deduced molecular mass, on WT-Csn (and on Chim-Csn) is explicable by the removal of the first methionine, which is common for proteins produced in *E. coli*.

Identification of the disulfide bond in Chim-Csn

As shown in Fig. 1D, two peptide loops, each containing a cysteine residue, were inserted in opposite walls of the substrate-binding cleft. These two cysteine residues were

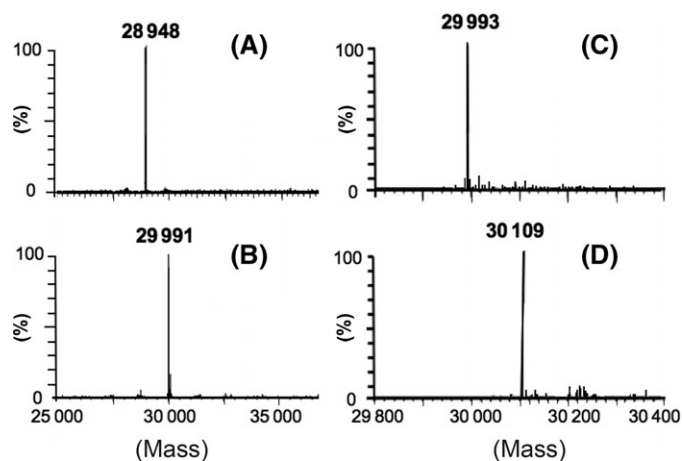


Fig. 3. Mass spectra of recombinant chitosanase and IAA-treated chitosanase. The measured molecular masses of WT-Csn (A) and Chim-Csn (B) are 28948 and 29991 u, respectively. The measured molecular masses of Chim-Csn treated with IAA only (C) and with both DTT and IAA (D) are 29993 u (expected value 29991 u) and 30109 u (expected value 30107 u), respectively.

designed to form a disulfide bond crossing the protruding loop. The existence of a disulfide bond was examined by chemical labeling and further analyzed by colorimetry or ESI-MS. As expected, when Chim-Csn was treated with DTNB, no UV change at OD_{412 nm} was detected. Further treatment with IAA showed no alteration of the molecular mass (Fig. 3C). These results indicate that there was no accessible free -SH group in Chim-Csn or WT-Csn. In contrast, when Chim-Csn was pre-treated with DTT and further with IAA, a 116-dalton increment in the molecular mass of Chim-Csn was observed (Fig. 3D), indicating that the disulfide bond on the roof was destroyed and the two free -SH groups became available for IAA labeling reaction. One more disulfide bond between Cys⁹² and Cys¹⁶⁶ (Saito *et al.*, 1999), which is buried inside the protein, cannot be labeled, like the case that modification with IAA WT-Csn failed under the same conditions. These results show that the two

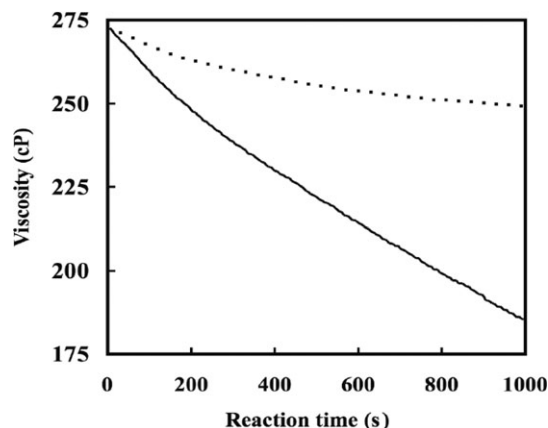


Fig. 4. Monitoring the viscosity variation of reactions catalyzed with WT-Csn (bold line) and Chim-Csn (dotted line). Activities of both enzymes, based on DNS assay, were controlled at a similar level. The viscosity was continuously monitored with a Brookfield V-II viscometer using chitosan (0.5% w/v) as the initial substrate.

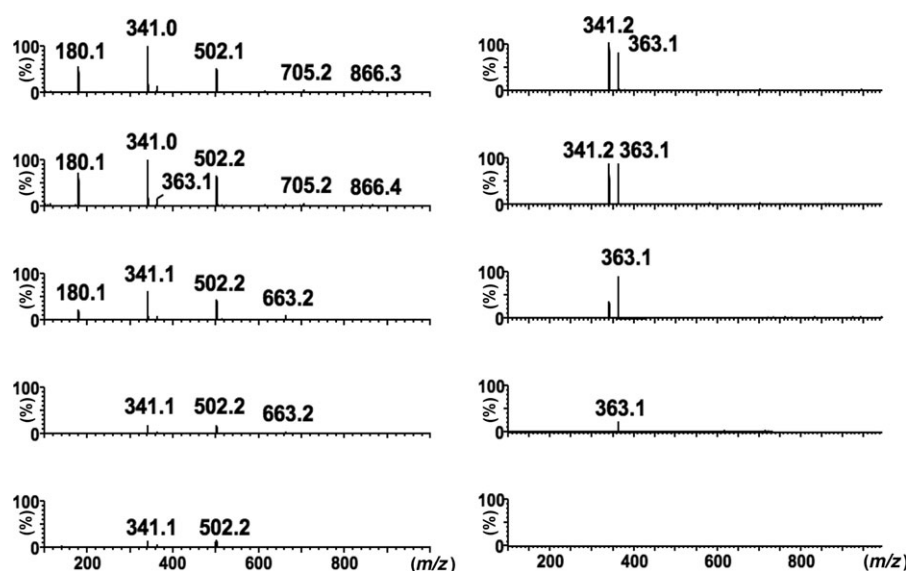


Fig. 5. MS analyses of products derived from hydrolysis of chitosan by WT-Csn and Chim-Csn at various durations of reaction. The mass spectra shown from bottom to top pertain to samples taken at 5, 50, 100, 200 and 300 min of the reaction. In WT-Csn catalysis (left column), glucosamine (DP1: m/z 180.1), GlcN₂ (DP2: m/z 341.0 and DP2 + Na⁺: m/z 363.1), GlcN₃ (DP3: m/z 502.2) and GlcN₄ (DP4: m/z 663.2) were produced concurrently. In Chim-Csn catalysis (right column), GlcN₂ (DP2: m/z 341.2 and DP2 + Na⁺: m/z 363.1) was detected as the dominant product throughout the hydrolysis.

added cysteines formed a disulfide bond, which might alter the binding topology of the substrate and consequently convert the endo-chitosanase into an exo-type enzyme.

Viscometric assay

To distinguish the conversion of endo- to exo-type mechanisms, we monitored the variation of viscosity of chitosan (0.5% w/v), monitored in the presence of WT-Csn and Chim-Csn, separately. It is important to add a similar amount of enzyme activity in the viscometric assay to ensure a comparable rate of production of the reducing sugar. The slow initial decrease of viscosity indicates an exo-type of enzymatic catalysis. As Chim-Csn was found to exhibit activity 3% of that of WT-Csn by DNS assay, 50-fold more concentrated Chim-Csn, relative to the employed WT-Csn, was applied in this work. As shown in Fig. 4, WT-Csn decreased the viscosity of chitosan solution greatly at an early stage of the reaction with a rate 5.48 cp/min, whereas Chim-Csn decreased the viscosity at a much smaller rate, 1.48 cp/min. This result indicates that the splitting pattern of substrate-catalyzed Chim-Csn differs from the endo-type of WT-Csn. The viscosity measurements provided a positive clue showing that Chim-Csn promoted the hydrolysis of chitosan in an exo-type manner, but the results are also explicable with an assumption that the enzyme became processive as discussed in the cases of GH-18 chitinase (Horn *et al.*, 2006; Sikorski *et al.*, 2006), GH-6 cellobiohydrolase II and GH-7 cellobiohydrolase I (Boisset *et al.*, 2000).

ESI-MS analysis of the enzymatic product

In general, an exo-type chitosanase can hydrolyze chitosan into a specific product, and an endo-type chitosanase hydrolyzes chitosan to form a mixture of chito-oligosaccharides. To confirm Chim-Csn is an exo-hydrolase and to analyze its hydrolytic product, we performed a temporal course experiment, monitoring the initial products with ESI-MS.

As expected, WT-Csn acting as an endo-chitosanase simultaneously releases various oligosaccharides, such as GlcN, GlcN₂ and GlcN₃, from chitosan (Fig. 5), consistent with published results (Fukamizo *et al.*, 2005). In contrast, during the entire catalytic reaction of Chim-Csn, GlcN₂ was the dominant product. This result not only confirms that Chim-Csn is an exo-hydrolase, but also identifies the enzymatic product as GlcN₂.

Funding

National Science Council and the MOE-ATU Program in Taiwan provided financial support.

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**Received September 24, 2007; revised April 23, 2008;
accepted May 7, 2008**

Edited by Dick Janssen