# Studies on the active site of chitosanase from *Paenibacillus fukuinensis* and its functional modification for utilizing chitosan

**Danya ISOGAWA**

**2014**

# Contents

# Abbreviations

| | |
|---|---|
| **BSA** | Bovine serum albumin |
| **CWPs** | Cell wall proteins |
| **DNS** | 3, 5-Dinitrosalicylic acid |
| ***E. coli*** | *Escherichia coli* |
| **ER** | Endoplasmic reticulum |
| **GPI** | Glycosylphosphatidylinositol |
| **HPLC** | High performance liquid chromatography |
| **MATα** | Mating-type α |
| **MATa** | Mating-type a |
| **PBS** | Phosphate-bufferd saline |
| **PCR** | Polymerase chain reaction |
| **PI-PLC** | Phosphatidylinositol-specific phospholipase C |
| ***S. cerevisiae*** | *Saccharomyces cerevisiae* |

# Introduction

Today, there are various biomass such as cellulose, chitin, and chitosan around the world and it is claimed that we have to utilize and recycle them effectively. Cellulose is one polysaccharide that can be obtained mainly from plant cell wall and is composed of β-1, 4 linkages of D-glucose. The amount of it is the most in biomass and it is annually produced about 10 billion ton. It is used mainly as wood, paper, and fiber, and the method of utilization of waste cellulose is widely investigated. Recently, the research of ethanol production from it is very popular and it becomes to utilize in various fields.

Chitin and chitosan are also natural polysaccharide that can be obtained mainly from crustaceans shell (Fig. 1). The amount of existing chitin is relatively abundant and is next to that of cellulose on earth. However it is annually produced as well as the amount of cellulose production, only a few percent of that are currently utilized. Therefore, it is necessary to promote utilization of that as "the last" biomass remained on earth. Chitin is an insoluble polymer composed of β-1, 4 linkages of *N*-acetyl-D-glucosamine and has a rigid structure for utilization (1). Chitosan is a partly deacetylated form of chitin and composed of β-1, 4 linkages of *N*-acetyl-D-glucosamine and D-glucosamine. It is a soluble polymer, and thus, chitosan can be utilized more easily than chitin (2, 3).

**Variation in bioactivity between chitosan polymer and chitosan oligosaccharides**

Chitosan polymers have various bioactivities such as anti-bacterial activity by inhibiting formation of bacterial cell wall, blood coagulation activity by activating enzymes related blood coagulation, and cholesterol lowering activity by binding to fatty acid (Fig. 2A). These bioactivities are mainly due to its positive charge of amino group in chitosan structure. In addition to such activities, on waste water treatment, chitosan is utilized as aggregation agent because amino group forms chelate bond to heavy metal ions. Therefore, chitosan is very attractive biomass and has prospect for utilizing various fields.

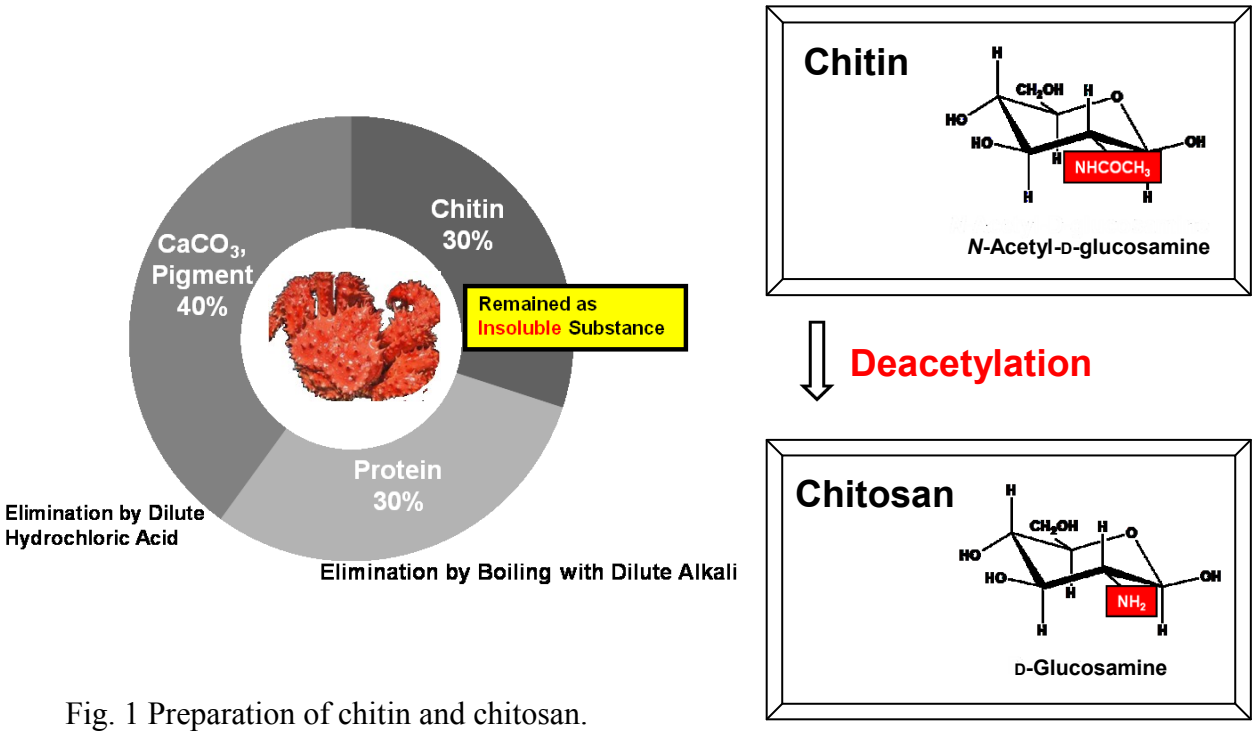


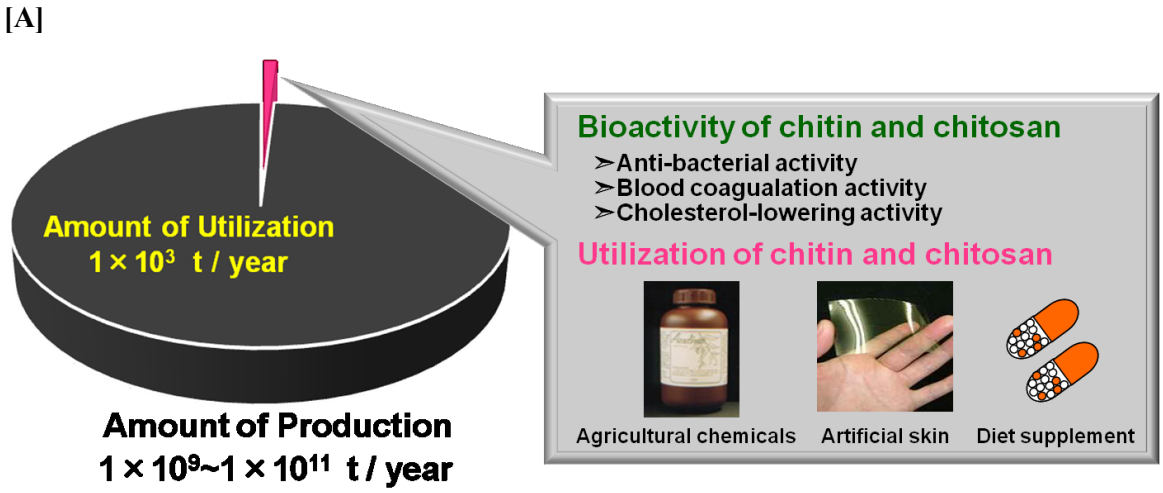

Fig. 1 Preparation of chitin and chitosan.

Chitosan oligosaccharides obtained after hydrolysis of chitosan polymers have also bioactivities such as antitumor activity and anticancer activity, in addition to bioactivities that chitosan polymer has (4, 5). The type of activity differs with the length of polymerization of glucosamine, a monomer of chitosan. Moreover, it is reported that such bioactivities of chitosan oligosaccharides are higher than that of chitosan polymer.

However chitosan oligosaccharides are such a useful biomaterial, the utilization is got behind since their high manufacturing cost. If we could produce desired valuable chitosan oligosaccharides selectively, it may contribute to reduce the underuse biomass and improve the rate of utilization of chitin and chitosan.

Chitosan is mainly degraded by chemical method for production of oligosaccharides. By the method, the degree of degradation can't be easily controlled and emitted chemical detergent is difficult issue (Fig. 2B). Similarly, by enzymatic method, chitosan is finally degraded to short chain such as dimer or trimer and can't obtain useful oligosaccharides easily using natural chitosansase. Although by using

natural chitosanase we can't obtain them, if we construct chitosanase mutants by genetic engineering method, we may control the degree of chitosan degradation (Fig. 2B). Therefore, the methods and clue of solution for producing them should be seeking.



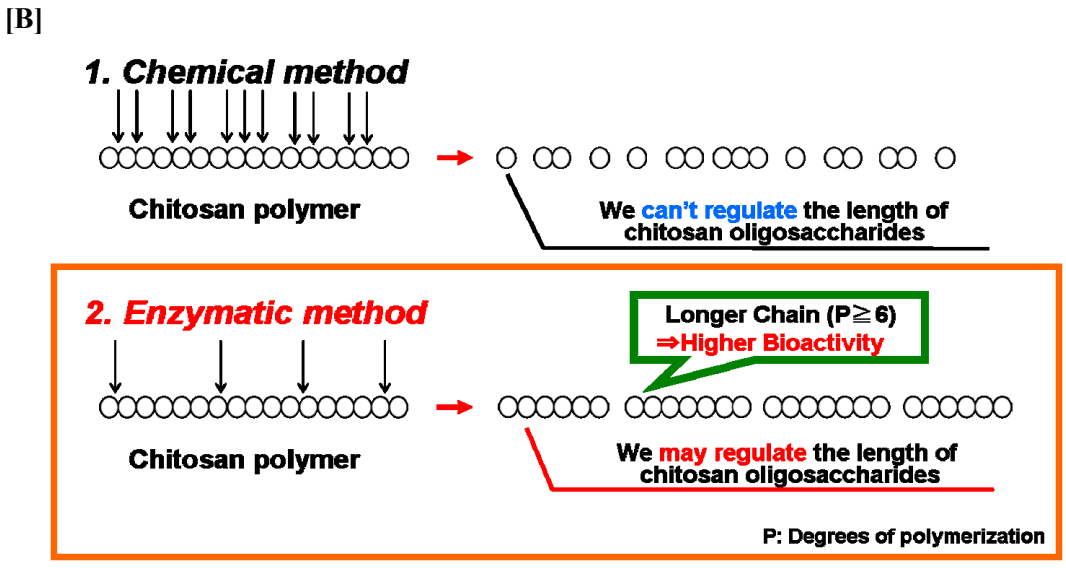




Fig. 2 [A] Bioactivity and utilization of chitin and chitosan [B] Difference of production of chitosan oligosaccharide between chemical method and enzymatic method.

***Paenibacillus fukuinensis***

Chitin hydrolysis enzyme "chitinase" (EC 3.2.1.14) catalyzes endo- or exo-hydrolysis of β-1, 4 linkages between two *N*-acetyl-D-glucosamines and chitosan hydrolysis enzyme "chitosanase" (EC 3.2.1.132) catalyzes exo- or endo-hydrolysis of β-1, 4 linkages between *N*-acetyl-D-glucosamine and D-glucosamine residues in partly acetylated chitosan. *Paenibacillus fukuinensis* screened in Fukui prefecture is gram-positive facultatively anaerobic rod bacteria (Fig. 3A). In the previous study, it was revealed that both chitinase and chitosanase were secreted by *P. fukuinensis*. Almost bacteria usually secrete either of them and, however, *P. fukuinensis* secretes both of them. Furthermore, some bacteria that have similar property to *P. fukuinensis* discovered previously and these bacteria are very unique. However, it has not already done enough investigation why they have both enzymes and the role of them.

**Chitosanase from *P. fukuinensis***

Glycoside hydrolases are classified into 133 families on the basis of their amino acid sequence (6-9). General chitosanase belongs to the family 46 glycoside hydrolase group (GH-46), as determined by sequence-based classification of glycosyl hydrolases (10, 11). The enzymes classified into family 8 glycoside hydrolase (GH-8) on the basis of homology of amino acid sequences are chitosanase, cellulose, xylanase, and lichenase (Fig. 3B). Representative enzyme in family 8 is cellulase A from *Clostridium thermocellum* (CelA) and it is conventional monofunctional enzyme (12). Besides these monofunctional enzymes, bifunctional enzymes that have both chitosanase activity and glucanase activity are present in family 8. For example, the enzymes from *Myxobactor* AL-1 (13, 14), *Bacillus* sp. *number* 7-M (15), *Streptomyces griseus* (16), *Bacillus circulans* WL-12 (17), and *Bacillus* sp. strain KCTC 0377BP (18) are bifunctional enzymes. However it is an interesting point, it was not made cleared why such diversity of enzymatic activity occurred in family 8 although amino acid sequences and structures are very similar.

Chitosanase from *P. fukuinensis* belongs to the family GH-8 glycoside hydrolase group (GH-8) by comparison of amino acid sequence and is a bifunctional enzyme, that is, it possesses both chitosanase and β-1, 4 glucanase activities (19) (Fig. 3C). Moreover, this chitosanase was secreted with two discoidin domains. It was clarified that these domains were functionalized as binding chitosan and glucan specifically and it was essential for degrading zygomycete cell wall (20). No chitosanase containing discoidin dimain was discovered previously and, therefore, it is very unique character.

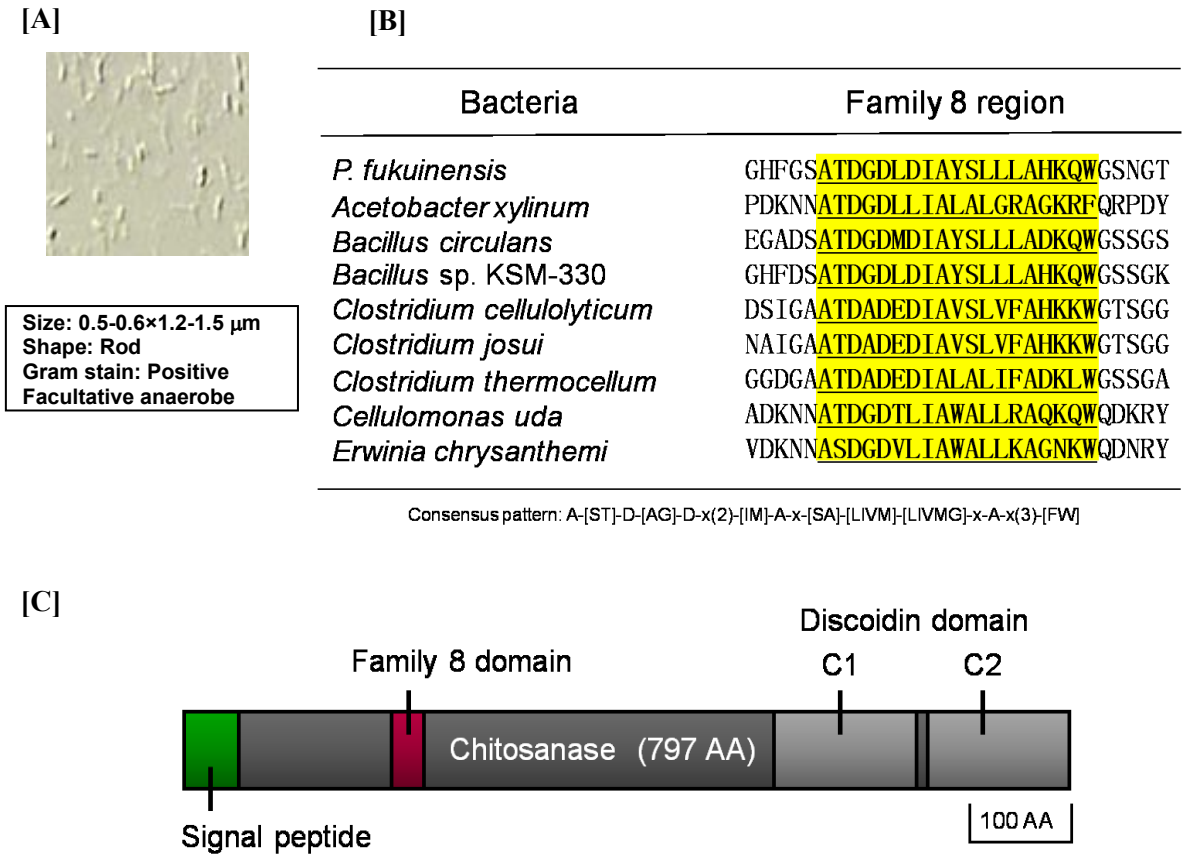



Fig. 3 [A] Micrograph of *P. fukuinensis* and its features [B] Sequence alignment of family 8 region among glycoside hydrolases belonging to family 8 [C] Domain structure of chitosanase from *P. fukuinensis*.

Comparison of amino acid redsidues of catalytic base between cellulase A from *Clostridium thermocellum* (CelA) and chitosanase from *P. fukuinensis* classified into family 8, we can observe the significant difference in the area of catalytic base (Fig. 4). These differences may bring new enzymatic activity beside the activity that the enzyme originally contains and is the reason of development of bifunctional enzyme. Therefore, the elucidation of the difference of catalytic amino acid residues of chitosanase from *P. fukuinensis* is very important and may be the key research area in enzymatic evolution as obtaining new enzymatic activities.

| Function | Cellulase A from *C. thermocellum* | Chitosanase from *P. fukuinensis* |
|---|---|---|
| Catalytic Acid | Glu95 | Glu115 |
| Substrate Recognition Site | Asp152 | Asp176 |
| Catalytic Base | No corresponding amino acid residue | Glu302 |
| Catalytic Base | Asp278 | Asn312 |

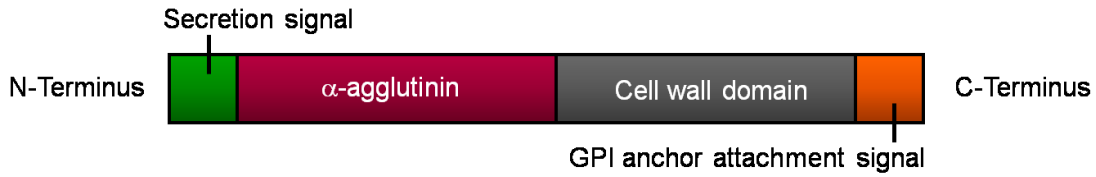
Fig. 4 Difference of amino acid residues of catalytic base between cellulase A from *C. thermocellum* (monofunctional) and chitosanase from *P. fukuinensis* (bifunctional) in family 8.

**Yeast cell-surface displaying system**

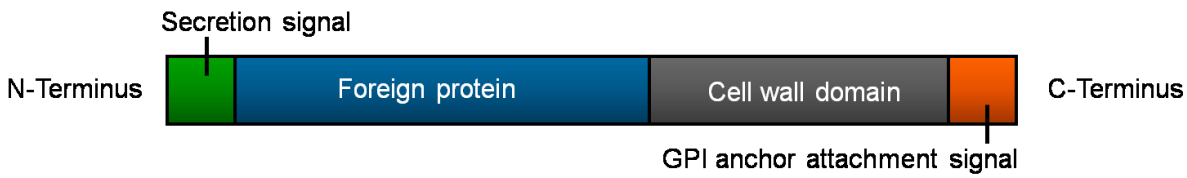
The yeast cell-surface displaying system is implemented in the use of the molecular information of yeast cell wall proteins (CWPs). Among the CWPs, α-agglutinin (Sag1p), which is produced during in mating-type α (MATα) yeast cells and mediates direct cell-cell sexual aggregation with mating-type a (MATa) yeast cells (21), is extensively used for the yeast cell surface displaying system (22). α-Agglutinin is composed of secretion signal, an active domain, and a cell wall binding domain containing glycosylphosphatidylinositol (GPI) anchor attachment signal (Fig. 5A). To produce a target protein on the yeast cell surface, the N-terminal side of the protein is

fused to an appropriate secretion signal and the C-terminal side of the protein is fused to the C-terminal half of α-agglutinin, which is the cell wall binding domain containing GPI anchor attachment signal (Fig. 5B). The fusion protein can be displayed on the cell surface by following pathway. After transcription and translation, the precursor fusion protein is transported to the endoplasmic reticulum (ER) by recognizing the recreation signal sequence and retained on the ER lumen. Then the GPI anchor attachment signal sequence is recognized and cleaved. The newly formed C-terminal side of the protein is fused to ethanolamine in a GPI anchor. The GPI-anchored protein is transported onto the plasma membrane by exocytosis via Golgi bodies and secretory vesicles. After the cleavage of the GPI anchor by phosphatidylinositol-specific phospholipase C (PI-PLC), the protein is released to the cell wall and finally fixed to it through covalent bonding by transglycosylation (Fig. 5C). Through this molecular mechanism, diverse proteins can be displayed on the yeast cell surface (22).

**[A]**




**[B]**



[C]

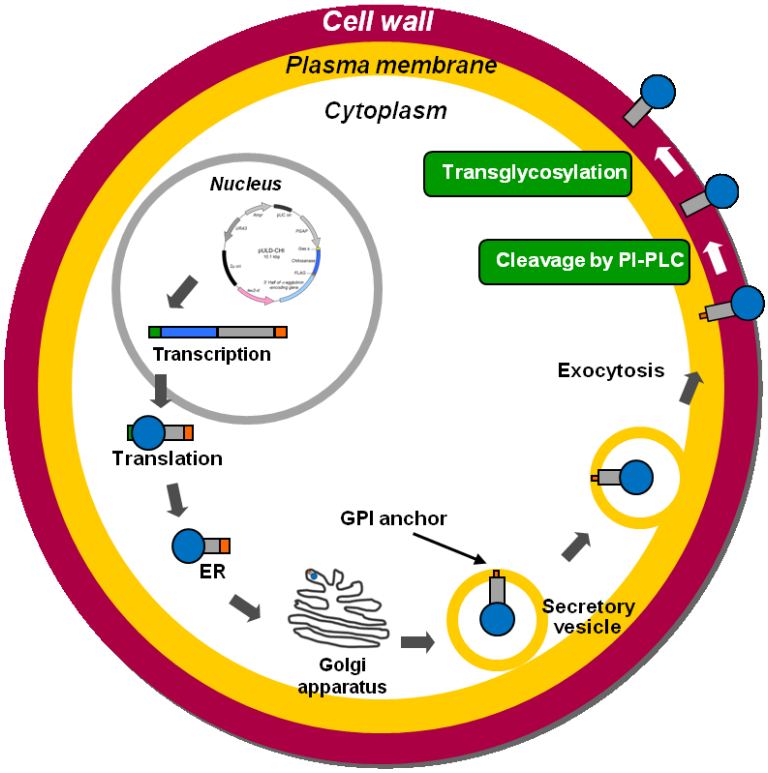


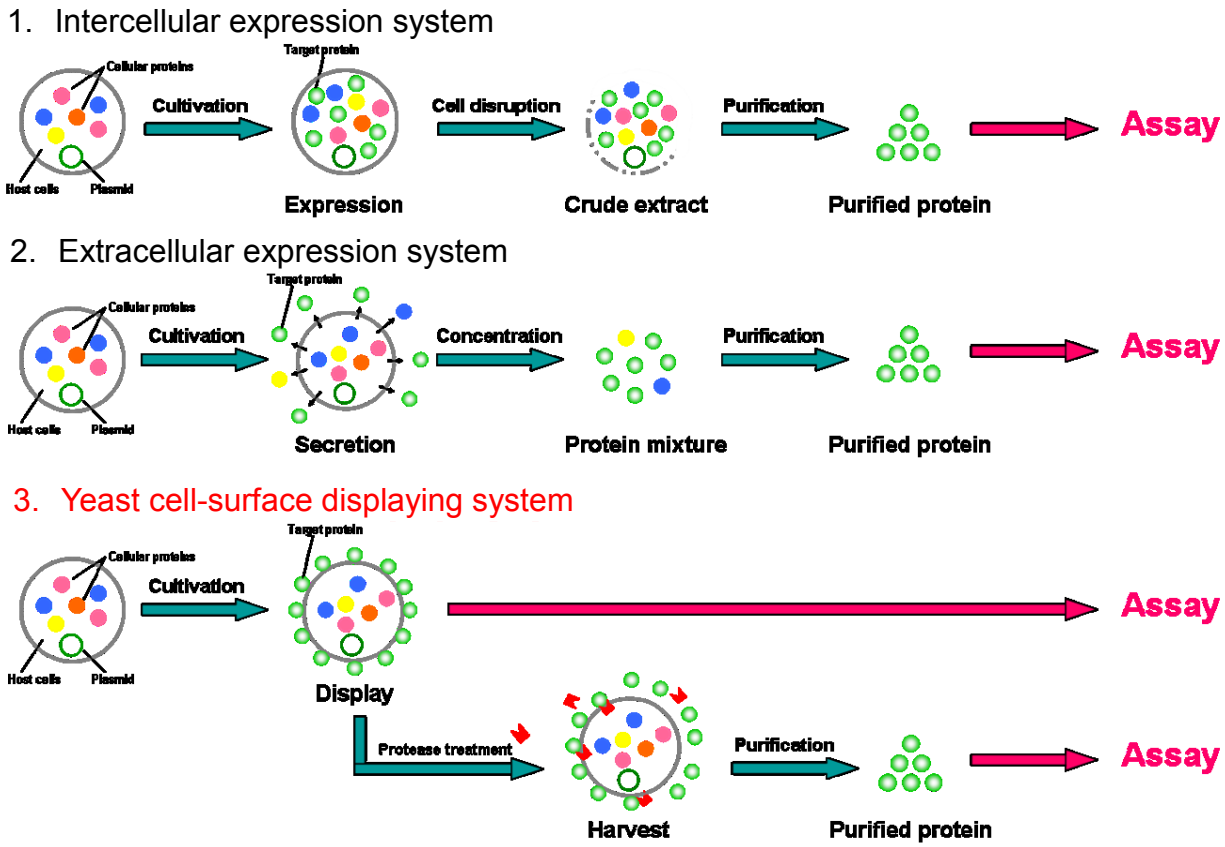

Fig. 5 Schematic representation of the yeast cell-surface displaying system [A] Protein molecule of α-agglutinin [B] Fusion protein molecule for the display of foreign protein on yeast cell-surface [C] Display of foreign proteins on the yeast cell-surface thorough the eukaryotic secretory pathway.

**Contribution to protein engineering of yeast cell-surface displaying system**

To prepare the recombinant protein, intracellular or extracellular expression system using *Escherichia coli* or yeast was usually employed. In such conventional methods, it takes a long time and much labor to collect the recombinant proteins by the many complicated purification steps and it doesn't ensure the rate of sufficient recovery for using on experiment. Yeast cell surface displaying system can display desired protein on yeast cell surface and the amount of displayed proteins were $10^5$~$10^6$

molecules per cell. By employing this system, we can utilize yeast as desired protein cluster and construct displaying yeast without many time-consuming purification steps (Fig. 6A). This system enables to construct of a large mutant library rapidly and to make high-throughput screening for obtaining useful mutants (23, 24) (Fig. 6B).

**[A]**



**[B]**

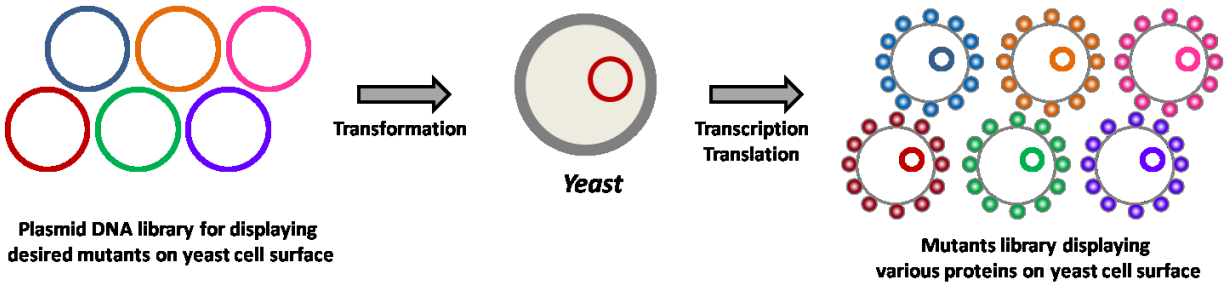



Fig. 6 Advantages of yeast cell surface displaying system [A] Comparison of methods of protein expression among conventional methods and yeast cell-surface engineering [B] High-throughput construction of desired mutants library.

# Chapter I

## Yeast cell-surface expression of chitosanase from *Paenibacillus fukuinensis*

Chitosanase from *P. fukuinensis* has interesting features such as containing both chitosanase activity and β-1, 4 glucanase activity. To clarify the reason, we have to verify the effect of amino acid residues in catalytic cleft comprehensively.

In this chapter, for efficient verification of amino acid residues in this chitosanase, chitosanase-displaying yeast was constructed using yeast cell-surface displaying system.

## Materials and Methods

**Construction of plasmid for chitosanase-displaying**

A DNA fragment containing the region encoding the chitosanase gene (1,152 base pairs, bp) from *P. fukuinensis* was prepared by polymerase chain reaction (PCR) (primers, 5'-GAAGATCTCCATGGACAAGAGATGACGACAAGGCCGGCGAGATG ATGCCGTTC-3' *Bgl*II site underlined, and 5'-CCGCTCGAGCCCGGCTCCTACCAG TTGCCCG-3' *Xho*I site underlined) using cDNA of *P. fukuinensis* chitosanase as the template, and inserted into the *Bgl*II–*Xho*I sites of the multi-copy-type yeast cell-surface displaying cassette vector pCAS1 (25). *Escherichia coli* DH5α [F⁻, ϕ80d*lacZ* ΔM15, Δ(*lac*ZYA-*arg*F)U169, *hsd*R17 ($r_k^-$, $m_k^+$), *rec*A1, *end*A1, *deo*R, *thi*-1, *sup*E44, *gyr*A96, *rel*A1, λ⁻] (Toyobo, Osaka, Japan) was used as a host cell for recombinant DNA manipulation. *E. coli* transformants were grown in Luria-Bertani (LB) medium [1% w/v tryptone, 0.5% w/v yeast extract, and 0.5% w/v sodium chloride] containing 50 μg/ml ampicillin. The resulting plasmid was named pCAS-CHI-LFL (Fig. 1). The DNA of pCAS-CHI-LFL was sequenced using a PCR Dye Terminator Cycle Sequencing Ready

Reaction Kit (Applied Biosystems, Foster City, CA). The PCR products were analyzed with a DNA sequencer (ABI Prism 373A, Perkin-Elmer/Applied Biosystems, Foster City, CA).

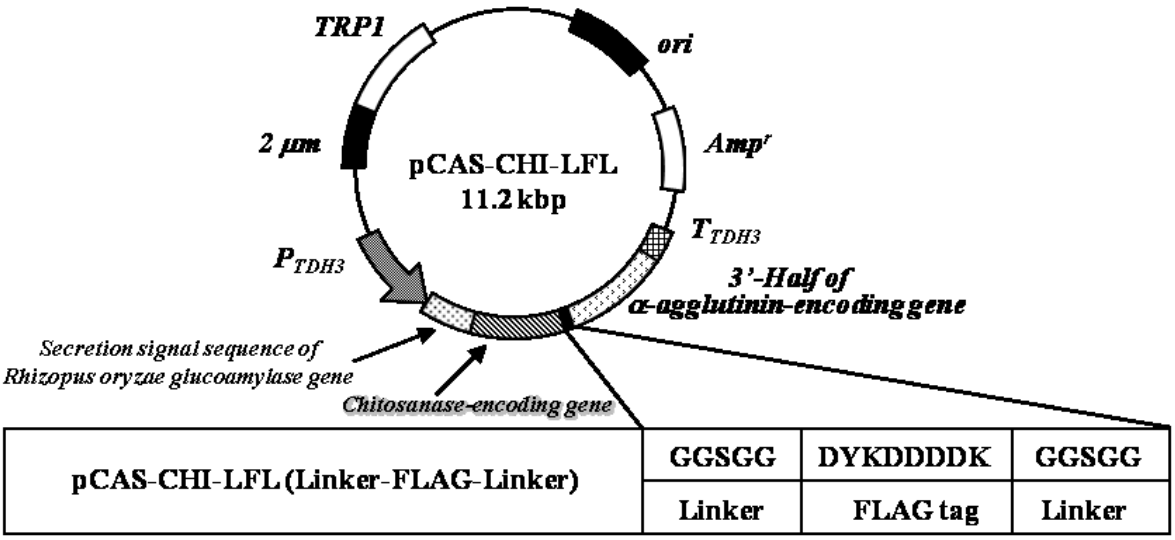



Fig. 1 Construction of the plasmid for displaying chitosanase from *P. fukuinensis* on yeast cell surface.

**Transformation of *S. cerevisiae***

*S. cerevisiae* strain MT8-1 (*MAT*a, *ade, his3, leu2, trp1, ura3*) was transformed with pCAS-CHI-LFL by the lithium acetate method using the EZ-yeast transformation kit (BIO 101; Qbiogene, Vista, CA, USA). The yeast cells were grown either in YPD medium (1% (w/v) yeast extract, 2% (w/v) polypeptone, and 2% glucose) or SDC-W medium (0.67% (w/v) yeast nitorogen base without amino acid (Difco, MI,

USA) and 2% (w/v) glucose with 0.002% (w/v) adenine sulfate, 0.002% (w/v) L-histidine, 0.003% (w/v) L-leucine, 0.002% (w/v) uracil) containing 2% (w/v) casamino acids. Transformants were isolated by incubation at 30˚C for 48 h on a plate of SDC-W medium.

**Immunofluorescence microscopy**

The localization of the mutated chitosanases on yeast cell surface was individually confirmed by the immunofluorescence labeling of cells (26). After incubation in SDC-W medium at 30˚C for 48 h, yeast cells were collected and washed with PBS; pH 7.4. Cells were fixed with 3.7% formaldehyde for 1.5 h at room temperature and resuspended in PBS containing 1% (w/v) BSA for 30 min at room temperature. The yeast cells were then incubated for 1.5 h in PBS containing 1% (w/v) BSA with the mouse monoclonal antibody against FLAG peptide (Sigma Chemical Co., St. Louis, MO, USA) used as the primary antibody at dilution rate 1:500, and then they were incubated for 1.5 h at room temperature with the secondary antibody, Alexa Fluor® 488-conjugated goat anti-mouse IgG (H+L) (Molecular Probes Inc., Eugene, OR, USA) at dilution rate 1:300. After washing with PBS, the green fluorescence on the cells was observed by fluorescence microscopy. Fluorescence was detected with the inverted microscope IX71 (Olympus, Tokyo, Japan) through a UMNIBA2 mirror unit with a 470-490 excitation filter, DM505 dichroic mirror, and a BA510-550 emission filter (Olympus). Live images were obtained using the Aqua Cosmos 2.0 software (Hamamatsu Photonics, Shizuoka, Japan) to control a digital charge-coupled device camera (C4742-95-12ER, Hamamatsu Photonics).

**Measurement of chitosanase activity with the Rondle-Morgan method**

The chitosanase activity was measured by the Rondle-Morgan method (27, 28). Substrate solution of chitosanase assay was prepared by dissolving glycol chitosan (Sigma Chemical, St. Louis, MO, USA) in PBS at concentration of 1.0%. Acetylacetone reagent was prepared by adding 1 ml of acetylacetone to 50 ml of 250 mM sodium carbonate solution. Ehlrich reagent was prepared by dissolving 1.3 g of *p*-dimetylaminobenzaldehyde in 50 ml of 99.5% ethanol and adding 50 ml of hydrochloric acid. Yeast cells were cultivated in 100 ml of SDC-W medium and collected by centrifuging at 900×*g*, 4°C for 5 min. After the cells ($A_{600}$ = 20.0) were washed with PBS, the reaction was initiated by adding 1 ml of substrate solution. After incubating at 30°C, the reaction solution was centrifuged at 900×*g*, 4°C for 5 min. The supernatant (0.5 ml) and distilled water (0.5 ml) were added to 0.5 ml of acetylacetone reagent and then placed in boiling water bath for 20 min. After cooling the solution, ethanol (3.0 ml) and Ehlrich reagent (0.5 ml) were added in it. Amounts of the reducing sugers liberated from glycol chitosan were measured with spectrophotometer at 530 nm.

**Measurement of β-1, 4 glucanase activity with the 3, 5-dinitrosalicylic acid (DNS) method**

The β- 1, 4 glucanase activity was measured with the 3, 5-dinitrosalicylic acid (DNS) method (29, 30, 31). The DNS reagent, containing 0.5% (w/v) DNS, 1.6% (w/v) NaOH and 30% (w/v) sodium potassium tartrate, was prepared by dissolving each component in the distilled water. Carboxymethyl cellulose sodium salt (CMC-Na), as a substrate of glucanase, was prepared by dissolving in PBS at a concentration of 0.7%. The supernatant (0.5 ml) of the reaction solution was added to 1 ml of DNS reagent, and then placed in boiling water bath for 5 min. After cooling the solution to room temperature, amounts of the reducing sugers liberated from CMC-Na were measured with spectrophotometer at 525 nm.

# Results

**Chitosanase expression on yeast cell surface**

The plasmid pCAS-CHI-LFL for displaying chitosanase on the yeast cell surface was constructed. To confirm the localization of the chitosanase on the yeast cell surface, immunofluorescence labeling was performed (Fig. 2). No fluorescence signal was observed on the cell surface of the MT8-1 strain, which was the negative control. Yeast cells expressing the chitosanase were confirmed by the presence of Alexa Fluor® 488, indicating that chitosanase was displayed on the yeast cell surface.

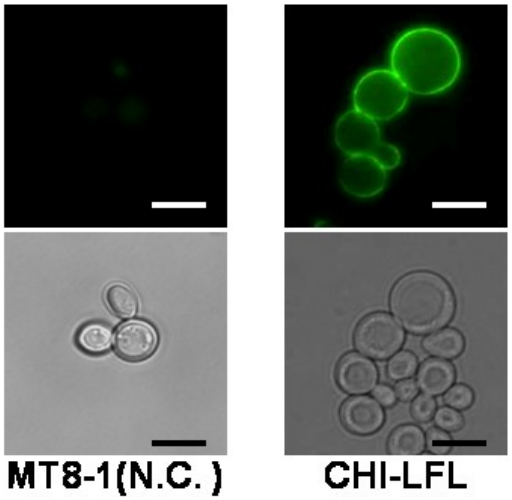
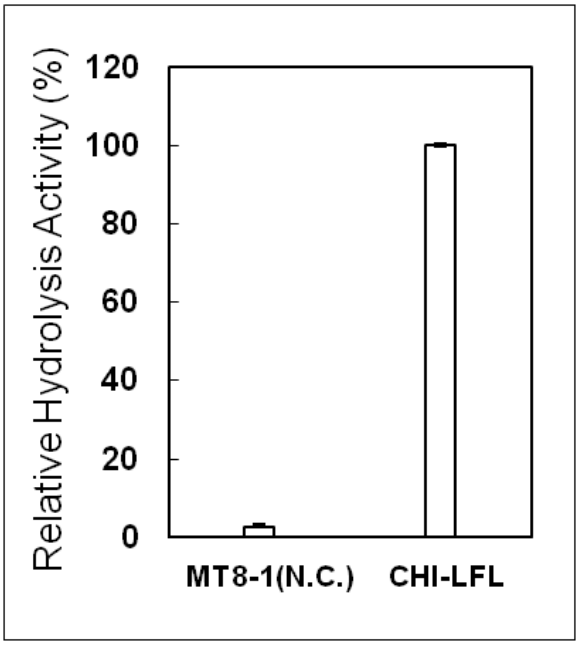



Fig. 2 Immunofluorescence microscopy. Bars, 5 μm.

**Measurement of hydrolysis activity of chitosanase activity and β-1, 4 glucanase activity**

Chitosanase activity was measured by Rondle-Morgan method (Fig. 3A). The chitosanase activity was detected on chitosanase-displaying yeast. Furthermore, β-1, 4 glucanase activity assay was measured by DNS method (Fig. 3B). The β-1, 4 glucanase activity was also detected on chitosanase-displaying yeast. Both activities did not be observed on the MT8-1 strain, which was the negative control. From these results, the chitosanase-displaying yeast was properly constructed.

**[A]**




**[B]**

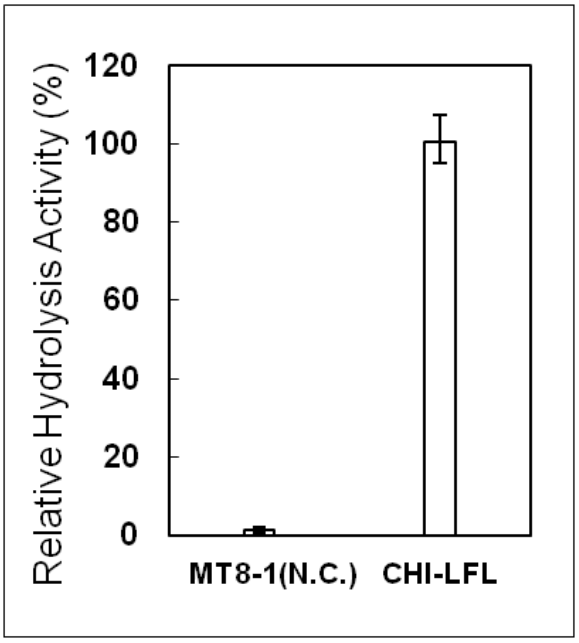



Fig. 3 Hydrolysis activity assay of chitosanase (A) and β-1, 4 glucanase (B).

## Discussion

**Yeast cell surface expression of chitosanase from *P. fukuinensis***

A constructed plasmid pCAS-CHI-LFL for displaying chitosanase from *P. fukuinensis* on yeast cell surface was constructed (Fig. 1) and observed fluorescence signal around the displaying yeast cell (Fig. 2). From the results of activity assays of chitosanase and β-1, 4 glucanase on chitosanase-displaying yeast, both activities were observed (Fig. 3).

Therefore, chitosanase-displaying yeast was correctly constructed and it will be able to perform analysis of catalyzing mechanism of this chitosanase using this system.

## Summary

By yeast cell-surface displaying system, we could construct chitosanase-displaying yeast CHI-LFL, and the chitosanase and β−1, 4 glucanase activities were become able to evaluate on the yeast cell surface. Chitosanase-displaying yeast has many advantages as a biocatalyst for bio-reaction process as compared with conventional immobilization methods for enzymes. For example, it can be used as a whole-cell that is a self-immobilized enzyme without any enzyme immobilized procedures.

# Chapter II

## Demonstration of catalytic proton acceptor of chitosanase from *Paenibacillus fukuinensis* by comprehensive analysis of mutant library

Carbohydrate was hydrolyzed by the action of the amino acid residues working as catalytic acid (proton donor) and catalytic base (proton acceptor) in glycoside hydrolase. Chitosanase from *P. fukuinensis* belongs to the family 8 glycoside hydrolase group (GH-8). The enzymes in family GH-8 can be further classified into three subfamilies, subfamily GH-8a, subfamily GH-8b, and subfamily GH-8c, on the basis of these proton acceptors in the enzymatic reaction and they can be classified into the following two systems (32). In subfamily GH-8a, Asp comprising the active site is conserved as a proton acceptor and in subfamilies GH-8b and GH-8c, Glu in the additional inserted loop domain at the active site is conserved (Fig. 1). Interestingly, in subfamilies GH-8b and GH-8c, Asp which acts as a proton acceptor in family GH-8a might be changed to Glu and/or Asn. We speculate that the Asn residue may assist in the hydrolysis as a proton acceptor to some extent.

| Function | Subfamily GH-8a | In case of Cellulase A from *Clostridium thermocellum* | Subfamily GH-8b | In case of Chitosanase from *Paenibacillus fukuinensis* |
|---|---|---|---|---|
| Catalytic Acid | Glu | (Glu95)[a] | Glu | (Glu115)[b] |
| Substrate Recognition Site | Asp | (Asp152)[a] | Asp | (Asp176)[b] |
| Catalytic Base | Asp | (Asp278)[a] | Glu | (Glu302?)[c] |
| Assistance of Catalytic Base | No corresponding amino acid residue | No corresponding amino acid residue | Asn? | (Asn312?)[c] |

Fig. 1 Comparison of amino acid residues related to catalytic activity in enzyme of subfamily GH-8a and GH-8b. (a) Alzari *et al.* (12) (b) Kimoto *et al.* (19) (c) This study.

Cellulase A from *Clostridium thermocellum* (CelA) classified into subfamily GH-8a has Glu95 and Asp152 as a proton donor and a substrate recognition site, respectively (12). The proton acceptor of CelA is Asp278, but the loop domain observed in subfamilies GH-8b and GH-8c are not present (Fig. 2A). Chitosanase from *P. fukuinensis* classified into subfamily GH-8b has Glu115 and Asp176 as a proton donor and a substrate recognition site, respectively (19). We can observe the presence of that Glu302 which is the putative proton acceptor in the inserted loop domain, and Asn312 which is conserved at the point of Asp acting as a proton acceptor in family GH-8a (Fig. 2B). However, Glu302 in the inserted loop domain as a proton acceptor for exhibiting chitosanase activity and Asn312 for mediating in the hydrolysis of chitosan have not been clarified in terms of their roles in the enzymatic reaction of chitosanase from *P. fukuinensis*. Therefore, we focused on these two amino acid residues. In the chapter I, we generated *P. fukuinensis* chitosanase-displaying yeast cells using a yeast cell-surface displaying system.

In this chapter, we demonstrated that Glu302 was essential as a putative proton acceptor for holding chitosanase activity, and the effects of Glu302 and Asn312 on chitosanase activity and β-1, 4 glucanase activity were clarified by comprehensive analysis of a mutant library.

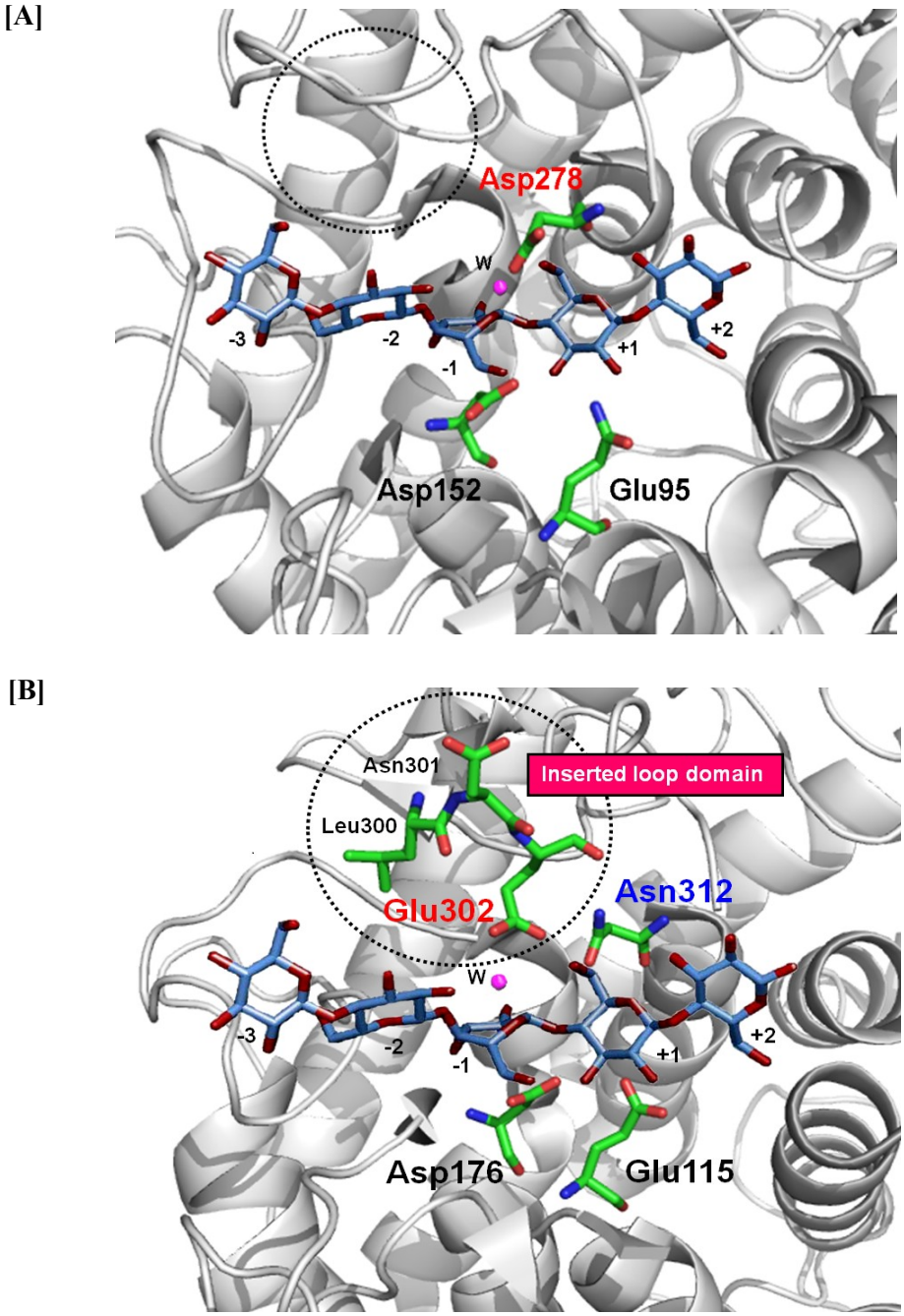



Fig. 2 Difference of catalytic amino acid residues contained in the catalytic cleft between cellulase (monofunctional) and chitosanase (bifunctional) in family 8 [A] *Clostridium thermocellum* CelA [B] *P. fukuinensis* chitosanase (The program PyMOL was used to produce these figures. Blue: substrate [A] cellulose [B] chitosan, Green: catalytic amino acid residues, W: bound water molecules).

# Materials and Methods

## Construction of plasmids for displaying mutated chitosanases on the yeast cell surface

In the chapter I, we constructed a pCAS-CHI-LFL plasmid vector for desplaying chitosanase from *P. fukuinensis* on yeast cell surface. In order to replace to comprehensive amino acid residues in Glu302 and Asn312 of chitosanase from *P. fukuinensis*, the plasmid vector pCAS-CHI-LFL was used as a template for site-directed mutagenesis using QuikChange® Site-Directed Mutagenesis Kit (Stratagene, CA, USA). Using primers are shown in Fig. 3. To confirm the introduction of mutation into Glu302 and Asn312, DNA sequencings of DNA of constructed plasmids were carried out using an ABI Prism 310 genetic analyzer using the BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA, USA).

| Name of primer | Sequence of primer | Name of primer | Sequence of primer |
|---|---|---|---|
| E302A-Forward | 5'-CCAGAATGGTACTTGAATGCATTCCAGC-3' | E302R-Forward | 5'-CCAGAATGGTACTTGAATCGCTTCCAGC-3' |
| E302C-Forward | 5'-CCAGAATGGTACTTGAATTGCTTCCAGC-3' | E302S-Forward | 5'-CCAGAATGGTACTTGAATAGCTTCCAGC-3' |
| E302D-Forward | 5'-CCAGAATGGTACTTGAATGACTTCCAGC-3' | E302T-Forward | 5'-CCAGAATGGTACTTGAATACCTTCCAGC-3' |
| E302F-Forward | 5'-CCAGAATGGTACTTGAATTTCTTCCAGC-3' | E302V-Forward | 5'-CCAGAATGGTACTTGAATGTGTTCCAGC-3' |
| E302G-Forward | 5'-CCAGAATGGTACTTGAATGGCTTCCAGC-3' | E302W-Forward | 5'-CCAGAATGGTACTTGAATTGGTTCCAGC-3' |
| E302H-Forward | 5'-CCAGAATGGTACTTGAATCACTTCCAGC-3' | E302Y-Forward | 5'-CCAGAATGGTACTTGAATTACTTCCAGC-3' |
| E302I-Forward | 5'-CCAGAATGGTACTTGAATATCTTCCAGC-3' | E302-Reverse | 5'-GCGTTATAATAATAAGCATTCGTTTGCTGG-3' |
| E302K-Forward | 5'-CCAGAATGGTACTTGAATAAGTTCCAGC-3' | N312A-Forward | 5'-CGAATGCTTATTATTATGCAGCAGCACG -3' |
| E302L-Forward | 5'-CCAGAATGGTACTTGAATCTGTTCCAGC-3' | N312D-Forward | 5'-CGAATGCTTATTATTATGACGCAGCACG -3' |
| E302M-Forward | 5'-CCAGAATGGTACTTGAATATGTTCCAGC-3' | N312K-Forward | 5'-CGAATGCTTATTATTATAAGGCAGCACG -3' |
| E302N-Forward | 5'-CCAGAATGGTACTTGAATAACTTCCAGC-3' | N302R-Forward | 5'-CGAATGCTTATTATTATCGCGCAGCACG -3 |
| E302P-Forward | 5'-CCAGAATGGTACTTGAATCCGTTCCAGC-3' | N312-Reverse | 5'-CCATGACGATGCGGAGCGGCACACGTGC -3 |
| E302Q-Forward | 5'-CCAGAATGGTACTTGAATCAGTTCCAGC-3' | | |

Fig. 3 Synthetic oligonucleotide primers used for site-directed mutagenesis. The 19 kinds of E302-forward primers and E302-reverse primer were used for substitution of Glu302. The 4 kinds of N312-forward primers and N312-reverse were used for substitution of Asn312.

**Measurement of fluorescence intensity**

To quantify the amount of displaying chitosanase on the yeast cell surface, the fluorescene intensity was evaluated (33). After immunofluorescence labeling, the cells were precipitated by centrifugation at 900×*g* and concentration of yeast was adjusted $OD_{600}$=3.0 with PBS. The fluorescence intensity was measured with Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) using a tissue-culture plate (353072 Multiwell 96-well; Becton-Dickinson Labware, NJ, USA).

# Results

**Individual display of comprehensive mutants of chitosanase prepared on yeast cell surface**

The plasmids for displaying comprehensive mutants of chitosanase on the yeast cell surface were constructed using pCAS-CHI-LFL as a template. Nineteen mutants in which Glu302 was substituted by other amino acid residues (E302A, E302C, E302D, E302F, E302G, E302H, E302I, E302K, E302L, E302M, E302N, E302P, E302Q, E302R, E302S, E302T, E302V, E302W, and E302Y), two mutants in which N312 was substituted by other amino acid residues (N312A and N312D), eight double mutants in which Glu302 and Asn312 were substituted by other amino acid residues (E302K/N312A, E302K/N312D, E302K/N312K, E302K/N312R, E302R/N312A, E302R/N312D, E302R/N312K, and E302R/N312R) were obtained by site-directed mutagenesis. To determine whether mutation was introduced in Glu302 and Asn312 of chitosanase from *P. fukuinensis*, DNA sequencing was performed. As a result of DNA sequencing, the preparations of all mutated plasmids were successful. *S. cerevisiae* strain MT8-1 was individually transformed with the mutated plasmids. The constructed mutants were employed in the following experiments.

**Immunofluorescence microscopy on constructed chitosanase mutants**

The individual display of chitosanase mutants on the yeast cell surface was observed and evaluated by immunofluorescence staining (Fig. 4). Immunofluorescence staining with a monoclonal antibody against the FLAG peptide as the primary antibody and AlexaFluor488-conjugated goat anti-mouse IgG as the secondary antibody was performed to confirm the presence and localization of chitosanase on the yeast cell surface. Fluorescence was observed on the cell surface of MT8-1/pCAS-CHI-LFL and all MT8-1 strains harboring mutated plasmids, excluding the MT8-1 strain without the plasmid as the negative control. It was demonstrated that chitosanase was indeed anchored on the yeast cell wall.

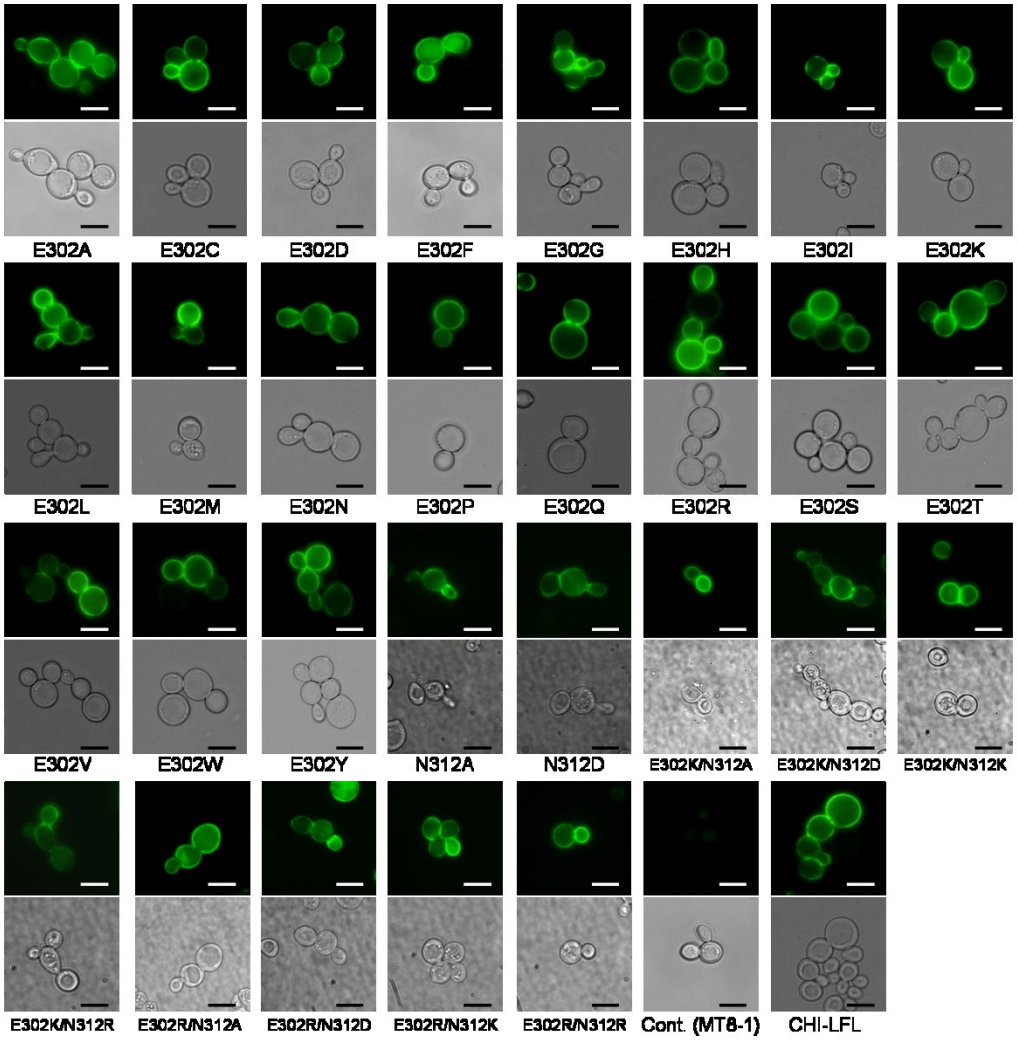



Fig. 4 Immunofluorescence microscopy. Bars, 5 μm.

**Measurement of fluorescence intensity**

To examine the effects of mutation on the amount of chitosanase displayed on the yeast cell surface, we measured fluorescence intensity of all mutants after immunofluorescence staining. Fluorescence intensity was obtained by subtracting the fluorescence intensity of the negative control MT8-1 from measured fluorescence intensity. As a result, a significant difference in fluorescence intensity was not observed between the parent and constructed mutants (Fig. 5). This result indicated that the amount of chitosanase displayed on the yeast cell surface was not affected by mutation, so that mutated chitosanase could be normally displayed on the yeast cell surface. Because the activities were normalized by the individual fluorescence intensities, the difference in activity in the subsequent activity assay was considered to be the effect of not the amount of displayed chitosanase but the mutation itself.

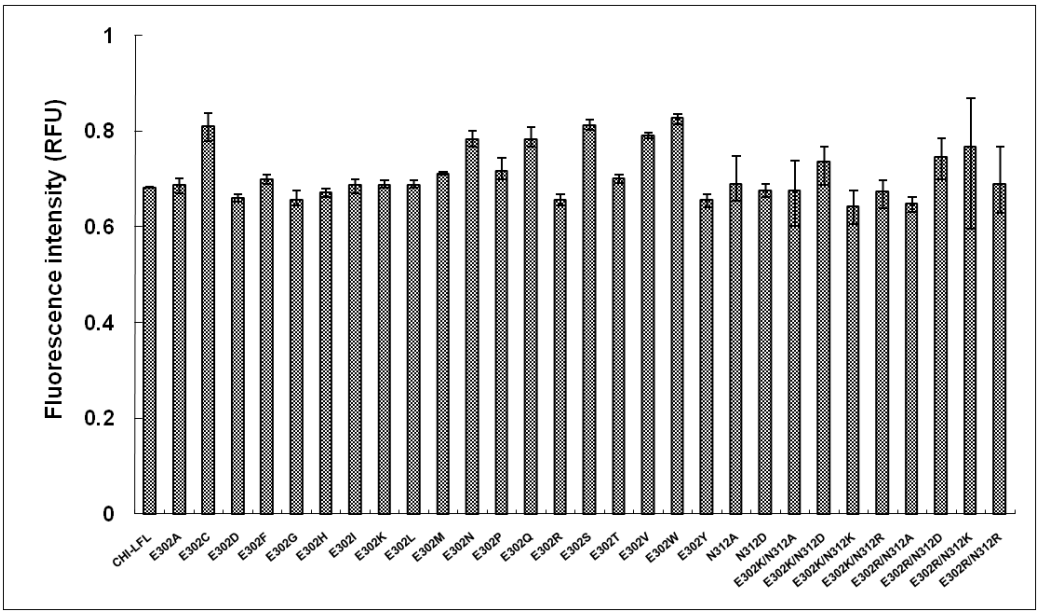



Fig. 5 Immunofluorescence intensity of constructed individual chitosanase displaying cells. After all cells were incubated in SDC-W medium at 30°C for 48 h, the fluorescence intensity was measured with immunofluorescence labeling. MT8-1 was used as a negative control. Values represent the means ± standard deviations of the results from three independent experiments.

**Measurement of hydrolysis activity of chitosanase by Rondle-Morgan method**

The hydrolysis activity of chitosanase was measured by the Rondle-Morgan method, and relative hydrolysis activities are shown in Fig. 6. Chitosanase activity in nearly all of the Glu302 chitosanase mutants remained approximately 70% of that of the parent strain at 144 h, but substrate degradation rate decreased to some extent. In the comparison of chitosanase activity among the constructed mutants, we observed that the mutants with basic amino acid and aromatic amino acid at the No. 302 residue, such as E302F, E302K, E302P, E302R, E302W, and E302Y, exhibited a lower activity than the parent strain with the original Glu, that is, approximately 50% of that of the parent strain. These results are probably due to the repulsion of positive poles among the basic amino acid, proton in the active water, and amino group of the substrate, chitosan, and due to the steric hindrance of the side chain of the amino acid in the active center.

Furthermore, we predicted that the conserved Asn312 contributed to β-1, 4 glucanase activity, although the degradation rate obtained by the substitution of Asn312 also decreased similarly to that obtained by the substitution of Glu302. As a result, we demonstrated that Asn312 certainly participated in hydrolysis of chitosan. Therefore, to analyze the correlation between Glu302 and Asn312, we constructed eight Glu302/Asn312 double mutants and verified their chitosanase activity. The activities of all constructed double mutants were nearly lost (approximately 15% compared with that of the parent). From these results, Asn312 probably participated in hydrolysis of the substrate and was the important amino acid as well as Glu302.

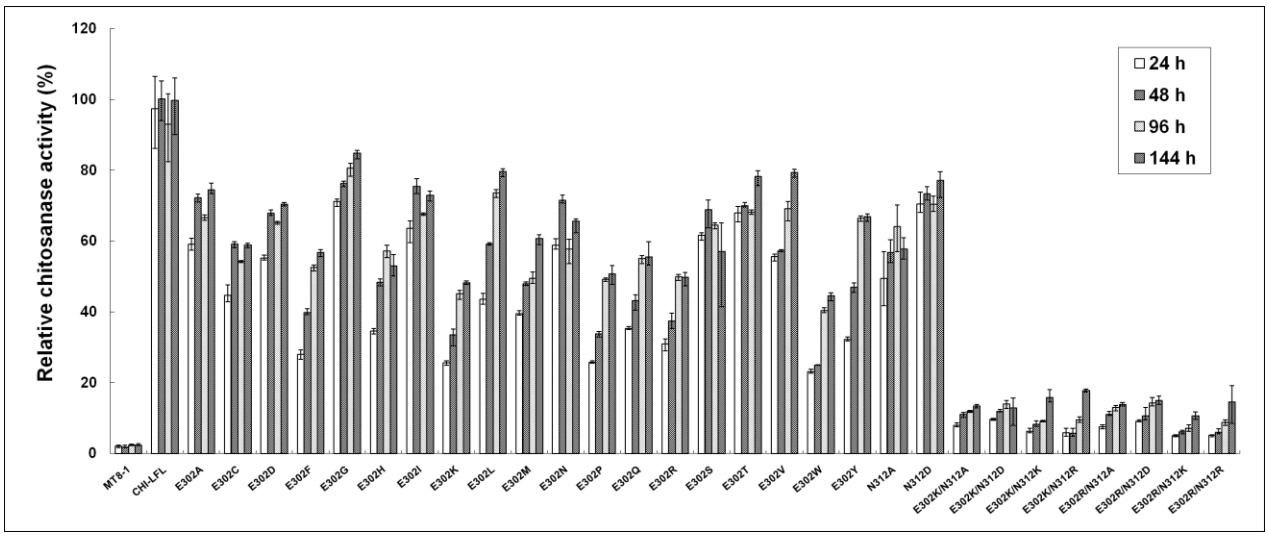



Fig. 6 Chitosanase activity assay. The value of the parent was regarded as 100% in 24, 48, 96, and 144 h, and relative ratios of values of mutants to that of the parent (pCAS-CHI-LFL) were calculated. MT8-1 was used as a negative control. To normalize the amount of displayed chitosanase, among parent and constructed mutants, the values of individual chitosanase activity were divided by the values of individual fluorescence intensity and represented as percentage. The values represent the means ± standard deviations of the results from three independent experiments.

**Measurement of hydrolysis activity of β-1, 4 glucanase by DNS method**

The hydrolysis activity of β-1, 4 glucanase was measured by the 3, 5-dinitrosalicylic acid (DNS) method, and relative hydrolysis activities are shown in Fig. 7. We demonstrated that the conserved Asn312 contributed to not only β-1, 4 glucanase activity but also chitosanase activity. Similarly, we attempted to demonstrate whether Glu302 also contributed to β-1, 4 glucanase activity. From the results, in comparison with the parent strains, β-1, 4 glucanase activity remained in nearly all of the chitosanase mutants substituted for Glu302 in 144 h, but the degradation rate was decreased to some extent. We also observed tendencies similar to those observed in the

activity assay of chitosanase, as mentioned in the previous section. In the comparison of β-1, 4 glucanase activity among the constructed mutants, we observed that the mutants with basic amino acid at the No. 302 residue, such as E302K and E302R, exhibited a lower activity than the other single mutants, that is, approximately 40% of that of the parent. However, the effect of substitution at the No. 302 residue by an aromatic amino acid was not observed. An asparagine at the No. 312 residue in chitosanase from *P. fukuinensis* is conserved as aspartate in subfamily GH-8a. We predicted that if the asparagine is substituted by aspartate, the β-1, 4 glucanase activity will be higher than that of the parent. Therefore, β-1, 4 glucanase activities of the mutants substituted for Asn312 were also observed. From the results, however, the glucanase activity of N312D was lower than to that of the parent. Interestingly, in comparison with the activities of N312A and N312D, the activity of N312D was higher than that of N312A, owing to substitution by acidic amino acid residues. Similarly, to analyze the correlation between Glu302 and Asn312, the β-1, 4 glucanase activity of Glu302/Asn312 double mutants was measured. To verify the effect of Asn312 only on β-1, 4 glucanase, we constructed eight double mutants. In the preparation of double mutants, we employed E302K and E302R as templates because the No. 302 residue cannot act as proton acceptor in these mutants (Fig. 6). The β-1, 4 glucanase activity of prepared E302K/N312K, E302K/N312R, E302R/N312A, E302R/N312K, and E302R/N312R was nearly lost (approximately 30% compared with that of the parent), as similarly observed for chitosanase activity assay. However, the β-1, 4 glucanase activity of E302K/N312A, E302K/N312D, and E302R/N312D was higher than that of other double mutants (approximately 40% compared with that of the parent). Except for E302K/N312A, these tendencies were the same as those observed in mutants of substitution of Asn312 only (Fig. 6 and Fig. 7). Therefore, substitution of asparagine by acidic amino acid residues made Asn312 slightly effective as a proton acceptor compared with substitution by nonpolar amino acid residues. Consequently, Glu302 and Asn312 probably contribute to both chitosanase activity and β-1, 4 glucanase activity and are essential amino acid residues for having those activities.

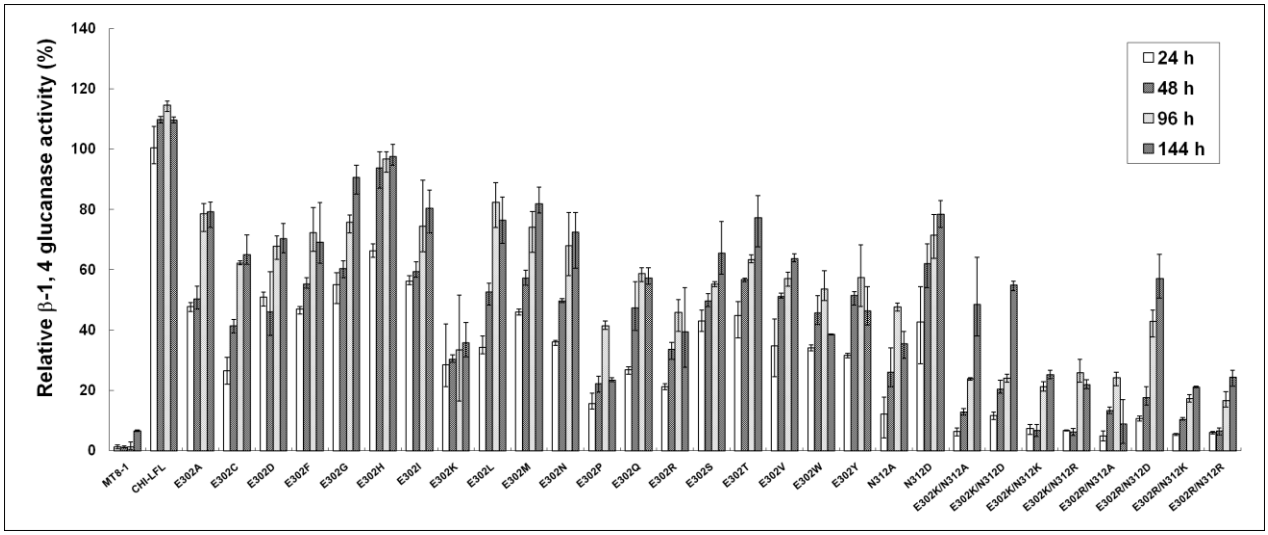



Fig. 7 β-1, 4 Glucanase activity assay. The value of the parent was regarded as 100% in 24, 48, 96, and 144 h, and relative ratios of values of mutants to that of the parent (pCAS-CHI-LFL) were calculated. MT8-1 was used as a negative control. To normalize the amount of displayed chitosanase, among parent and constructed mutants, the values of individual β-1, 4 glucanase activity were divided by the values of individual fluorescence intensity and represented as percentage. The values represent the means ± standard deviations of the results from three independent experiments.

## Discussion

The enzymes in family GH-8 can be further divided into three subfamilies, subfamilies GH-8a, GH-8b, and GH-8c, depending on the position of the proton acceptor (32). The bifunctional enzymes were classified into family GH-8b. Chitosanase from *P. fukuinensis* is a bifunctional enzyme having both chitosanase and β-1, 4 glucanase activities and classified into subfamily GH-8b. Glu302 is a putative proton acceptor of chitosanase and Asn312 is the conserved aspargine residue corresponding to Asp, which acts as a proton acceptor in subfamily GH-8a. In this study, we constructed a mutant library of chitosanase in which Glu302 and Asn312 were comprehensively substituted by the other nineteen amino acid residues and analyzed both chitosanase and β-1, 4 glucanase activities. As the results, we demonstrated that Glu302 acted as a proton acceptor and Asn312 contributed to hydrolysis of chitosan. In subfamily GH-8b, chitosanase having only chitosanase activity, glucanase having only glucanase activity, and bifunctional enzymes having both chitosanase and glucanase activities are reported (34, 35). Despite that these enzymes have almost similar structures, such diversity in activity was observed. Moreover, the enzymes classified into subfamily GH-8b have a loop domain containing a proton acceptor. Although the structural character of the enzyme classified into subfamily GH-8a was almost similar to that of the enzyme classified into subfamily GH-8b, the loop domain is not present in subfamily GH-8a (12, 36-38). Therefore, it was suggested that the loop domain is inserted into the structure of subfamily GH-8a, and glutamate in the inserted loop domain will become a new proton acceptor in the relationship of steric location in the catalytic cleft. On the other hand, from the results that the chitosanase activity of mutants of Asn312 substituted by the other amino acid residues was decreased, the conserved asparagine, Asn312, corresponding to Asp acted as a proton acceptor in subfamily GH-8a, is still associated with hydrolysis of chitosan. In this investigation, we could demonstrate that the proton acceptors of chitosanase from *P. fukuinensis* were Glu302 and Asn312. Therefore, we clarified that the catalytic amino acid residues of chitosanase were Glu302 as a proton acceptor and Asn312 for contributing to hydrolysis of the substrate, and the functions of

both Glu302 and Asn312 may be associated with the bifunctional potential of chitosanase from *P. fukuinensis*.

## Summary

To clarify the amino acid residues of putative proton acceptor (Glu302 and Asn312), we evaluate the effects to chitosanase activity and β-1, 4 glucanase activity on mutant library constructed by yeast cell-surface displaying system. From the results of activity assay of chitosanase and β-1, 4 glucanase, both activities almost lost only on double mutants. These results suggest that Glu302 and Asn312 were essential for holding both chitosanase activity and β-1, 4 glucanase activity.

# Chapter III

## Evaluation of chitosan-binding amino acid residues of chitosanase from *Paenibacillus fukuinensis*

It catalyzes endohydrolysis of chitosan and cellulose and produces dimeric and trimeric degradation products. Chitosan polymers and chitosan oligosaccharides have various bioactivities, such as anti-tumor activity (4, 5). The type of bioactivity shown differs with the extent of polymerization of the chitosan monomer (glucosamine). Compared to polymers or shorter oligosaccharides such as the monomers or dimers, chitosan oligosaccharides longer than a hexamer have higher bioactivity.

In chapter II, we identified the catalytic amino acid residues of chitosanase from *P. fukuinensis*. Through modification of the chitosanase, we attempted to obtain useful mutants that selectively produce desired oligosaccharides possessing high bioactivities. To obtain useful chitosanase mutants, we introduced mutations at the chitosan-binding amino acid residues and evaluated their significance. When seeking candidate amino acid residues for substrate binding in the chitosanase, we referred to chitosanase from *Bacillus* sp. K17 (32), which appear to be similar to chitosanase from *P. fukuinensis*. In that strain, Trp166, Tyr318, Trp335, and Phe413 have been indicated as the amino acid residues for substrate binding. The corresponding amino acid residues in the chitosanase from *P. fukuinensis* were Trp159, Trp228, Tyr311, and Phe406 (Fig. 1). It was suggested that these aromatic amino acid residues facilitated substrate binding by stacking the rings between amino acid residues and substrate sugars. The role of these amino acid residues has not been clarified. Therefore, we investigated the role that these amino acid residues play in substrate binding, and attempted to find clues for modifying the manner of substrate binding. To evaluate the correlation of substrate binding among the amino acid residues, we employed a yeast cell-surface displaying system for constructing a mutant library. Here, we constructed plasmid pULD-CHI as *P. fukuinensis* chitosanase-displaying yeast cells, and attempt to introduce mutations on

amino acid residues, engaging substrate binding, by using the plasmid for *P. fukuinensis* chitosanase-display as a template. To assess the constructed mutants, we performed a chitosanase activity assay and examined the role of substrate binding by HPLC analysis.

In this chapter, it was shown that the "center group" (Trp159 and Phe406) may be more significant than the "outer group" (Trp228 and Tyr311) for substrate binding, and that substrate binding pattern can be modified by substitution of Trp228 and Tyr311.

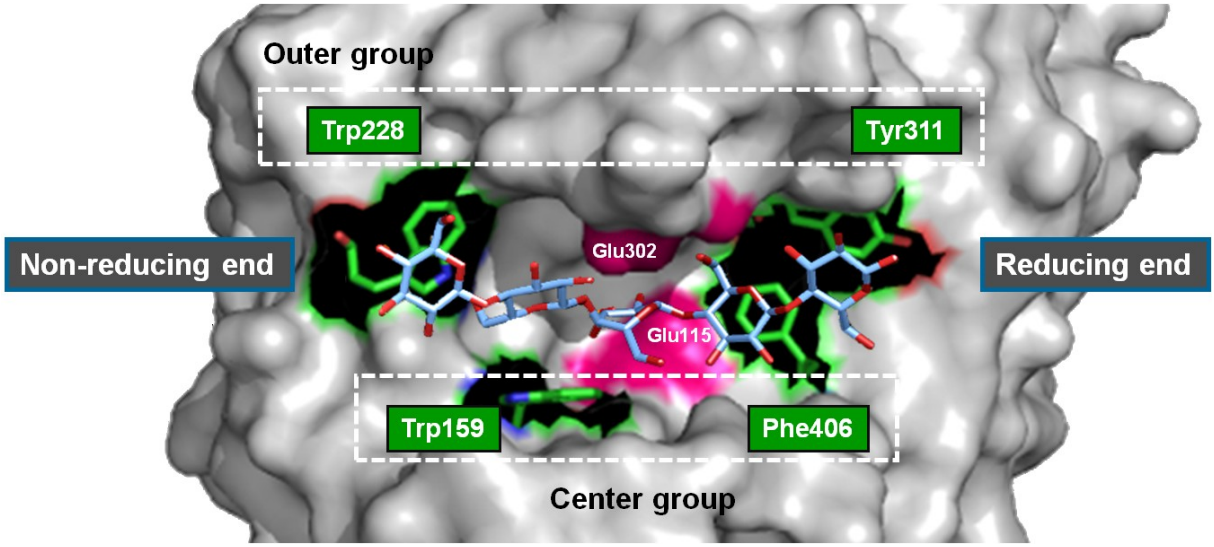



Fig. 1 Four substrate binding amino acid residues with chitosan pentamer accommodated in the catalytic cleft of chitosanase from *P. fukuinensis*. We divided the four amino acid residues engaged in substrate binding into two groups; one designated as the "outer group" (Trp228 and Tyr311) and the other designated as the "center group" (Trp159 and Phe406). Because the crystal structure of chitosanase from *P. fukuinensis* has not been resolved, we referred to the crystal structure of chitosanase from *Bacillus* sp. K17, and as reported by Adachi *et al.* (32). The program PyMOL was used to produce these figures. Magenta color indicates the catalytic domain (Glu115 and Glu302) and Blue color indicates substrate chitosan.

# Materials and Methods

## Strains and materials

Yeast strain, *S. cerevisiae* BY4741/*sed1*Δ (*MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *YDR077w::KanMX4*; EUROSCARF, Frankfurt, Germany), a high-efficiency displaying yeast, was used to display chitosanase on the cell surface (39). The yeast cells were grown in SDC+HML medium [0.67% w/v yeast nitorogen base without amino acid (Difco, MI, USA) and 2% w/v glucose with 0.002% w/v l-histidine, 0.003% w/v l-Methionine, 0.003% w/v l-leucine containing 2% w/v casamino acids].

## Construction of plasmids for displaying mutated chitosanase on the yeast cell surface

In this chapter, we constructed the pULD-CHI plasmid vector for chitosanase-displaying with a FLAG tag on the yeast cell surface using a yeast cell-surface display system (Fig. 2). The plasmid vector pULD-CHI was used as a template for site-directed mutagenesis using QuikChange® Site-Directed Mutagenesis Kit (Stratagene, CA, USA) (Table 1, 2) in order to replace by Ala and Lys at Trp159, Trp228, Tyr311, and Phe406 of chitosanase from *P. fukuinensis*. In chapter II, the chitosanase activity hasn't completely lost by alanine substitution. On the other hand, chitosanase activity was almost lost by lysine substitution. Therefore, these amino acid residues were replaced by Ala and Lys. To confirm the introduction of mutation, the DNA of the constructed plasmids was sequenced out using an ABI Prism 310 genetic analyzer using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

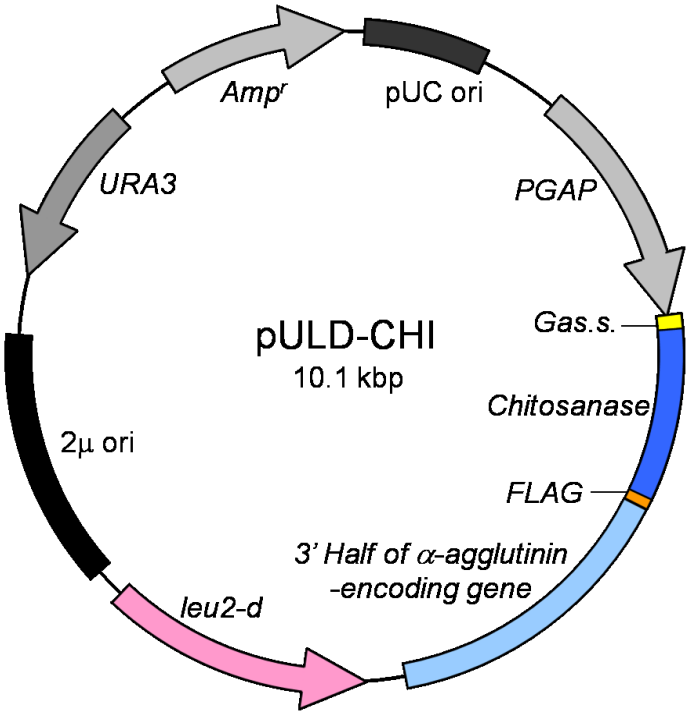



Fig. 2 The plasmid pULD-CHI for display of chitosanase with a leu2-d marker on yeast cell surface.

**HPLC analysis of degradation products**

Substrate solution for HPLC analysis was prepared by dissolving glycol chitosan and chitosan-hexamer (Seikagaku Co., Tokyo, Japan) in PBS at a concentration of 5 mg/ml. Yeast cells were cultivated in 100 ml of SDC+HML medium and collected by centrifugation at 900×*g* and 4°C for 5 min. After the cells ($A_{600}$ = 20.0) were washed with PBS, the reaction was initiated by adding 300 μl of the substrate solution (1% glycol chitosan or 5 mg/ml chitosan-hexamer). After incubating at 30°C, the solution was centrifuged at 900×*g* and 4°C for 5 min and the supernatant was recovered. HPLC analysis was performed to detect the degradation products of glycol chitosan and the chitosan-hexamer, using Prominence HPLC System (Shimadzu, Kyoto, Japan) equipped with Coulochem III Electrochemical Detector (Thermo Scientific). An analytical column (TSKgel $NH_2$-60, TOSOH) was used with a flow rate of 1 ml / min. A sample (4 μL) was injected and separated by the isocratic mode (60% acetonitrile) at 35°C.

Table 1 List of constructed mutants

| Name | Remarks |
|---|---|
| Substitution of center group | |
| pULD-CHI-W159A | W159 single mutant |
| pULD-CHI-W159K | W159 single mutant |
| pULD-CHI-F406A | F406 single mutant |
| pULD-CHI-F406K | F406 single mutant |
| pULD-CHI-W159A/F406A | W159/F406 double-mutant |
| pULD-CHI-W159A/F406K | W159/F406 double-mutant |
| pULD-CHI-W159K/F406A | W159/F406 double-mutant |
| pULD-CHI-W159K/F406K | W159/F406 double-mutant |
| Substitution of outer group | |
| pULD-CHI-W228A | W228 single mutant |
| pULD-CHI-W228K | W228 single mutant |
| pULD-CHI-Y311A | Y311 single mutant |
| pULD-CHI-Y311K | Y311 single mutant |
| pULD-CHI-W228A/Y311A | W228/Y311 double-mutant |
| pULD-CHI-W228A/Y311K | W228/Y311 double-mutant |
| pULD-CHI-W228K/Y311A | W228/Y311 double-mutant |
| pULD-CHI-W228K/Y311K | W228/Y311 double-mutant |

Table 2 List of primers for construction mutants

| Name of primer | Sequence of primer |
|---|---|
| W159A-FORWARD | 5'- GGCAATCCCAATCTGATGGGCGCAGTCGTAGCG -3' |
| W159K-FORWARD | 5'- GGCAATCCCAATCTGATGGGCAAGGTCGTAGCG -3' |
| W159-REVERSE | 5'- CCGAAATGCCCTTGGGCATTAATATGGTCCGCTACG-3' |
| F406A-FORWARD | 5'- GGATGAAGAACAAGCAGGAGAACTACGCAAGCGATTCC -3' |
| F406K-FORWARD | 5'- GGATGAAGAACAAGCAGGAGAACTACAAGAGCGATTCC -3' |
| F406-REVERSE | 5'- CCCGTAATGAACAGCATCGTCATCAGGTTATAGGAATCG -3' |
| W228A-FORWARD | 5'- CGTCTGAACCTGGGCGATGCAGATTCCAAGAGC -3' |
| W228K-FORWARD | 5'- CGTCTGAACCTGGGCGATAAGGATTCCAAGAGC -3' |
| W228-REVERSE | 5'- GGACGCGTAGCCAGCGAGCTCTTGG -3' |
| Y311A-FORWARD | 5'- CGAATGCTTATTATGCAAACGCAGCACGTGTGCCGC -3' |
| Y311K-FORWARD | 5'- CGAATGCTTATTATAAGAACGCAGCACGTGTGCCGC -3' |
| Y311-REVERSE | 5'- CCATGACGATGCGGAGCGGCACACG -3' |

# Results

**Construction of chitosanase-displaying yeast and its mutants with substituted amino acid residues for substrate binding by Ala and Lys**

The pULD-CHI plasmid for displaying chitosanase was constructed and the display was confirmed by immunofluorescence labeling with anti-FLAG antibody and Alexa Fluor® 488 anti-mouse IgG (Fig. 3). Using this plasmid as a template, sixteen plasmids for displaying mutated chitosanase on the yeast cell surface were constructed. These plasmids in which four amino acid residues for substrate binding (Trp159, Trp228, Tyr311, and Phe406) were substituted by Ala and Lys were obtained by site-directed

mutagenesis (Table 1, 2). *S. cerevisiae* strain BY4741/*sed1*Δ was transformed with pULD-CHI and derivative plasmids containing mutated chitosanase-coding genes, and then we compared the properties of individual proteins prepared from yeasts harboring pULD-CHI as wild type (WT) or constructed mutants.

To evaluate the effect of the four amino acid residues, we divided the four amino acid residues engaged in substrate binding into two groups; one designated as the "outer group" (Trp228 and Tyr311) and the other designated as the "center group" (Trp159 and Phe406), and then constructed single or double mutants substituted by Ala and Lys.

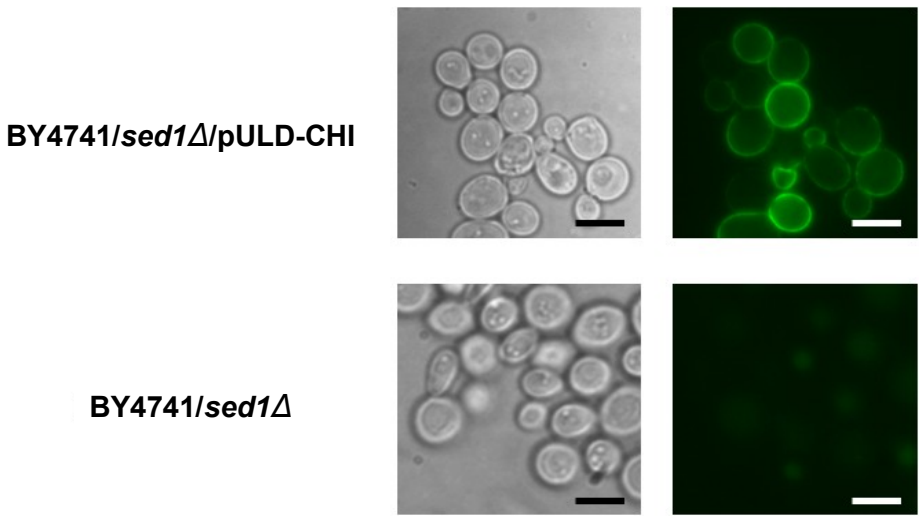



Fig. 3 Fluorescence observation of the cells after immunofluorescence labeling of BY4741/*sed1*Δ/pULD-CHI (WT), and BY4741/*sed1*Δ (negative control). Cells were labeled with anti-FLAG antibody and Alexa Fluor 488 anti-mouse IgG. Phase-contrast micrograph (*left column*), anti FLAG antibody and Alexa Fluor® 488 anti-mouse IgG (*right column*). Bars, 5 μm.

**Immunofluorescence microscopy on constructed chitosanase mutants**

The individual displays of chitosanase mutants on the yeast cell surface were observed and evaluated by immunofluorescence staining. Immunofluorescence staining with a monoclonal antibody against the FLAG peptide as the primary antibody and Alexa Fluor® 488-conjugated goat anti-mouse IgG as the secondary antibody was performed to confirm the presence and localization of chitosanase on the yeast cell surface. Fluorescence was observed on the cell surface of BY4741/*sed1*Δ/pULD-CHI and all BY4741/*sed1*Δ strains harboring mutated plasmids, excluding the BY4741/*sed1*Δ strain without the plasmid as the negative control (Fig. 4). It was demonstrated that chitosanase was indeed anchored on the yeast cell wall.

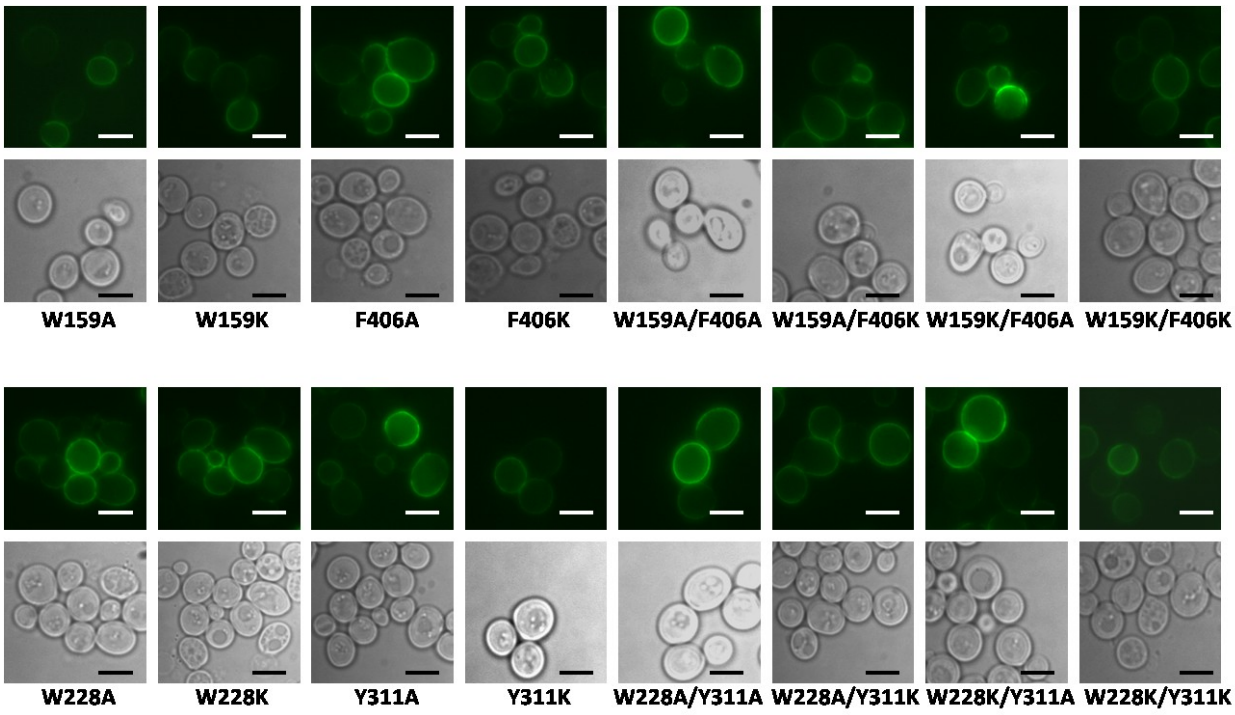



Fig. 4 Immunofluorescence microscopy. Bars, 5 μm.

**Measurement of fluorescence intensity**

To examine the effects of mutation on the amount of chitosanase displayed on the yeast cell surface, we measured fluorescence intensity of all mutants following immunofluorescence staining. Fluorescence intensity was obtained by subtracting the fluorescence intensity of the negative control, BY4741/*sed1*Δ, from the measured fluorescence intensity. As a result, a significant difference in fluorescence intensity was not observed between the parent and constructed mutants (Fig. 5). This result indicated that the amount of chitosanase displayed on the yeast cell surface was not affected by mutation. In the subsequent activity assays, chitosanase activities were normalized to the value of fluorescence intensity. Therefore, the difference in activity was considered not to be due to the amount of displayed chitosanase but due to the mutation itself.

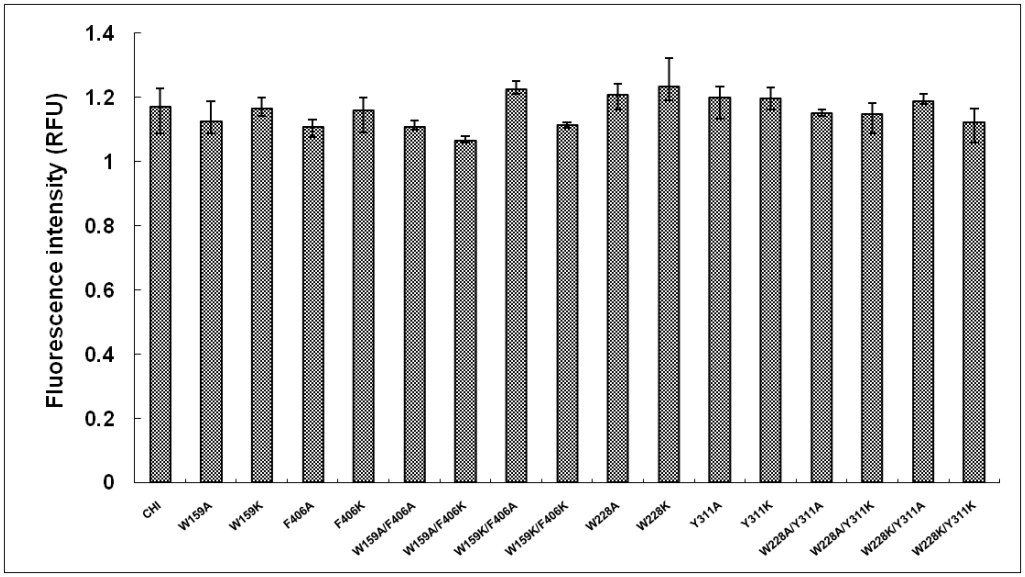



Fig. 5 Immunofluorescence intensity of constructed individual chitosanase displaying cells. After all cells were incubated in SDC+HML medium at 30°C for 48 h, the fluorescence intensity was measured with immunofluorescence labeling. BY4741/*sed1*Δwas used as a negative control. Values represent the means ± standard deviations of the results from three independent experiments.

**Measurement of hydrolysis activity of chitosanase by Rondle-Morgan method**

Hydrolysis activity of chitosanase was measured by the Rondle-Morgan method, and relative hydrolysis activities are shown in Fig. 6. As mentioned in the previous section, the values of individual chitosanase activities were divided by the values of individual fluorescence intensity and represented as a percentage to normalize the amount of displaying chitosanase among WT and constructed mutants. In the chitosanase activity assay of the WT, the hydrolysis reaction reached upper limit of colorimetric detection within 1 hour. However, as the hydrolysis reaction of all mutants may not be finished within 1 h, the reaction time of hydrolysis was set at 24 h. In the results of chitosanase activity assay among the sixteen mutants, the activity of the "center group" decreased more than that of the "outer group". Particularly, on double mutants of W159/F406, the chitosanase activity was almost lost. As demonstrated our results, W159 and F406 as the "center group" are essential for substrate binding. On the other hand, the activity of the "outer group" did not critically decrease. The activity was higher than that of the WT on W228A and remained about 70% on Y311A. However, on double mutants of W228/Y311 the chitosanase activity was decreased to about 30%. As indicated by our results, Y311 was more important in substrate binding than W228. W228 was not critical for maintaining the chitosanase activity, and it may work to assist binding of the end of substrate accommodated in the catalytic cleft.

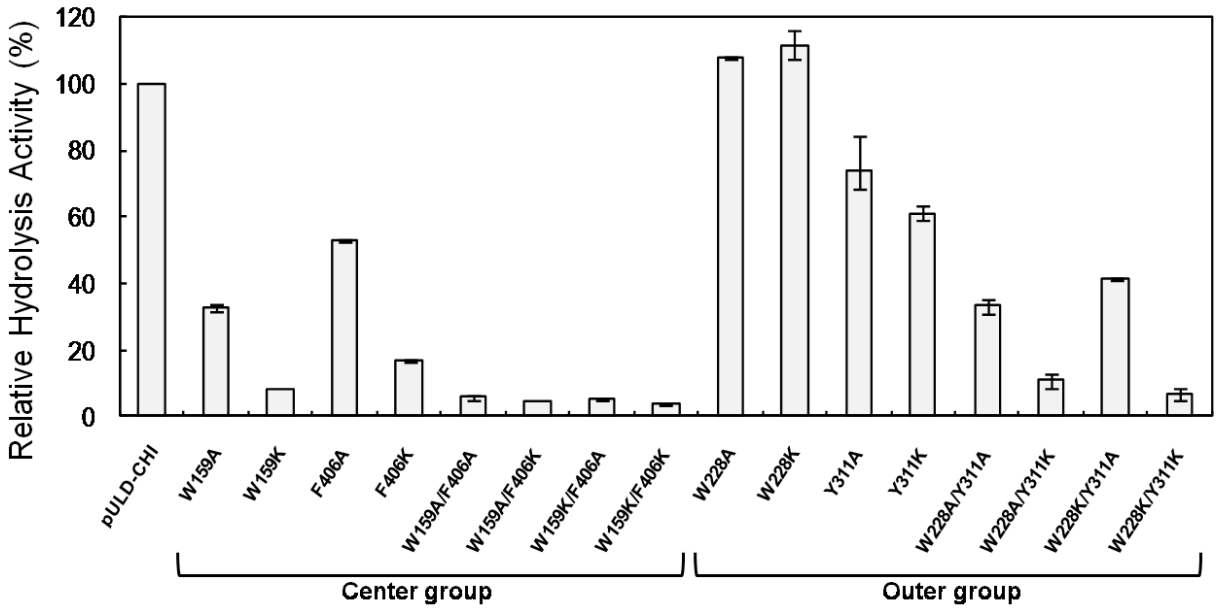
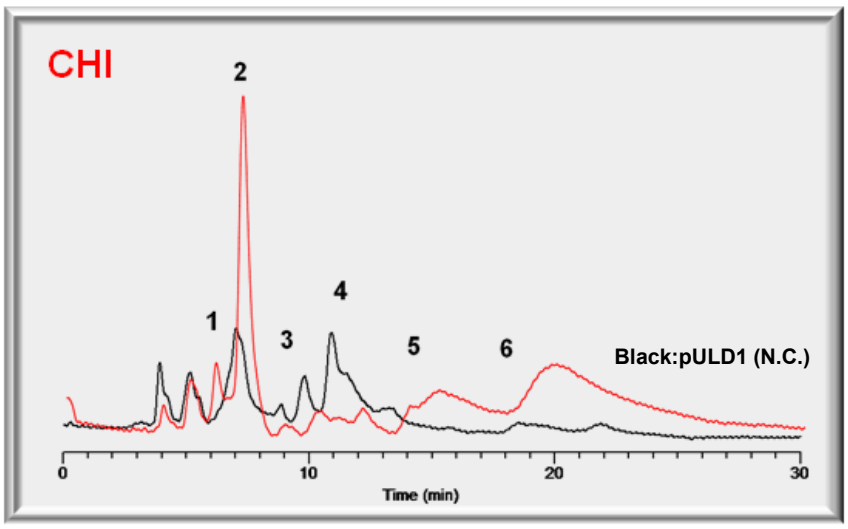



Fig. 6 Chitosanase activity assay. The value of pULD-CHI was regarded as 100% and relative ratios of values of mutants to that of WT (pULD-CHI) were calculated. Values represent the means ± standard deviations of the results from three independent experiments.

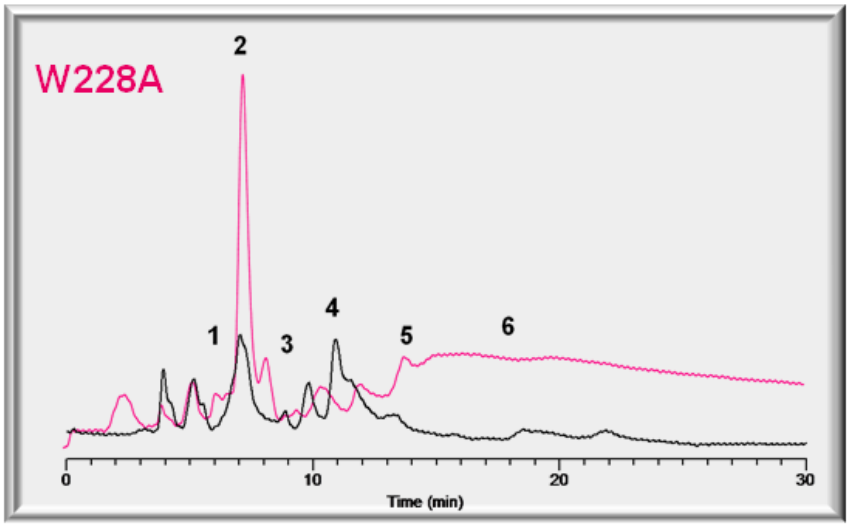
**Confirmation of degradation products from glycol chitosan as polymer and chitosan-hexamer by constructed mutants using HPLC**

From results of the activity assay, we determined the degree of significance of four amino acid residues engaged in substrate binding. To examine the manner of degradation by the constructed mutants, we performed HPLC analysis to detect degradation of the polymer and hexamer. Among the constructed mutants, W228A and Y311A were chosen for HPLC analysis because a significant effect was not observed with W228A and chitosanase activity remained about 70% with Y311A (Fig. 6). Other mutants that decreased chitosanase activity did not produce short oligosaccharides such as dimers, and trimers from the polymer and hexamer (data not shown). By degradation of the polymer, a dimer was mainly produced with wild type (WT), W228A, and Y311A chitosanases (Fig. 7).

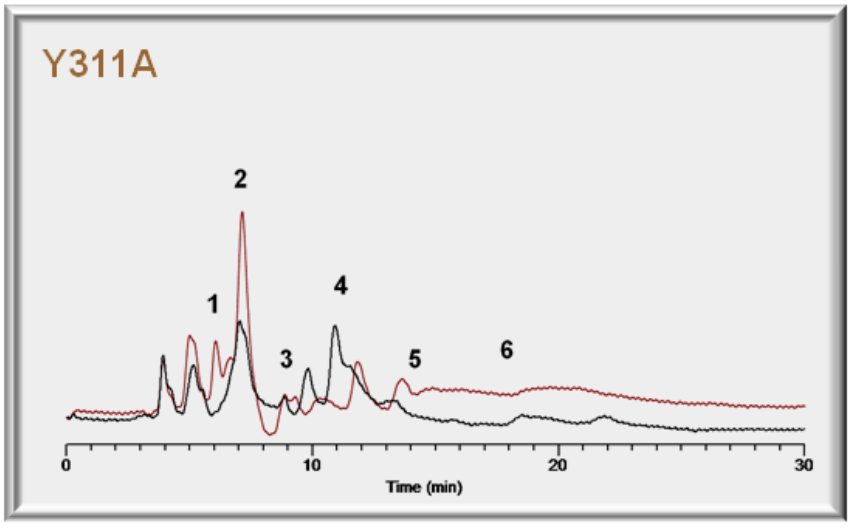
[A]


CHI
2
1
3
4
5
6
Black:pULD1 (N.C.)
0
10
20
30
Time (min)

[B]


W228A
2
1
3
4
5
6
0
10
20
30
Time (min)

[C]


Y311A
2
1
3
4
5
6
0
10
20
30
Time (min)

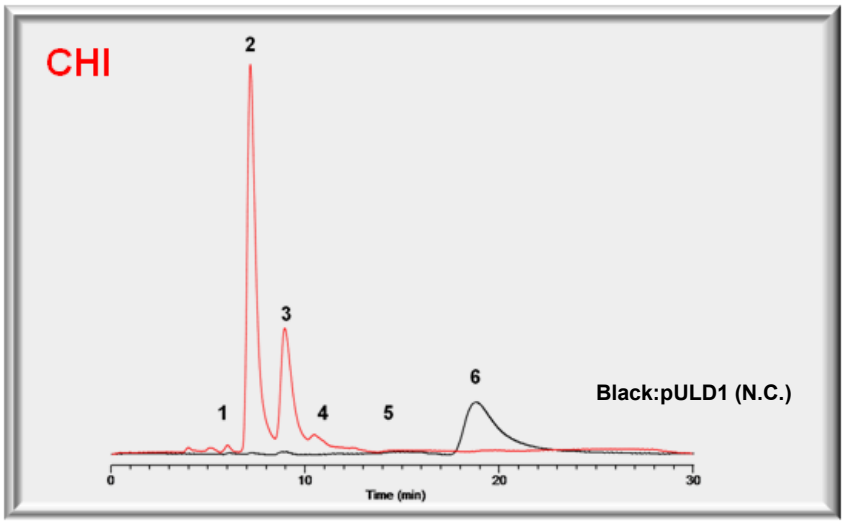
**[D]**

| Dimer | CHI | W228A | Y311A |
|---|---|---|---|
| nmol | 7.97 | 8.59 | 4.82 |
| % | 100 | 107.3 | 60.4 |

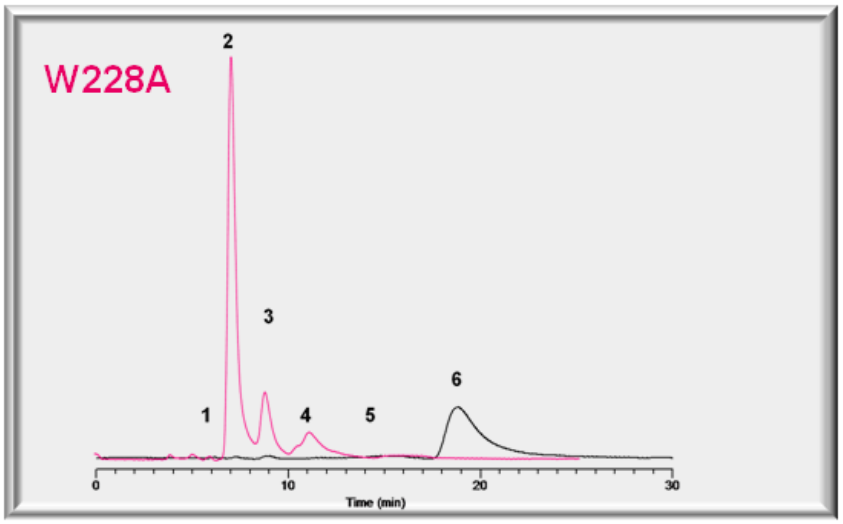
Fig. 7 HPLC analysis of degradation of glycol chitosan. (A) WT (pULD-CHI), (B) pULD-CHI-W228A, and (C) pULD-CHI-Y311A. (D) Comparison of dimer production (nmol). The value of pULD-CHI was regarded as 100% and relative ratios of values of mutants to that of WT (pULD-CHI) were calculated.

Since we could not precisely relate the effects of mutation to the manner of substrate binding by the degradation of polymer, we further examined the degradation of chitosan-hexamer and clarified the influence on the degradation manner. Trimer and tetramer production was detected by degradation of the hexamer with WT chitosanase. Additionally tetramer was reduced after degradation of the hexamer (Fig. 8). Because we have already confirmed that the monomer, dimer, and trimer were not degraded with WT (Fig. 9), the dimer and trimer were the main products with WT.

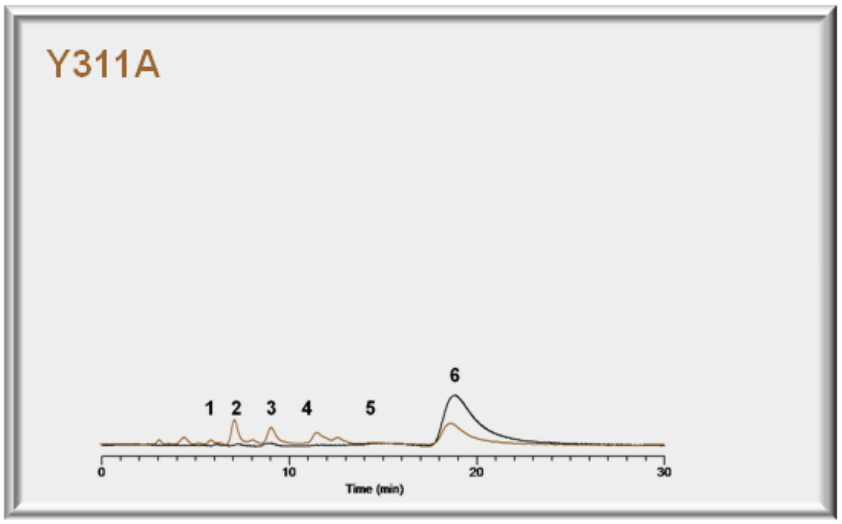
[A]


CHI
2
3
6
1
4
5
Black:pULD1 (N.C.)
0
10
20
30
Time (min)

[B]


W228A
2
3
6
1
4
5
0
10
20
30
Time (min)

[C]


Y311A
6
1
2
3
4
5
0
10
20
30
Time (min)

**[D]**

Production of oligomers (nmol)

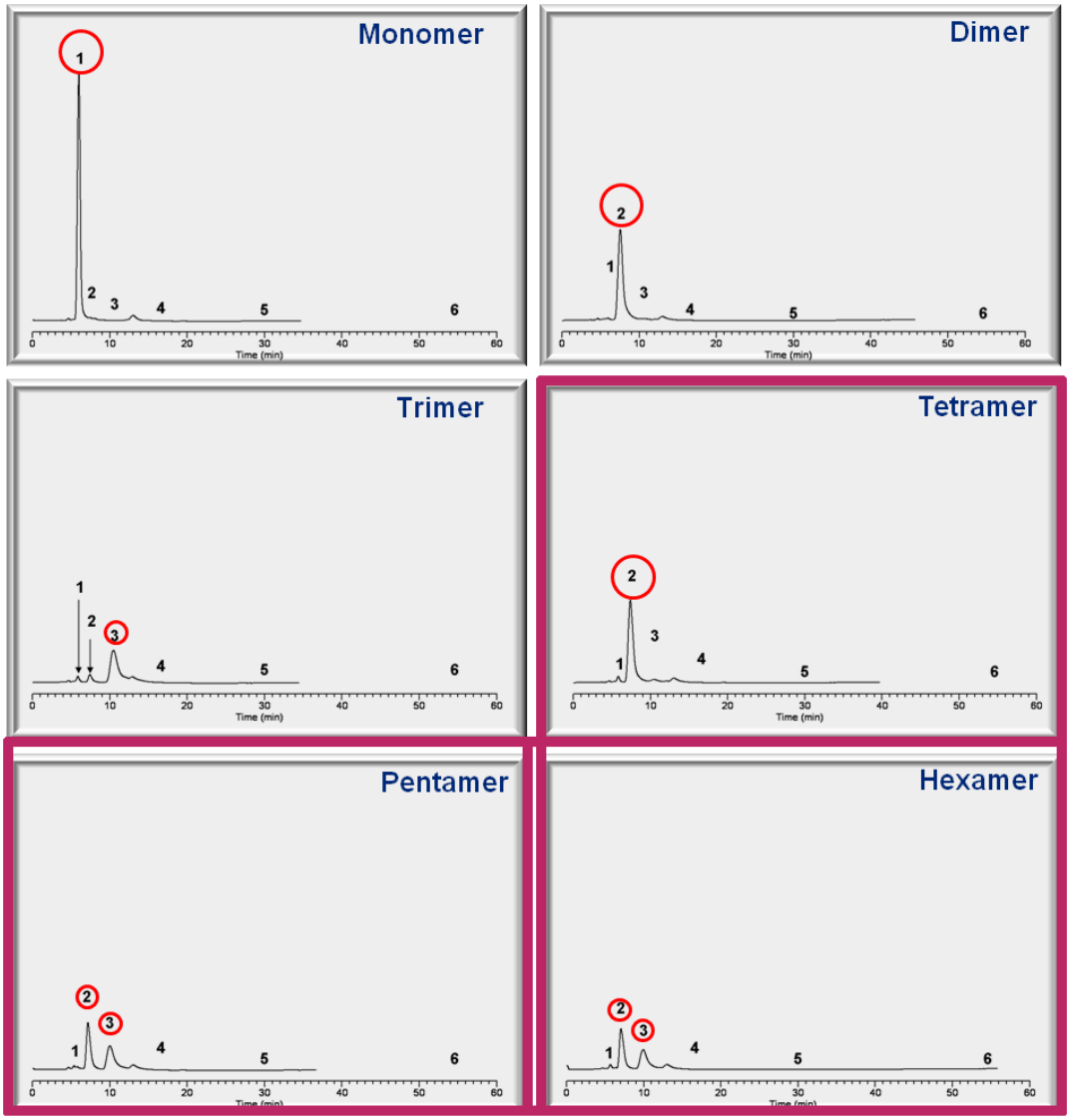
| | CHI | W228A | Y311A |
|---|---|---|---|
| Dimer | 15.1 | 15.4 | 0.19 |
| Trimer | 6.32 | 1.81 | ND |
| Tetramer | ND | 0.89 | ND |

*ND:Not Detected*

Fig. 8 HPLC analysis of degradation of chitosan-hexamer. (A) WT, (B) pULD-CHI-W228A, and (C) pULD-CHI-Y311A. (D) Comparison of produced chitosan oligosaccharides (nmol).

From the results, it was suggested that there are two conceivable types of degradation patterns by hexamer degradation. One is that the hexamer is primarily degraded to a dimer and tetramer, and the produced tetramer is finally degraded to two dimers [Pattern A]. Another is that the hexamer is degraded to two trimers [Pattern B]. Typically, it was suggested that both patterns occurred by a constant ratio with the WT. By degradation of the hexamer, dimer and trimer were finally produced on W228A. However, the degradation speed of the hexamer with W228A was slower than that of the WT because the hexamer was not been degraded within 8h (Fig. 10A). Degradation of the hexamer was not completed until 16 h when we could not detect its peak. The tetramer was then degraded to two dimers with W228A as well as degradation of the tetramer after 2 h with the WT (Fig. 10B).

[A]




[B]

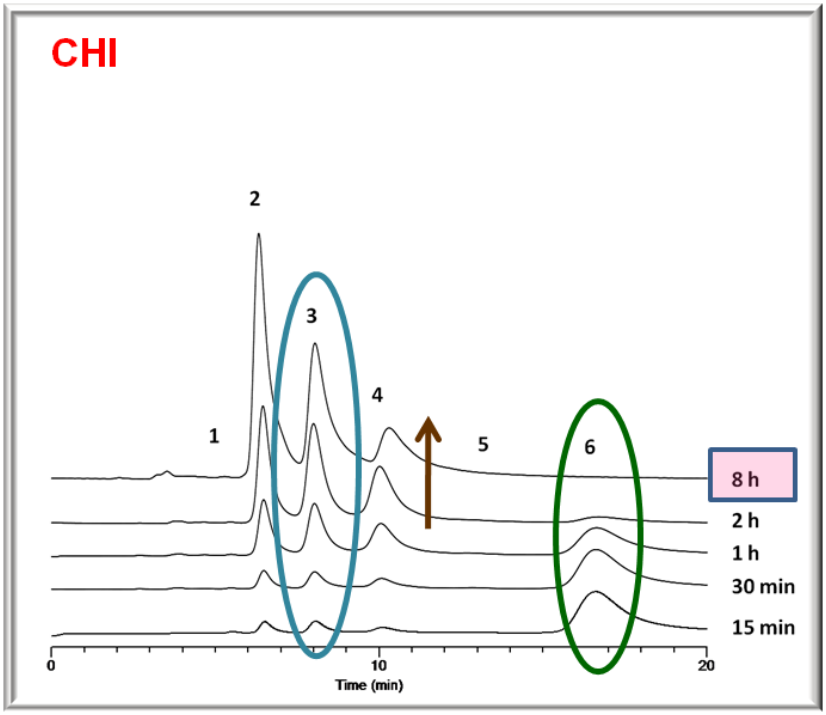
Amount of produced oligomer (nmol)

| Produced oligomer \ Reacted oligomer | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| monomer | 29.0 | ND | ND | ND | ND | ND |
| dimer | – | 12.2 | 0.87 | 11.0 | 6.30 | 5.42 |
| trimer | – | – | 8.84 | ND | 6.46 | 5.27 |
| tetramer | – | – | – | ND | ND | ND |
| pentamer | – | – | – | – | ND | ND |
| hexamer | – | – | – | – | – | ND |

*ND:Not Detected*

Fig. 9 HPLC analysis of degradation products with WT. (A) Degradation of monomer to hexamer (B) Comparison of degradation products (nmol).

[A]




[B]

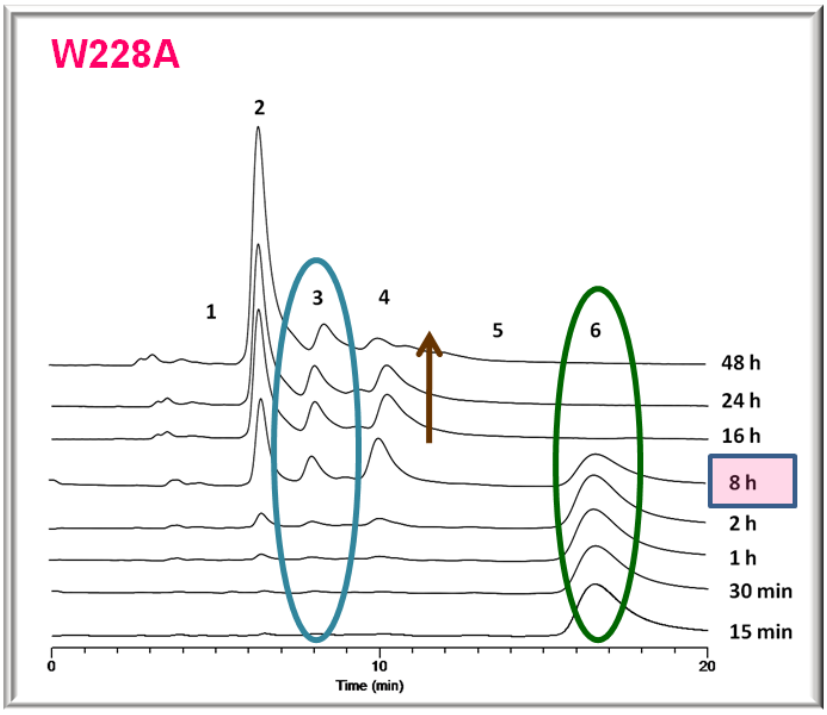



Fig. 10 HPLC analysis of degradation of chitosan-hexamer. Degradation time-course of (A) WT, (B) pULD-CHI-W228A.

Although both dimer and trimer increased after degradation of the hexamer with the WT, the trimer did not increase with W228A (Fig. 10B). Focused on the final degradation products of the hexamer, the amounts of the produced trimer were different between the WT and W228A. This was likely caused by abolishment of stacking interaction between the end of the hexamer and W228 around the non-reducing end, which would change the mechanism of accommodating the hexamer into the catalytic cleft. Therefore, binding of the chitosan hexamer was affected by mutation and it may be difficult to degrade in two trimers [Pattern B] (Fig. 11). In contrast to these results with the WT and W228A, production of the dimer, trimer, and hexamer was not observed by degradation of the hexamer with Y311A (Fig. 8).

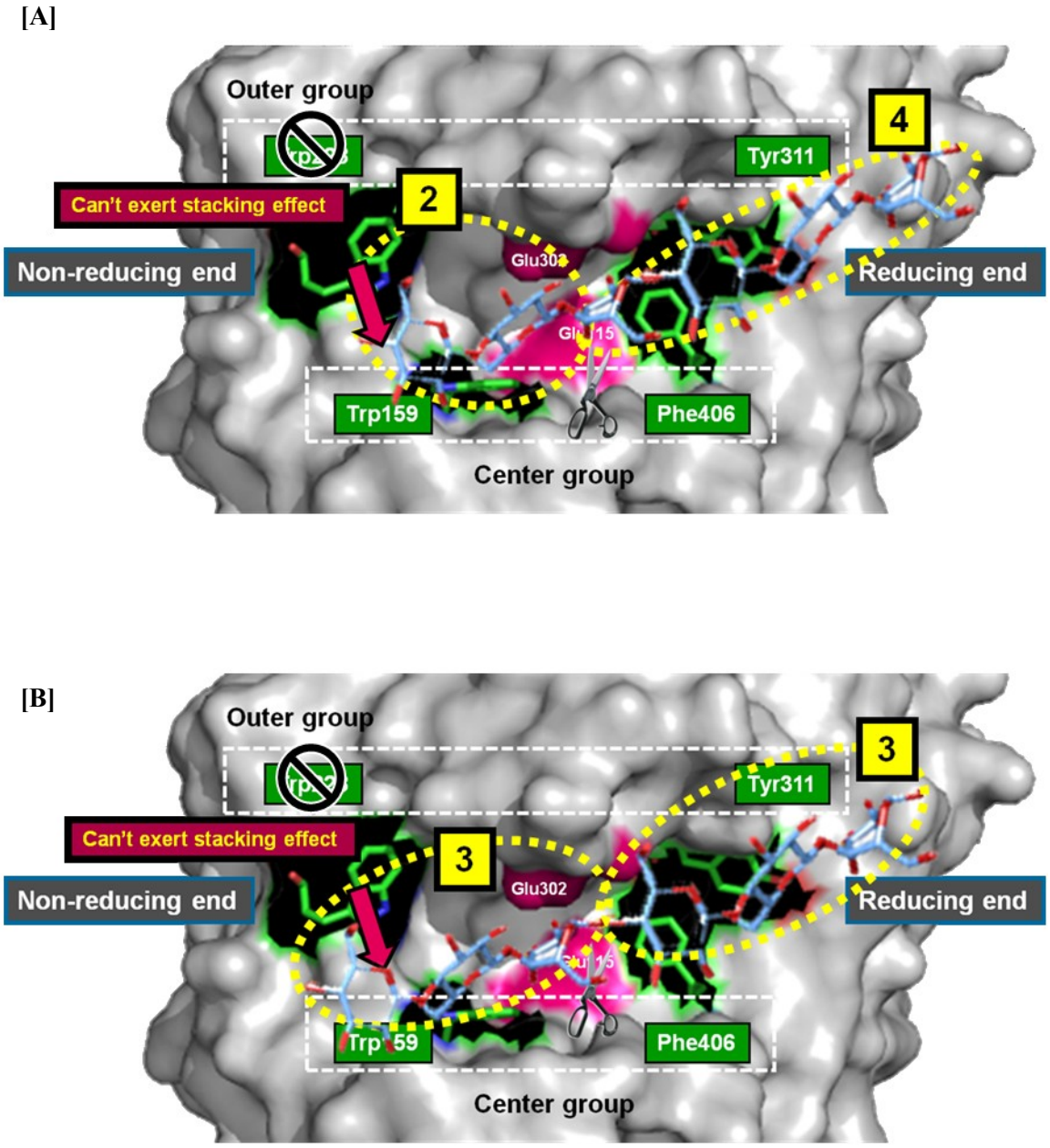



Fig. 11 Model of substrate binding by substitution of Trp228 (A) Pattern A, (B) Pattern B.

## Discussion

The amino acid residues for substrate binding of chitosanase from *P. fukuinensis* are four aromatic amino acid residues (Trp159, Trp228, Tyr311, and Phe406) referred by chitosanase from *Bacillus* sp. K17 (ChoK) (Fig. 1) (32). To investigate the action of amino acid residues, we constructed a mutant library of chitosanase. Primary, we constructed a plasmid (pULD-CHI) for high expression of chitosanase from *P. fukuinensis* on the yeast cell surface. We obtained chitosanase-displaying yeast BY4741/*sed1*Δ/pULD-CHI as the wild type (Fig. 2, 3). To evaluate the effect of these amino acid residues for substrate binding, we divided the four amino acid residues engaged in substrate binding into two groups ("center group" and "outer group") and then constructed single or double mutants substituted by Ala and Lys using pULD-CHI as the template (Table 1, 2). On the constructed sixteen mutants, we performed immunofluorescence microscopy and measured fluorescence intensity (Fig. 4, 5). Based on our results, we demonstrated that the degree of significance for substrate binding was different among the four amino acid residues. The "center group" (W159 and F406) is more significant than the "outer group" (W228 and Y311) for substrate binding, as indicated by the results of the chitosanase activity assay (Fig. 6). It was suggested that the amino acid residues of the "center group" exist near their active sites (Fig. 1) and, therefore, chitosanase activity was almost lost. On the other hand, the amino acid residues of the "outer group" were not as critical to retain chitosanase activity because they were assumed to act as support to bind to the end of the sugar chain. To examine the effect of the products by degradation of the hexamer, we performed HPLC analysis (Fig. 7). Because we could not precisely describe the effects of mutation with the results of the degradation of the polymer, we examined the products by degradation of the hexamer (Fig. 8). As the result of degradation of the hexamer, the dimer, trimer, and tetramer were initially produced with the WT and W228A, and the tetramer was then degraded to two dimers (Fig. 10). This was the reason for the aforementioned two conceivable degradation patterns ([Pattern A] and [Pattern B]). However, the degradation speed with W228A was slow compared to the

WT, and degradation of the hexamer finished within 16 h and the produced tetramer further degraded within 48 h (Fig. 10). The increase of the amount of dimer was stoichiometrically corresponded to the decrease of the amount of tetramer from 16 h to 48 h with W228A. Moreover, while trimer levels increased with the WT after degradation of the hexamer (2 h to 8 h), increase in the trimer levels after degradation of the hexamer (16 h to 48 h) with W228A was not observed (Fig. 10). In comparison with final products between the WT and W228A at 48 h, the production of trimer differed and it was about one third that of the WT (Fig. 8D). This was likely because it became hard to degrade the hexamer on [Pattern B] by abolishment of the stacking effect of W228. From these results, we demonstrated that it was effective to substitute the "outer group", not to substitute "center group", for modifying substrate binding manner so as not to lose chitosanase activity. Moreover, we observed the delay of degradation of hexamer by substitution of W228. This result showed that it became more difficult to degrade the hexamer than the WT (Fig. 11). To obtain useful chitosan oligosaccharides, such as longer than the hexamer, we may design a mutant that does not lose chitosanase activity, and is hard to degrade producing longer chitosan oligosaccharides holding higher bioactivities. With Y311A, we could not observe products by degradation of the hexamer (Fig. 8C). These results may possibly be due to the fact that the longer oligosaccharides produced by degradation of the polymer remained not to degrade by substitution of Y311. Based on the results from these assays with W228A and Y311A, it was determined that the amino acid residues of the "outer group" were significant for binding not to the polymer, but to longer oligosaccharides, such as the hexamer. It is suggested that the most effective strategy for designing useful mutants producing longer oligosaccharides, was to stop the degradation of produced longer oligosaccharides by introduction of mutation into W228 or Y311 as the "outer group".

In conclusion, it was confirmed the substrate binding amino acid residues of chitosanase from *P. fukuinensis* and evaluated the significance among them. These findings may propose to guideline for construction of useful mutants that produce oligosaccharides holding higher bioactivities.

## Summary

The previous chapter II indicated that the catalytic proton acceptors of chitosanase from *P. fukuinensis* were Glu302 and Asn312. From the results, catalytic amino acid residues for holding chitosanase activity and β-1, 4 glucanase activity were clarified. Therefore, we attempted to confirm the substrate binding amino acid residues, subsequently. Among the putative substrate binding amino acid residues (Trp159, Trp228, Tyr311, and Phe406), the "center group" (Trp159 and Phe406) was more significant than the "outer group" (Trp228 and Tyr311) to bind substrate by the reason of the distance from the active site. The effect to degradation pattern of chitosan-hexamer was observed by substitution of Trp228. Although chitosanase activity and dimer production was detected by polymer degradation, no degradation products from chitosan-hexamer were detected by substitution of Tyr311. This suggests that the amino acid residues of "outer group" were significant for binding not to chitosan polymer but to chitosan-hexamer.

## References


1. Svein JH, Pawel S, Jannicke BC, Gustav VK, Morten S, Bjørnar S, Gert V, Kjell MV, Vincent GHE (2006) *Proc. Natl. Acad. Sci. USA*. **103**, 18089-18094
2. Tsigos I, Martinou A, Kafetzopoulos D, Bouriotis V (2000) *Trends Biotechnol.* **18**, 305-312
3. Synowieck J, Al-Khateeb N (2003) *Crit. Rev. Food Sci. Nutr.* **43**, 145-171
4. Caiqin Q, Yumin D, Ling X, Zhan L, Xiaohai G (2002) *Int. J. Biol. Macromol.* **31**, 101-107
5. Huang R, Mendis E, Rajapakse N, Kim SK (2006) *Life Sci.* **78**, 2399-2408
6. Henrissat B (1991) *Biochem. J.* **280**, 390-316
7. Henrissat B, Bairoch A (1993) *Biochem. J.* **293**, 781-788
8. Davis G, Henrissat B (1995) *Structure* **3**, 853-859
9. Henrissat B, Bairoch A (1996) *Biochem. J.* **316**, 695-696
10. Fukamizo T, Amano S, Yamaguchi K, Yoshikawa T, Katsumi T, Saito J, Suzuki M, Miki K, Nagata Y, Ando A (2005) *J. Biochem.* **138**, 563-569
11. Katsumi T, Lacombe-Harvey ME, Tremblay H, Brzezinski R, Fukamizo T (2005) *Biochem. Biophys. Res. Commun.* **338**,1839-1844
12. Alzari PM, Souchon H, Dominguez R (1996) *Structure* **4**, 265-275
13. Monzingo AF, Marcotte EM, Hart PJ, Robertus JD (1996) *Nat. Struct. Biol.* **3**, 133-140
14. Pedraza-Reyes M, Gutierrez-Corona F (1997) *Arch. Microbiol.* **168**, 321-327
15. Ohtakara A (1988a) *Methods Enzymol.* **161**, 501-505
16. Ohtakara A (1988b) *Methods Enzymol*. **161**, 505-510
17. Mitsutomi M, Isono M, Uchiyama A, Nikaidou N, Ikegami T, Watanabe T (1998) *Biosci. Biotechnol. Biochem.* **62**, 2107-2114
18. Choi YJ, Kim EJ, Piao Z, Yun YC, Shin YC (2004) *Appl. Environ. Microbiol.* **70**, 4522-4531
19. Kimoto H, Kusaoke H, Yamamoto I, Fujii Y, Onodera T, Taketo A (2002) *J. Biol. Chem.* **277**, 14695-14702

20. Kimoto H, Akamatsu M, Fujii Y, Tatsumi H, Kusaoke H, Taketo A (2010) *J. Mol. Nicrobiol. Biotechnol.* **18**, 14-23
21. Lipke, PN, Kurjan J (1992) *Microbiol. Rev.* **56**, 180-194
22. Ueda M, Tanaka A (2000) *Biotechnol. Adv.* **18**, 121-140
23. Ueda M, Tanaka A (2000) *J. Biosci. Bioeng.* **90**, 125-136
24. Kondo A, Ueda M (2004) *Appl. Microbiol. Biotechnol.* **64**, 28-40
25. Shibazaki S, Ueda M, Iizuka T, Hirayama M, Ikeda Y, Kamasawa N, Osumi M, Tanaka A (2001) *Appl. Microbiol. Biotechnol.* **55**, 471-475
26. Kobori H, Sato M, Osumi M (1992) *Protoplasma* **167**, 193-204
27. Elson LA, Morgan WTJ (1933) *Biochem. J.* **27**, 1824-1828
28. Rondle CJM, Morgan WTJ (1955) *Biochem. J.* **61**, 586-589
29. Sengupta S, Jana ML, Sengupta D, Naskar AK (2000) *Appl. Microbiol. Biotechnol.* **53**, 732-735
30. Ramírez MG, Avelizapa LIR, Avelizapa NGR, Camarillo RC (2004) *J. Microbiol. Methods* **56**, 213-219
31. Fen LL, Illias RM, Kamaruddin K, Maskat MY, and Hassan O (2006) *Enzyme Microbiol. Technol.* **38**, 215-219
32. Adachi W, Sakihama Y, Shimizu S, Sunami T, Fukazawa T, Suzuki M, Yatsunami R, Nakamura S, Takenaka A (2004) *J. Mol. Biol.* **343**, 785-795
33. Kadonosono T, Kato-Murai M, Ueda M (2008) *Protein Eng. Des. Sel.* **21**, 507-513
34. Ozaki K, Sumitomo N, Hayashi Y, Kawai S, Ito S (1994) *Biochim. Biophys. Acta.* **1207**, 159-164
35. Kim PI, Kang TH, Chung KJ, Kim IS, Chung K (2004) *FEMS Microbiol. Lett.* **240**, 31-39
36. Guérin DMA, Lascombe M-B, Costabel M, Souchon H, Lamzin V, Béguin P, Alzari PM (2002) *J. Mol. Biol.* **316**, 1061-1069
37. Schmidt A, Gonzalez A, Morris R J, Costabel M, Alzari PM, Lamzin VS (2002) *Acta. Crystallog .sect. D* **58**,1433-1441
38. Petegem FV, Collins T, Meuwis MA, Gerday C, Feller G, Beeumen JV (2003) *J. Biol. Chem.* **278**,7531-7539

39. Kuroda K, Matsui K, Higuchi S, Kotaka A, Sahara H, Hata Y, Ueda M (2009) *Appl. Microbiol. Biotechnol.* **82**, 713-719

# Conclusion

The present study has been carried out to understand a degradation mechanism of chitosanase from *P. fukuinensis* and to design useful mutants that produce useful chitosan oligosaccharides.

In chapter I, *P. fukuinensis* chitosanase-displaying yeast was constructed using yeast cell-surface displaying system. On constructed chitosanase-displaying yeast, we determined chitosanase activity. Therefore, comprehensive analysis by constructing chitosanase mutant library based on this chitosanase-displaying yeast will be able to be performed.

In chapter II, to clarify proton acceptor of chitosanase from *P. fukuinensis*, chitosanase mutant library was constructed. Glu302 was a proton acceptor for chitosanase activity and β−1, 4 glucanase activities and Asn312 also participated in the hydrolysis of chitosan and cellulose.

In chapter III, to confirm the effect of substrate binding amino acid residues of chitosanase from *P. fukuinensis*, Trp159, Trp228, Tyr311, and Phe406 were evaluated. The four amino acids were divided into two groups; “center group” (Trp159 and Phe406) and “outer group” (Trp228 and Tyr311) and constructed mutants. Measurement of chitosanase activity assay indicated that the “center group” was more important for binding substrate than “outer group”. HPLC analysis of degradation of hexamer indicated that two degradation patterns. One is that the hexamer is primarily degraded to a dimer and tetramer, and the produced tetramer is finally degraded to two dimers [Pattern A]. Another is that the hexamer is degraded to two trimers [Pattern B]. Because stacking interaction between aromatic ring of amino acid residue and sugar ring of substrate could not be formed by introduction of mutation in Trp228 in “outer group”, the [Pattern B] was hard to occur. Although degradation of polymer was observed on Tyr311 substituted mutant, the degradation products from hexamer could not be observed. This suggests that the amino acid residues in “outer group” were important for binding not to polymer but to chitosan oligomers such as hexamer. From these results, it is effective for constructing the mutants that cannot degrade produced

oligosaccharides subsequent to polymer degradation and for obtaining useful oligosaccharides to introduce mutation into amino acid residues of “outer group”.

## Acknowledgments

This thesis is submitted by the author to Kyoto University for the Doctor degree of Agriculture. The studies presented here have been carried out under the direction of Professor Mitsuyoshi Ueda in the Laboratory of Biomacromolecular Chemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, during 2008-2014. I would like to express my sincere gratitude to Professor Mitsuyoshi Ueda for his continuous guidance and support throughout the course of this study. I am also indebt to Professor Shin-ichiro Suye. If there were not their encouragement, I couldn't accomplish this study. I am deeply grateful to Associate Professor Kouichi Kuroda for his invaluable suggestions, discussion, and help throughout my study. I am also deeply grateful to Associate Professor Hironobu Morisaka for his experimental suggestion and support. I am also grateful to Professor Hisashi Kimoto for his experimental support. I would also like to express my appreciation to Secretary Fukuko Suzuki for her lovely support of all my life. My deepest appreciation goes to all the members in this laboratory for their constant encouragement and support of this work. Especially, I would like to express my gratitude to Mr. Kota Tsuchiyama, Dr. Takeshi Fukuda, Dr. Tetsuya Kadonosono, Mr. Hiroaki Fukada, Mr. Jun Fuchimoto, and Mr. Naoki Tokumoto they are my master in my life. I would also like to express my appreciation to Mr. Masaru Ozawa, manager of department of clinical technology, Kyoto College of Health and Hygiene, for his continuous support always. I would also like to express my great gratitude to my parents Mr. Mitsuo Isogawa and Ms. Junko Isogawa for their kind understanding of my career decision, moral support, and warm encouragement. Finally, I would like to thank Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists that made it possible to complete this study.

**Danya Isogawa**

Laboratory of Biomacromolecular Chemistry
Division of Applied Life Science
Graduate School of Agriculture
Kyoto University

# Publication

Chapter I

Fukuda T, Isogawa D, Takagi M, Kato-Murai M, Kimoto H, Kusaoke H, Ueda M, Suye S

Yeast cell-surface expression of chitosanase from *Paenibacillus fukuinensis*

*Biosci. Biotechnol. Biochem.* **71** (11), 2845-2847 (2007)

Chapter II

Isogawa D, Fukuda T, Kuroda K, Kusaoke H, Kimoto H, Suye S, Ueda M

Demonstration of catalytic proton acceptor of chitosanase from *Paenibacillus fukuinensis* by comprehensive analysis of mutant library

*Appl. Microbiol. Biotechnol*. **85**, 95-104 (2009)

Chapter III

Isogawa D, Morisaka H, Kuroda K, Kusaoke H, Hisashi K, Suye S, Ueda M

Evaluation of chitosan-binding amino acid residues of chitosanase from *Paenibacillus fukuinensis*

Submitted

**Other publication**

**In English**

Whole-cell biocatalyst for utilization of chitosan by yeast cell surface engineering of chitosanase

Isogawa D, Kuroda K, Ueda M

Handbook of Chitosan Research and Application (Nova Science Publisher), 425-434 (2011)

**In Japanese**

◆書籍

五十川團哉、黒田浩一、植田充美, 「リサイクルバイオテクノロジーの最前線」（植田充美監修）, 『カニ廃棄物からの有用オリゴ糖生産技術（p.150-158)』シーエムシー出版, 2013.