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Enzymatic Syntheses of Novel Oligosaccharides Using Haloarchaeal Glycosidases

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Introduction

Chitin is a β-1,4-linked polymer of *N*-acethyl-D-glucosamine (GlcNAc) and is the second most abundant biopolymer in nature after cellulose. Chitinases (EC 3.2.14) are enzymes, which hydrolyze β-1,4-glycosidic bonds of chitin. In the more than 80 families of glycosyl hydrolases that have been defined on the basis of amino acid sequence homology, chitinases are classified into two glycosidase families, 18 and 19.[1] In contrast to bacterial, fungal, plant and insect chitinases, studies on chitinase from the third kingdom, archaea, are very few with the following exceptions: Chitinase genes from hyperthermophile *Thermococcus kodakaraensis* KOD1[2] and *Pyrococcus furiosus*[3] were isolated, cloned and expressed in *Escherichia coli*, and membrane-associated chitinase from *Thermococcus chitonophagus* was purified and characterized as a native enzyme.

Halobacterium species are extremely halophilic archaea that thrive in environments nearly saturated with salt (4–5 M NaCl). So far, characterization of chitinases from halophilic archaea has not been reported. Recently, the complete genome sequence of *Halobacterium* sp. NRC-1 was reported,[4] and this research revealed an open reading frame which is predicted to encode a chitinase homolog, which we termed ChiN1. ChiN1 contained three putative domains: a chitin-binding domain (ChBD), a polycystic kidney disease I domain (PKD), and a catalytic domain (CatD).

Haloarcula japonica strain TR-1 is an extremely halophilic archaeon and has large amount of glycoprotein on its cell surface.[5)] Previously, the gene encoding the cell surface glycoprotein (CSG) was cloned and sequenced.[6)] Because of the large amount of CSG in *Har. japonica*, the promoter of CSG gene is expected to be powerful. Thus, it may be possible to construct high expression systems of foreign genes in *Har. japonica* by employing the CSG promoter.

In this study, we report an expression of ChiN1 gene from *Halobacterium* sp. NRC-1 including its signal sequence by *Har. japonica* using the promoter sequence of CSG gene, and characterization of ChiN1 produced by *Har. japonica*. Furthermore, to reveal the function of ChBD and PKD, a truncated ChiN1 ($ChiN1_{CatD}$), which lacked the polypeptide region between ChBD and PKD, has been constructed. Characterization of $ChiN1_{CatD}$ revealed that one of the physiological function of ChBD and/or PKD might be to enhance the hydrolysis of insoluble chitin.

Results and Discussion

Sequence analysis and protein domains

Genome sequence of *Halobacterium* sp. NRC-1 revealed an open reading frame of 1,638 nucleotides which is predicted to encode a chitinase homolog which we termed ChiN1. The ChiN1 gene encoded 546 amino acid (aa). However, the predicted signal sequence was found in the *N*-terminal 28 aa portion, the removal of which would result in a mature protein of 57 kDa. By comparing of deduced amino acid sequence among ChiN1 and other chitinases, ChiN1 seemed to contain three putative domains, a chitin binding domain (ChBD), a polycystic kidney disease I domain (PKD), and a catalytic domain presumably classified in family 18 of glycosyl hydrolases (CatD) (Fig. 1).

Recently, a novel translocation pathway for protein secretion was discovered. Because signal peptides in proteins using this pathway contain a typical twin-arginine motif, it was denoted the twin-arginine translocation (Tat) pathway. In total, 103 proteins with putative signal peptides were identified in *Halobacterium* sp. NRC-1, and more than 60% of these contain a twin-arginine motif. There was a twin-arginine motif in the signal peptide of ChiN1, so ChiN1 is expected to be secreted by Tat pathway.

According to classification of carbohydrate-binding modules (CBMs), the putative ChBD of ChiN1 belongs to family V CBM. Enzymes that have family V CBM are almost chitinases, but a few of chitosanases, xylanases and cellulases also contain CBM of this family.

PKD was first identified in human polycystin-1, the primary protein responsible for polycystic kidney disease. The PKD was an immunoglobulin (Ig)-like fold mainly composed of ß-sheets. This structure can be found in fibronectin type III domain which is prevalent in chitinases and cellulases, and presumably function as enzyme-carbohydrate interactions. Due to the similarity of their structure,

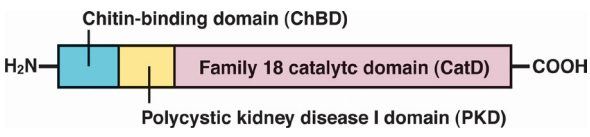

Fig. 1. Shematic structure of ChiN1.

PKD in chitinases are expected to act like fibronectin type III domain.

The deduced amino acid sequence of CatD of ChiN1 showed moderate homology to that of chitianse A of *Thermococcus kodakaraensis* and chitinase A1 of *Bacillus circulans*, both of which belong to family 18 chitinases. This suggests that ChiN1 belongs to family 18 chitinases.

Expression of chiN1 gene in Har. japonica

The *chiN1* gene was subjected to be expressed in *Har. japonica* by using its CSG promoter. *Har. japonica* was transformed with expression plasmid pChiN9, encoding a chiN1 gene downstream of the CSG promoter of *Har. japonica*. SDS-PAGE analysis of *Har. japonica* transformants revealed the presence of a large amount of protein corresponding to about 70 kDa only in the culture supernatant. SDS-PAGE usually yielded high molecular mass values for halophilic proteins than calculated molecular mass, due to the reduced ability of them to bind SDS because of its high acidic residues content. The predicted mature ChiN1 contains larger proportion of acidic amino acids (17%) compared to reported chitinases from mesophilic organisms (3～10%). Chitinase activity of this protein was detected by activity staining followed by SDS-PADE, indicating that ChiN1 gene was expressed in *Har. japonica*. *N*-terminal amino acid sequence of the purified ChiN1 revealed the first 12 amino acids as ADTPPEWDPDTV. This sequence was corresponding to the predicted first amino acid of the mature form of ChiN1 after cleavage of the signal peptide.

Characterization of the purified ChiN1

Effects of pH and temperature

To evaluate the roles of additional domains ChBD and PKD, domain-truncated derivative of ChiN1 ($ChiN1_{CatD}$) was prepared and characterized. The pH- and temperature-activity profiles of ChiN1 and $ChiN1_{CatD}$ were almost similar; optimum pH (Fig. 2) and temperature (Fig. 3) of enzyme were around pH 4.5 and 50°C, respectively.

Effects of NaCl

Maximal chitinase activity of ChiN1 was detected at 1 M NaCl (pH 6.0, 37°C) (Fig. 4). ChiN1 retained only 50% of optimal activity at 60 mM NaCl, indicating a salt requirement for its activity. Chitinase activity was very stable over a wide NaCl range from 1–4.5 M (24 h, pH 6.0, 4°C) (Data not shown). In general, halophilic enzymes require high salt concentration for retaining their structure and function. Because of its unusual content of acidic residues, ChiN1 was predicted to be a halophilic enzyme, and these profiles of ChiN1 and $ChiN1_{CatD}$ were almost similar.

Chitin binding activity

Binding activity toward insoluble chitin was compared for ChiN1 and $ChiN1_{CatD}$. ChiN1 bound to insoluble chitin. However, the deletion of ChBD and PKD abolished the binding activity to insoluble chitin (Date not shown). This result suggested that these regions were responsible for the chitin binding. The chitinase activity of ChiN1 and $ChiN1_{CatD}$ was compared when soluble or insoluble chitin

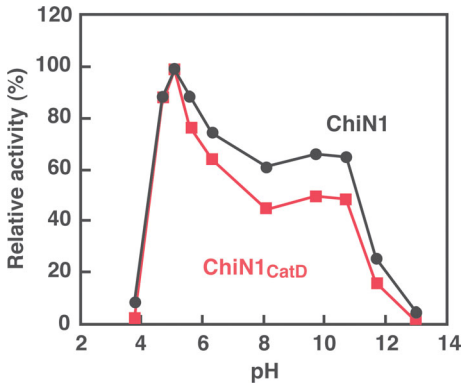

Fig. 2. Effect of reaction pH on the activity of ChiN1 and $ChiN1_{CatD}$.

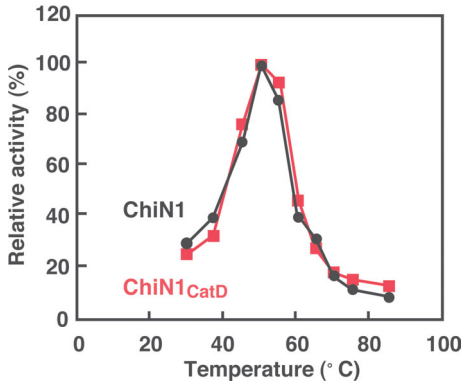

Fig. 3. Effect of reaction temperature on the activity of ChiN1 and $ChiN1_{CatD}$.

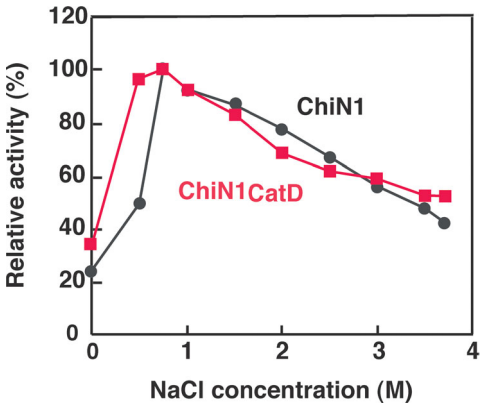

Fig. 4. Effect on NaCl concentration on activity.

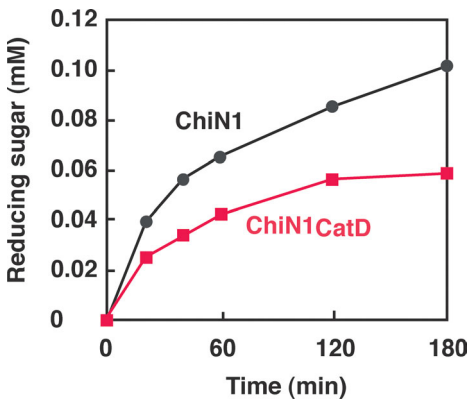

Fig. 5. Time course of insoluble chitin degradation by ChiN1 and $ChiN1_{CatD}$.

was used as substrate. Both enzymes showed almost equal activity toward soluble chitin, however, ChiN1 hydrolyzed insoluble chitin more efficiently than $ChiN1_{CatD}$. did (Fig. 5). These results indicated that one of the physiological function of ChBD and/or PKD might be to enhance the hydrolysis of insoluble chitin.

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