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Substrate binding to the inactive mutants of *Streptomyces* sp. N174 chitosanase: indirect evaluation from the thermal unfolding experiments

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Abstract Oligosaccharide binding to chitosanase from *Streptomyces* sp. N174 was indirectly evaluated from thermal unfolding experiments of the protein. Thermal unfolding curves were obtained by fluorescence spectroscopy in the presence of D-glucosamine oligosaccharides ($(GlcN)_n$, $n = 3,4,5$, and 6) using the inactive mutant chitosanase in which the catalytic residue, Glu^{22}, is mutated to glutamine (E22Q), aspartic acid (E22D), or alanine (E22A). The midpoint temperature of the unfolding transition (T_m) of E22Q was found to be 44.4°C at pH 7.0. However, the T_m increased upon the addition of $(GlcN)_n$ by 1.3°C ($n = 3$), 2.5°C ($n = 4$), 5.2°C ($n = 5$), or 7.6°C ($n = 6$). No appreciable change in T_m was observed when $(GlcNAc)_6$ was added to E22Q. The effect of $(GlcN)_n$ on the thermal stability was examined using the other protein, RNase T1, but the oligosaccharide did not affect T_m of the protein. Thus, we concluded that the stabilization effect of $(GlcN)_n$ on the chitosanase results from specific binding of the oligosaccharides to the substrate binding cleft. When E22D or E22A was used instead of E22Q, the increases in T_m induced by $(GlcN)_6$ binding were 2.7°C for E22D and 4.2°C for E22A. In E22D or E22A, interaction with $(GlcN)_6$ seems to be partly disrupted by a conformational distortion in the catalytic cleft.

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Key words: Chitosanase; Glucosamine oligosaccharide; Substrate binding; Thermal unfolding

1. Introduction

Substrate binding site of carbohydrolases has been recognized to consist of several subsites, each of which can accommodate one sugar residue of the corresponding substrate. It is important to identify the subsite structure of carbohydrolases because the specificity of the enzymatic reaction is mostly determined by characteristics of the subsites. We have been studying the structure and function of chitosanase from *Streptomyces* sp. 174, and identified its catalytic site and the reaction mechanism [1,2]. From energy-minimization procedure, the most probable substrate binding cleft has been estimated based on the X-ray crystal structure of the enzyme [3]. However, no experimental evidence was obtained for substrate binding, hence for the subsite structure of the enzyme. Oligosaccharide binding experiments for carbohydrolases have been conducted using physicochemical methods, such as fluorescence, CD, and NMR spectroscopy [4–6]. In particular, tryptophan fluorescence observation is very useful for the binding study because of its high sensitivity. However, a suitable localization of the tryptophan residues in the protein molecule is required in order to observe a change in the fluorescence spectrum upon the saccharide binding. In the crystal structure of *Streptomyces* sp. N174 chitosanase, tryptophan residues are not localized in the surface of the substrate binding cleft, but in the hydrophobic core region or in an outer surface of the probable binding cleft. This indicates that it is difficult to observe the fluorescence change upon the substrate binding. Actually, in our preliminary experiments, we have failed to observe a change in the fluorescence spectrum of the chitosanase upon the addition of oligosaccharide. On the other hand, it has been recognized that ligand binding to a protein stabilize the protein structure through a conformational change resulting from the interaction between them [7,8]. The stabilization may afford some information about the ligand binding. In this paper, we have tried to evaluate indirectly the substrate binding to *Streptomyces* sp. N174 chitosanase from thermal unfolding curve obtained by means of tryptophan fluorescence. The unfolding curves of the chitosanase were obtained in the presence of D-glucosamine oligosaccharides, and the oligosaccharide binding was evaluated from increases in T_m or differences in free energy change of the unfolding.

2. Materials and methods

2.1. Materials

The wild-type *Streptomyces* sp. N174 chitosanase and its inactive mutants (E22Q, E22D, and E22A) were obtained by the method previously described [1]. D-Glucosamine oligosaccharides ($(GlcN)_n$, n=3, 4, 5, and 6], and *N*-acetyl-D-glucosamine hexasaccharide ($(GlcNAc)_6$) were purchased from Seikagaku Kogyo Co. RNase T1 from *Aspergillus oryzae* was from Sigma. Other reagents were analytical grade commercially available.

2.2. Fluorescence measurements and thermal unfolding

Tryptophan fluorescence of these proteins was measured in 50 mM sodium phosphate buffer, pH 7.0, with excitation wavelength of 295 nm using a Hitachi F3010 spectrofluorometer. For obtaining thermal unfolding curves of the chitosanases, fluorescence intensity at 330 nm was monitored raising the solution temperature at the rate of 1°C/min. The solution temperature was directly measured by setting thermocouple in the cell using a thermometer model DP-500 (Rikagaku kogyo). Reversibilities of the unfolding transition were estimated from comparison of fluorescence intensity obtained after annealing with that obtained before raising the temperature, and were more than 75% for all proteins tested. For facilitating comparison between the unfolding curves obtained, the experimental data were normalized as follows. The fraction of unfolded protein was calculated from the

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Abbreviations: GlcN, 2-amino-2-deoxy-D-glucopyranose; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of n; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of 2-acetamido-2-deoxy-D-glucopyranose with a polymerization degree of n

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fluorescence intensity by linearly extrapolating the pre- and post-transition base lines into the transition zone, and plotted against temperature. Analysis of the thermal unfolding curves was done according to the method of Pace et al. [9]. The temperature at the midpoint of the transition curve (T_m) was calculated from a least-square curve-fitting procedure of the unfolding curve on the assumption that the unfolding equilibrium of the chitosanases follows a two-state mechanism. The enthalpy and entropy changes at T_m (ΔH_m and ΔS_m) were calculated by van't Hoff analysis. The difference in the free energy change of unfolding (at T_m of the free wild-type protein) from that of the free wild-type protein ($\Delta\Delta G_m$) was estimated by the relationship, $\Delta\Delta G_m = \Delta T_m \cdot \Delta S_m$ (wild type), given by Becktel and Schellman [10], where ΔT_m is the difference in T_m from that of the free wild-type protein and ΔS_m (wild type) is the entropy change of the free wild-type protein at T_m.

3. Results and discussion

3.1. Thermal unfolding of Glu^{22}-mutated chitosanase

As reported in the previous paper [1], Glu^{22}-mutated chitosanases are completely inactive, but their CD spectra are almost the same as that of the wild type. This indicates that no global conformational change of the protein structure is induced by the Glu^{22} mutation and that the inactivity results only from loss of the catalytic potential of Glu^{22}. However, the Glu^{22} mutation may affect the thermal stability of the protein. Before the thermal unfolding experiments in the presence of the substrate, therefore, we investigated the thermal stability of each mutated chitosanase itself. Fig. 1A shows the thermal unfolding curves of wild-type chitosanase, E22Q, E22D, and E22A. The unfolding transition occurred in between 40 and 48°C. The normalized unfolding curves are shown in Fig. 1B. Each of the normalized unfolding curves were well fitted to the theoretical unfolding curves obtained with assuming a simple two-state transition mechanism. This enabled us to determine the values of T_m and the thermodynamic parameters for the unfolding process of the chitosanase. The T_m values obtained were 43.2°C for the wild-type, 44.4°C for E22Q, 42.5°C for E22D, and 42.6°C for E22A. Although the Glu^{22} mutation to aspartic acid or alanine somewhat destabilized the protein, the mutation to glutamine rather stabilized the protein by 1.2°C. At first, therefore, we performed the unfolding experiments in the presence of $(GlcN)_n$ using the most stable mutant protein, E22Q.

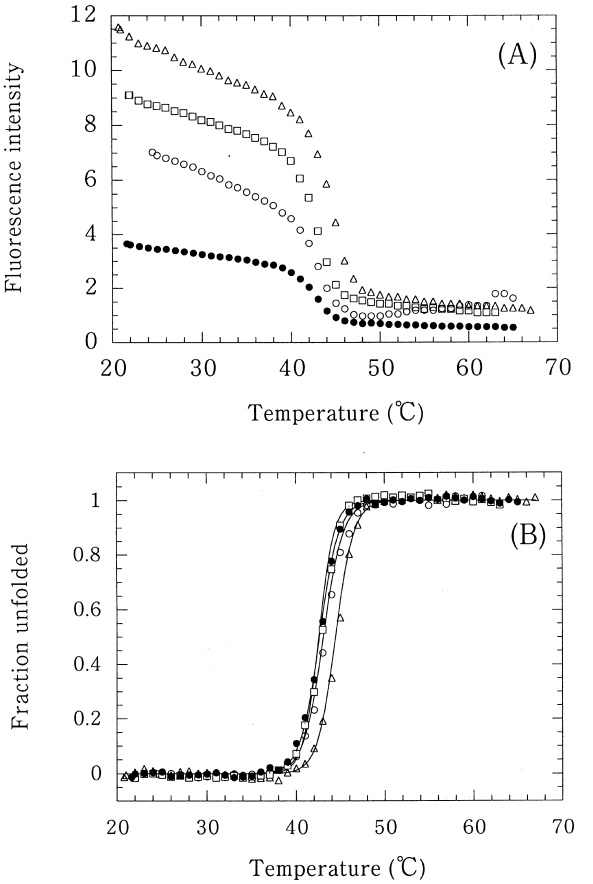

Fig. 1. A: Thermal unfolding curves obtained by monitoring fluorescence intensity at 330 nm with excitation wavelength at 295 nm. Each protein was dissolved in 50 mM sodium phosphate buffer, pH 7.0. The protein concentrations were 2.0 μM for the wild type, 3.4 μM for E22Q, 2.8 μM for E22D, and 1.0 μM for E22A. The symbols represent the experimental points for the wild type (○), E22Q (△), E22D (□), and E22A (●). B: The normalized unfolding curves obtained from the results shown in (A). The symbols are the same as in (A). The solid lines indicate the theoretical unfolding curves obtained with assuming the two-state transition mechanism.

3.2. Effect of $(GlcN)_n$ on the unfolding curve of E22Q

The unfolding curves of E22Q were obtained in the presence of $(GlcN)_n$ ($n = 3$, 4, 5, or 6). The ratio of the molar concentration of $(GlcN)_n$ added to that of the protein was 110∼120. From the initial velocity analysis using the wild-type enzyme, the chitosanase was found to be saturated with $(GlcN)_n$ in such a condition of the molar concentration ratio (Honda et al., unpublished). As shown in Fig. 2, a significant increase in the thermal stability was observed when $(GlcN)_n$ was added to E22Q. The T_m value increased upon the addition of $(GlcN)_n$ by 1.3°C ($n = 3$), 2.5°C ($n = 4$), 5.2°C ($n = 5$), or 7.6°C ($n = 6$). The increase in T_m appeared to be parallel to the polymerization degree of the substrate added. The stabil-

Table 1
Thermodynamic parameters for the unfolding transition of wild-type and Glu^{22}-mutated chitosanase from *Streptomyces* sp. N174

Enzyme	T_m (°C)	ΔT_m (°C)	ΔH_m (kcal·mol^{-1})	ΔS_m (kcal·mol^{-1}·°C^{-1})	$\Delta\Delta G_m$ (kcal·mol^{-1})
Wild-type	43.2	0	160.64	0.508	0
E22D	42.5	−0.7	225.02	0.713	−0.50
E22D+(GlcN)6	45.4	+1.8	207.74	0.652	+1.18
E22A	42.6	−0.6	180.39	0.572	−0.34
E22A+(GlcN)6	46.8	+3.6	243.47	0.761	+2.74
E22Q	44.4	+1.2	194.30	0.612	+0.73
E22Q+(GlcN)3	45.7	+2.5	234.09	0.735	+1.84
E22Q+(GlcN)4	46.9	+3.7	184.50	0.577	+2.13
E22Q+(GlcN)5	49.6	+6.4	215.64	0.668	+4.28
E22Q+(GlcN)6	52.0	+8.8	217.51	0.669	+5.89

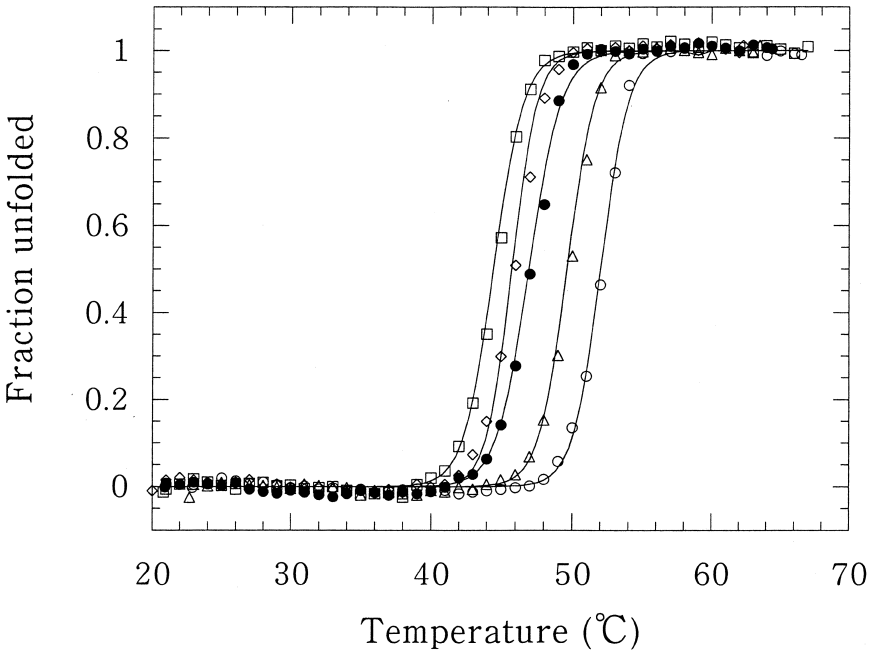

Fig. 2. The normalized unfolding curves of E22Q in the presence of $(GlcN)_n$. The protein concentration was 3.2 μM and the concentration of each oligosaccharide added was 0.36 mM. The reaction conditions were the same as in Fig. 1. The symbols represent E22Q (□), E22Q+$(GlcN)_3$ (◇), E22Q+$(GlcN)_4$ (●), E22Q+$(GlcN)_5$ (△), and E22Q+$(GlcN)_6$ (○). The solid lines are the theoretical curves.

ization effect induced by $(GlcN)_n$ seems to be derived from the specific binding of $(GlcN)_n$ to the chitosanase.

3.3. *Control experiments of the saccharide effects on the thermal stability of E22Q*

In order to ascertain the cause of stabilization effect of $(GlcN)_n$, a similar experiment was conducted using $(GlcNAc)_6$ instead of $(GlcN)_n$. $(GlcNAc)_6$ did not affect the unfolding curve of E22Q (Fig. 3). Effect of $(GlcN)_n$ on the protein stability was also investigated using RNase T1 instead of E22Q. No appreciable change in the unfolding curve of RNase T1 was observed upon the addition of $(GlcN)_6$ (data not shown). All of these results indicated that the stabilization effect of $(GlcN)_n$ results from the specific binding to the substrate binding cleft of the chitosanase. Thus, we concluded that the increases in T_m obtained from the unfolding curves reflect the strength of $(GlcN)_n$ binding to the chitosanase, and that the substrate binding to the chitosanase can be indirectly evaluated from the thermal unfolding curve of the protein.

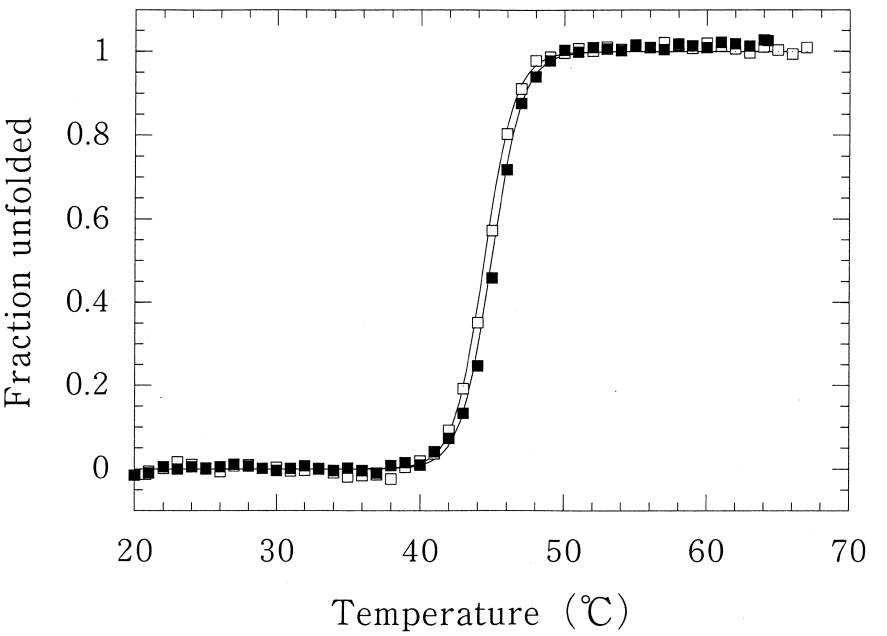

Fig. 3. The normalized unfolding curves of E22Q in the presence of $(GlcNAc)_6$. The reaction conditions were the same as in Fig. 2. The symbols represent E22Q (□) and E22Q+$(GlcNAc)_6$ (■). The solid lines are the theoretical curves.

3.4. Effect of $(GlcN)_6$ on unfolding curve of E22D or E22A

Similar experiments were conducted using E22D or E22A instead of E22Q. As shown in Fig. 4, effect of $(GlcN)_6$ on the thermal stability of each protein was smaller than that of E22Q. Increases in T_m induced by $(GlcN)_6$ binding were 2.7°C for E22D and 4.2°C for E22A. This indicates that the interaction of $(GlcN)_6$ with the chitosanase is partly disrupted in E22D or E22A. The length of the side chain at 22nd residue is smaller in E22D and E22A than in the wild-type enzyme and E22Q. This situation may cause the conformational distortion in the catalytic cleft, resulting in the partial disruption of the enzyme-oligosaccharide interaction.

3.5. Differences in the free energy change of unfolding

Thermodynamic parameters were calculated from the experimental unfolding curves and are listed in Table 1. The difference in the free energy change from that of the free wild-type protein, $\Delta\Delta G_m$, are summarized in Fig. 5. The stabilization energy should be derived from a conformational change of the chitosanase induced by the interaction with GlcN oligosaccharides. In the previous paper [1], we reported that the catalytic residues of the chitosanase are Glu^{22} and Asp^{40}. The crystal structure of the chitosanase revealed that the distance between the two catalytic residues is 13.8 Å [3].

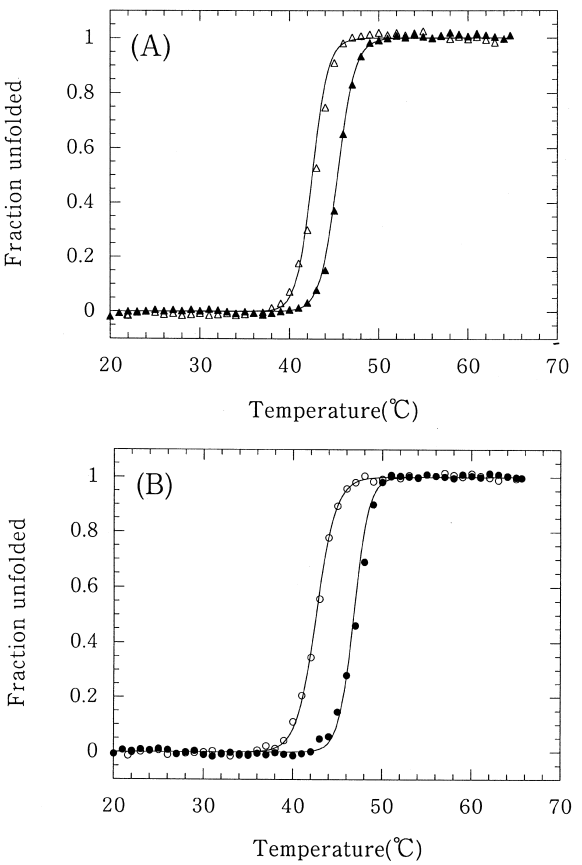

Fig. 4. A: The normalized unfolding curves of E22D in the presence of $(GlcN)_6$. The concentrations of the protein and the saccharide were 2.8 μM and 0.33 mM, respectively. B: The normalized unfolding curves of E22A in the presence of $(GlcN)_6$. The concentrations of the protein and the saccharide were 1.0 μM and 0.12 mM, respectively. The other conditions were the same as in Fig. 1. The symbols represent E22D (△), E22D+$(GlcN)_6$ (▲), E22A (○), and E22A+$(GlcN)_6$ (●). The solid lines are the theoretical curves.

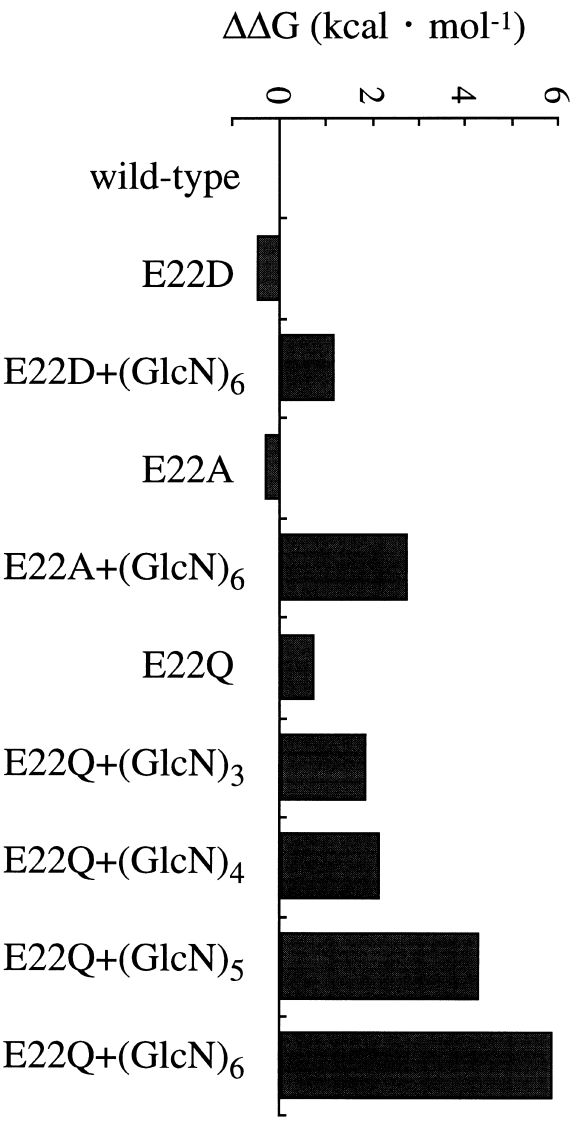

Fig. 5. The difference in the free energy change of unfolding transition from that of the free wild-type enzyme. The values were calculated from the experimental unfolding curves as described in the text.

This is somewhat longer than the average distance between two catalytic residues in the inverting β-glycosidases (9.0∼9.5 Å, [11]). Upon the saccharide binding, therefore, the active site cleft must undergo a conformational change that would narrow the binding cleft. The conformational change induced by the substrate binding would make the protein structure more compact and stabilize it.

The oligosaccharide binding to hen egg white lysozyme have been intensively studied by various spectroscopic methods, and the free energy changes of the binding were found to be larger in the longer chain length oligosaccharide [12,13]. This would be true for the *Streptomyces* sp. N174 chitosanase because the energy minimization based on the X-ray crystal structure of the chitosanase suggested that the substrate binding cleft of the enzyme is similar to that of the lysozyme [3]. In fact, Fig. 5, where $\Delta\Delta G_m$ increase from $(GlcN)_3$ to $(GlcN)_6$, shows a close resemblance to the binding data obtained for the lysozyme. It is likely that the chitosanase has at least six

subsites in its substrate binding cleft. Pace and McGrath reported that T_m of hen egg white lysozyme increased by 5.3°C upon the addition of 10 times molar equivalent of $(GlcNAc)_3$ [14]. This corresponds to an increase in the stability of the lysozyme of 1.3 kcal/mol. In this study, the stability increase of the chitosanase by $(GlcN)_3$ is calculated to be 1.8 kcal/mol. The trisaccharide binding ability of the chitosanase would be comparable to that of hen egg white lysozyme.

The direct observation of the saccharide binding to the chitosanase has been unsuccessful because of the unsuitable positioning of the chromophoric side chains in the chitosanase molecule. Nevertheless, in this paper, we reported a convenient method for indirect evaluation of substrate binding to the chitosanase. The binding experiments are now being conducted using various mutant chitosanases in order to investigate the substrate binding mechanism.

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