CHARACTERISTICS OF *Bombyx mori* CHITINASES AND THEIR PROCESSING

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Abstract

We purified two chitinases from the integument of *Bombyx mori* by column chromatography. They are named 65-kDa chitinase and 88-kDa chitinase after their molecular weights. These chitinases were similar in cleavage pattern of *N*-acetylchitooligosaccharides, optimum pH and temperature, pH and thermal stability, and inhibition pattern by allosamidin. Kinetic analysis indicated that both chitinases are endo-type hydrolytic enzymes and prefer the longer substrates.

Furthermore, we found that the 88-kDa chitinase was processed to the 65-kDa chitinase and in turn to the 54-kDa protein during their storage. The *N*-terminal amino acid sequences of the 88-kDa and 65-kDa chitinases and the 54-kDa protein were all identical as ADSRARIVXYFSNWAVYRPG. These results suggest that *Bombyx mori* chitinases are processed from C-terminal side from the larger chitinases to the smaller chitinases or proteins during insect ecdysis.

By comparison of amino acid and nucleotide sequences, *B. mori* chitinases are classified into family 18 glycosyl hydrolases as well as *M. sexta* Chitinase.

Keywords: Chitinases, *Bombyx mori*.

Introduction

Insects have to degrade cuticular chitin, which is one of the main components of the integument, during the ecdysis. In order to clarify the mechanism of the insect ecdysis, we have investigated two kinds of chitinolytic enzymes such as chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) using the tobacco hornworm, *Manduca sexta*, and the silkworm, *Bombyx mori* (Dziadik-Turner *et al*, 1981; Koga *et al.*, 1982, 1983a and 1983b, Kramer and Koga, 1986). Chitinase and β-*N*-acetylglucosaminidase were induced in the integument by exogenous administration of 20-hydroxyecdysone to *B. mori* larvae: chitinase was induced by a high level of the hormone, whereas β-*N*-acetylglucosaminidase was induced even by a low level (Koga *et al.*, 1991). These results strongly suggest that chitinolytic enzymes are induced by 20-hydroxyecdysone *in vivo* for the onset of insect ecdysis.

Furthermore, the similarity in chitinolytic enzymes between *B. mori* and *M. sexta* were also investigated. The immunological analyses showed two types of chitinolytic enzymes are similar between both insects using polyclonal antibodies raised against the *B. mori* enzymes, respectively (Koga *et al.*, 1992). All these results suggest that the chitinolytic enzyme system is similar between *M. sexta* and *B. mori*. However, only *B. mori* chitinase has not been electrophoretically purified yet.

Therefore, we purified chitinases from integument of *B. mori*, and investigated the kinetic behavior, inhibition, partial amino acid and nucleotide sequences and some properties. Furthermore, we found the processing of these chitinases to the smaller one or protein during storage.

Materials and methods

Enzyme assay

Chitinase activity was measured using both glycolchitin and *N*-acetylchitooligosaccharides as the substrates. For the assay of glycolchitin, the reducing end group was measured colorimetrically at 420 nm with ferri-ferrocyanide reagent by the method of Imoto and Yagishita (1971). When *N*-acetylchitooligosaccharides were used, the reaction mixture was analyzed by HPLC using a Tosoh TSK Gel G2000 PW column by the method of Koga *et al.* (1990).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining.

SDS-PAGE was done by the method of Laemmli (1970). After electrophoresis, proteins were stained with Coomassie Brilliant Blue R250. Carbohydrates were stained by the periodic acid-Schiff (PAS) method according to Fairbanks *et al.* (1971).

Amino acid sequence

N-Terminal amino acid sequences were analyzed with a protein sequencer (Shimadzu PSQ2). Partial amino acid sequence was also analyzed using three main peptides that were obtained by digestion of a mixture of the 65-kDa and 88-kDa chitinases with trypsin and by reversed-phase HPLC with ODS Finepak Sil C18S.

Nucleotide sequence

The first stranded cDNA was prepared from the total RNA by using a Ready-To-GoTM T-Primed First-Strand kit (Pharmacia LKB). The cDNA encoding *B. mori* chitinase was amplified by polymerase chain reaction (PCR) using primers prepared by deduction from the analyzed amino acid sequences. The PCR product was subcloned into M13 mp18. The nucleotide sequence was determined by the method of Sanger *et al.* (1977) with a Hitachi DNA sequencer SQ-3000.

Results and discussion

Purification and properties of Bombyx mori *chitinases*

Two isozymes of chitinases were purified from the fifth-instar larval integument of *B. mori* by column chromatography of DEAE-Cellulofine A-500 hydroxylapatite, Butyl-Toyopearl 650M and Fractogel EMD DEAE 650(M). Their purities and molecular masses were investigated by SDS-PAGE. These isozymes were named 65-kDa chitinase and 88-kDa chitinase after their molecular masses. These molecular masses are similar to those of other insects. Both chitinases were glycoproteins. The 65-kDa and 88-kDa chitinases were purified about 558- and 262-fold with recoveries of 3.7 and 31.1%, respectively.

The optimum pHs toward the short substrate, $GlcNAc_5$, were pH 5.5 for the 65-kDa chitinase and pH 6.5 for the 88-kDa chitinase, whereas toward the long substrate, glycolchitin, both chitinases have high activity at pH 10 and low activity at pH 4, and their optimum pH was 8.0 in kcat/*K*m. This pH profile against glycolchitin was observed in that of *M. sexta* chitinase. (Koga *et al.*, 1983b). Regarding pH stability, the 65-kDa chitinase was stable from pH 5 to 11 at 4°C for 5 hr, while the 88-kDa chitinase was stable from pH 6 up to 12.

Both 65-kDa and 88-kDa chitinases showed high activities at 60°C. The 65-kDa chitinase was stable up to only 30°C, while the 88-kDa chitinase was stable up to 40°C. These results indicated that both chitinases were not so thermo-stable enzymes.

These and other properties of the 65-kDa and 88-kDa chitinases from *Bombyx mori* are summarized in Table I.

Kinetics of Bombyx mori *chitinases*

To investigate the enzymatic action, kinetic analysis was performed using the long substrate, glycolchitin, and the short substrate, $GlcNAc_5$. The double reciprocal plots are shown in Fig. 1, and the values of kinetic parameters obtained are also shown in Table I. The *K*m value of the 65-kDa chitinase is larger than that of the 88-kDa chitinase, meaning that the 88-kDa chitinase apparently has a two-fold higher affinity to glycolchitin than the 65-kDa chitinase. However, both chitinases are almost the same in the overall reaction (kcat/*K*m). When the short substrates, $GlcNAc_5$ and $GlcNAc_4$, were used, however, the negative slopes were obtained in the double reciprocal plots (Fig. 1). This is probably due to strong substrate inhibition, meaning that short substrates such as chitooligosaccharides are not real substrates *in vivo* but rather products for the chitinases. Therefore, their substrate specificities were instead investigated by measuring the initial velocities using *N*-acetylchitooligosaccharides from $GlcNAc_2$ to $GlcNAc_6$. The results are shown in Table II. Both

Table I. Properties of *Bombyx mori* Chitinases

	Bombyx mori	
	65-kDa chitinase	88-kDa chitinase
Molecular weight	65,000	88,000
Optimum pH (v_0, $GlcNAc_4$ or $GlcNAc_5$	5.5	6.5
Optimum pH (kcat/Km, glycol chitin)	8.0	8.0
Optimum temperature (°C)	60	60
pH stability	5-11	6-12
Temperature stability (°C)	to 30	to 40
Kinetics		
toward glycol chitin (pH 8, 32°C)		
K_m (mg/ml)	0.050	0.028
kcat (1/sec)	0.181	0.085
kcat/K_m (ml/mg/sec)	3.62	3.04
toward $GlcNAc_4$ and $GlcNAc_5$	endo-type substrate inhibition	endo-type substrate inhibition
Inhibition		
with 9 mM divalent cation	$HgCl_2$ (58%) $CuSO_4$ (43 %)	$HgCl_2$ (72%)
ID_{50} for allosamidin ($GlcNAc_5$)	3.5μM(pH5.5)	1.0μM(pH6.5)
ID_{50} for demethylallosamidin ($GlcNAc_5$)	2.5μM(pH5.5)	1.0μM(pH6.5)
K_i for allosamidin (glycolchitin, 32°C)	0.062μM (pH8)	0.023μM(pH8)
Antigenecity		
against anti-65kDa chitinase antibody	+	+
against anti-88kDa chitinase antibody	+	+
against anti-yam H chitinase antibody	+	+
N-terminal amino acid	Ala	Ala
Classification (Glycosyl hydrolase)	Family 18	Family 18

chitinases similarly cleaved *N*-acetylchitooligosaccharides in an endo-cleaving fashion except for $GlcNAc_2$, suggesting *B. mori* chitinases are endo-type chitinolytic enzymes. Furthermore, both chitinases prefer the longer substrates. The activity of the 65-kDa chitinase toward these *N*-acetylchitooligosaccharides was about 1.7-fold higher than that of the 88-kDa chitinase. These kinetic behavior are nearly the same as that of the *M. sexta* chitinases (Koga *et al.*, 1983b), suggesting that insect chitinase plays a role in the initiation of insect ecdysis by degrading cuticular chitin.

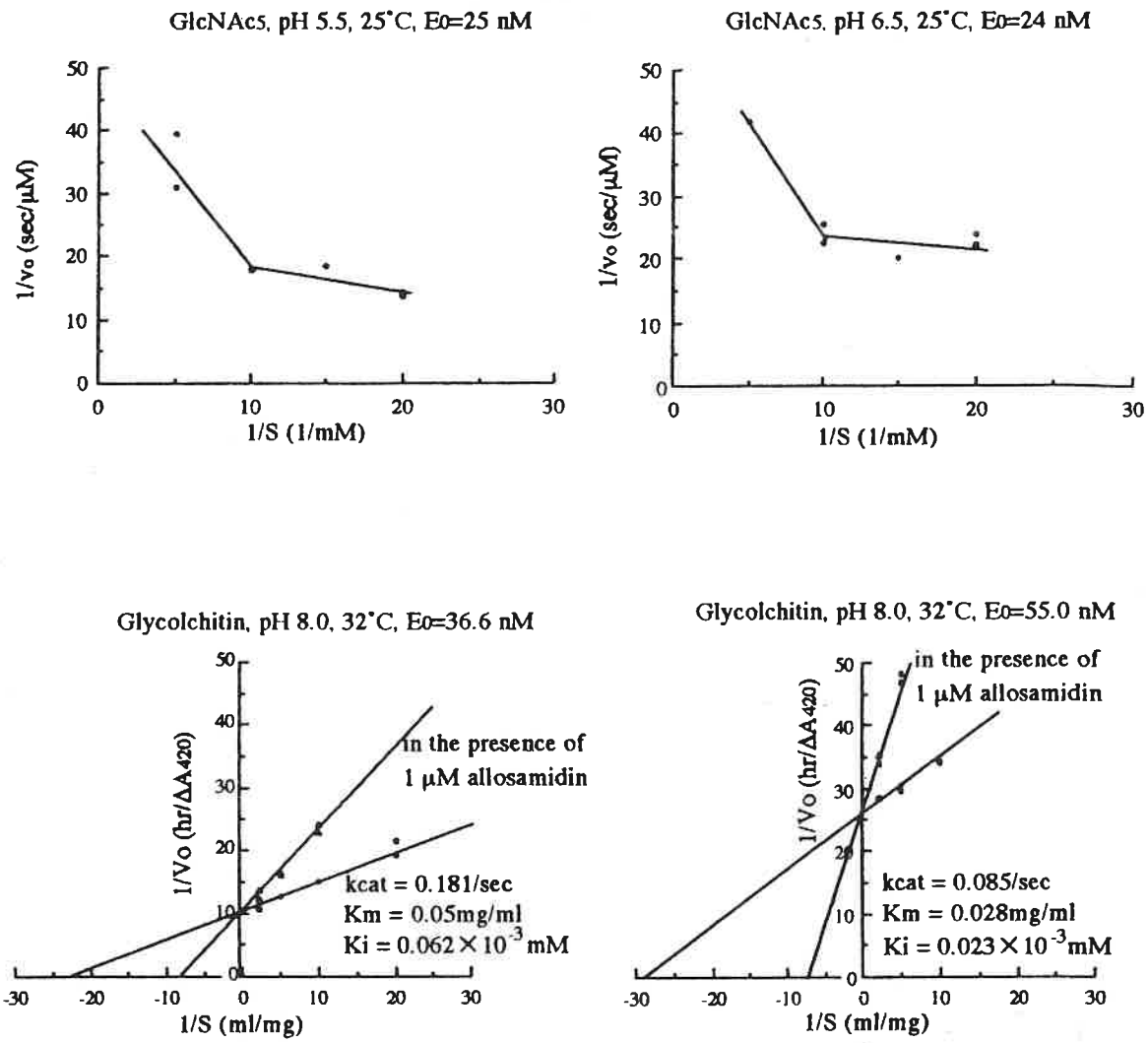

Fig. 1. Lineweaver-Burk Plots for *Bombyx mori* Chitinase.
Steady state-kinetic analysis were performed in the reaction of a short substrate, GlcNAc5, and a long substrate, glycolchitin, by the 65-kDa and 88-kDa chitinases.

Inhibition of Bombyx mori *chitinases by allosamidin*

The enzymatic reaction was done in the presence of allosamidin (0 to 1 mM) using glycolchitin as the substrate. The 65-kDa and 88-kDa chitinases were inhibited in a competitive fashion by allosamidin with *Ki* value of 0.062 and 0.023 μM at pH 8, respectively (Fig. 1).

Table II. Substrate Specificity of Bombyx mori Chitinases

Initial velocities were measured in the reaction of 0.1 mM*N*-acetyloligosacharides with 10 nM *Bombyx mori* chitinases in 50 mM sodium phosphate buffer, pH 5.5 for the 65-kDa chitinas or pH 6.5 for the 88-kDa chitinase at 25°C. The ratios of initial velocities to that for GlcNAc3 are shown in the parentheses and brackets.

Substrate	Reaction pattern	Initial velocity (nM/sec) 65-kDa chitinase		88-kDa chitinase	
GlcNAc2 (II)	No reaction	0		0	
GlcNAc3 (III)	III → II + I	3.4	(1)	2.0	[1]
GlcNAc4 (IV)	IV → 2 II	15.9	(4.7)	8.5	[4.3]
GlcNAc5 (V)	V → III + II	20.1	(5.9)	12.1	[6.1]
GlcNAc6 (VI)	VI →	25.2	(7.4)	14.1	[7.1]
	→ IV + II	16.5		9.0	
	→ 2 III	8.7		5.1	

I, GlcNAc

Processing of Bombyx mori *chitinases*

For storage of *B. mori* chitinases, the 65-kDa and 88-kDa chitinases were kept in ammonium sulfate-precipitated state at 4°C with several dialysis. After 3.5 months, SDS-PAGE indicated that the 88-kDa chitinase was processed a little to 65-kDa and 54-kDa proteins and the 65-kDa chitinase was processed to a 54-kDa protein. These all proteins were recognized by anti-88-kDa chitinase antibody. Furthermore, *N*-terminal amino acid sequences of these chitinases and the processed 54-kDa protein were analyzed and found to be identical as ADSRARIVXYFSN WAVYRPG, where X is an unidentified amino acid (Fig. 2). Considering these results together with the observation that the 88-kDa chitinase mainly appears at the early stage of one day before ecdysis, while the 65-kDa chitinase appears at the later stage, *B. mori* chitinases may be processed to the smaller chitinase *in vivo* from C-terminal side during insect ecdysis (Koga *et al.*, accepted). Such a processing mechanism might be related to the activation of chitinase (Koga *et al.*, 1989).

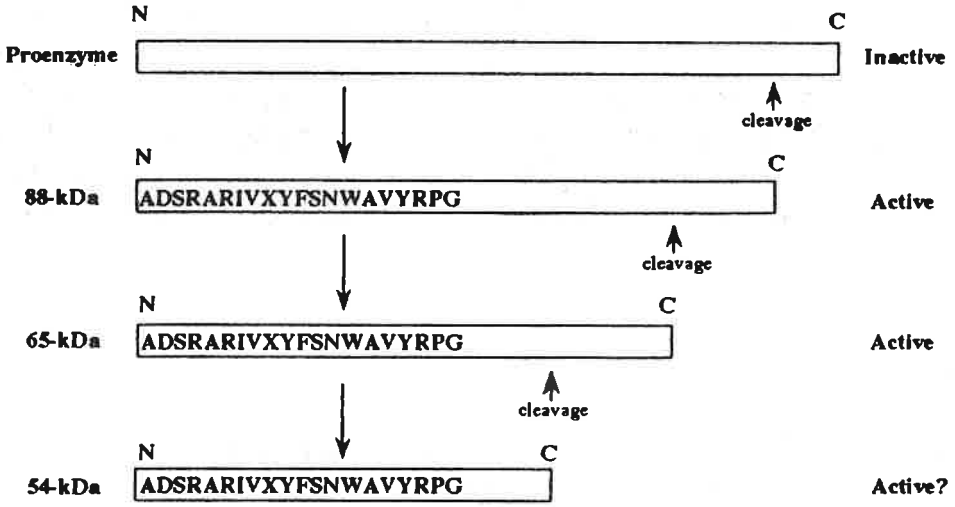

Fig. 2. Presumptive Scheme for Processing of *Bombyx mori* Chitinase.

Classification of Bombyx mori *chitinases*

To classify *B. mori* chitinases, the partial amino acid sequence and nucleotide sequence were analyzed (data not shown). By comparison with the sequence of *M. sexta* chitinase (Kramer *et al.* 1993), 6 amino acids are different from the corresponding 139 amino acids, and 49 nucleotides are different from the corresponding 278 nucleotides. The homology of *B. mori* chitinase to *M. sexta* chitinase in the region determined is 82.4% in nucleotide sequence and 95.7% in amino acid sequence. Two regions of amino acid sequence are identical to the conserved regions of the family 18 glycosyl hydrolases such as KXXX(S/A)XGG and (F/L/V/Y)(D/H)XXDXDXE, where X is a nonspecific amino acid (Terwissacha *et al.*, 1994). The homology between *B. mori* chitinases and *M. sexta* chitinases was previously suggested by the immunological study (Koga *et al.*, 1992). These results suggest that *B. mori* chitinases are classified into family 18 glycosyl hydrolases.

Conclusion

I. Two chitinases were purified from the integument of *Bombyx mori* by column chromatography. They are glycoproteins with molecular masses of 65-kDa and 88-kDa, and similar to each other in many properties. The 88-kDa chitinase has a two-fold higher affinity to glycolchitin than the 65-kDa chitinase. Both chitinases are endo-type chitinolytic enzymes and prefer the longer substrates.

II. The 88-kDa chitinase was processed to the 65-kDa chitinase and in turn to the 54-kDa protein from C-terminal side during storage. This suggests that *B. mori* chitinase is processed from the larger chitinase to the smaller one during insect ecdysis.

III. By comparison of amino acid sequence, *B. mori* chitinases are classified into family 18 glycosyl hydrolases as well as *M. sexta* chitinase.

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Purification and Properties of Chitinase from a Seaweed, *Gigartina mikamii*

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Abstract

A chitinase was purified from a seaweed, *Gigartina mikamii* (marine red algae), by ammonium sulfate fractionation and column chromatographies with DEAE Sepharose Fast Flow, Butyl-Toyopearl 650S, Macro-Prep Ceramic Hydroxyapatite, and DEAE-Toyopearl 650S. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis (PAGE), and its molecular mass was estimated to be 24kDa by SDS-PAGE. The isoelectric point was less than 3.5 by isoelectric focusing. The enzyme showed two optimum pHs at 3.5 and 9.0 using glycol chitin as the substrate. The optimum temperature was 60℃. The enzyme was stable after incubation at 60℃ for 60 min (pH6.2). The activity was inhibited by Mg^{2+} and Ag^{+}, and activated by Cu^{2+}, Ni^{2+}, and Sr^{2+}. The chitinase hydrolyzed glycol chitin and colloidal chitin, but not *p*Np-GlcNAc and *Micrococcus lysodeikticus*. The cleavage pattern was investigated using *N*-acetylchitooligosaccharides ($GlcNAc_n$, n=2 to 6). The enzyme hydrolyzed $GlcNAc_5$ to $GlcNAc_2$ plus $GlcNAc_3$, and $GlcNAc_6$ to two molecules of $GlcNAc_3$ (70%), and $GlcNAc_2$ plus $GlcNAc_4$ (30%). Hydrolysis of $GlcNAc_2$, $GlcNAc_3$, and $GlcNAc_4$ was not observed. These results suggest that this enzyme is an endo-type acidic chitinase.

Key words: Chitinase, acidic chitinase, seaweed, marine red algae, *Gigartina mikamii*, purification, properties, substrate specificity, cleavage pattern

Introduction

Chitin is a polysaccharide consisting of β-1,4 linked *N*-acetyl-D-glucosamine units and it is known to be a major component of exoskeleton of crustaceans and cell walls of fungi. Chitinase (EC 3.2.1.14) is known to decompose chitin to *N*-acetylchitooligosaccharides and to be distributed widely in nature. In marine organisms, it is distributed in the digestive organs and liver of fish [1,2], squid [3], and crustacean [4-8], in the crystalline style of shellfish [9,10], and in the saliva of octopus [11,12]. It is generally considered that the enzyme takes part in the digestion of chitin in the prey. Chitinase is also distributed in the blood of fish, and it is presumed that the chitinase plays a role in the defense toward fungus and parasites [13]. Furthermore, chitinase is distributed in the integument of crustaceans, and is known to be related to molting [4,14]. In marine organisms, the enzyme has been isolated from several fishes [15-18], crustaceans [5,6,8,19], octopus [12], and squid [20].

Recently, we also found the chitinase in seaweed [21], but the isolation of

chitinase from seaweed has not been done. In this paper, we describe the purification and properties of a chitinase from a seaweed, *Gigartina mikamii* (marine red algae).

Materials and Methods

Materials Marine red algae *Gigartina mikamii* (Gigartinales, Rhodophyta) were collected from shallow water on the shores of Katsuura (Chiba Prefecture in Japan). The red algae were stored in ice and then quickly transported to our laboratory, washed under running water, distilled water, and then preserved at –80℃.

Chemicals DEAE Sepharose Fast Flow was purchased from Pharmacia Biotech, Butyl-Toyopearl 650S and DEAE-Toyopearl 650S from Tosoh Co., and Macro-Prep Ceramic Hydroxyapatite from Bio-Rad Laboratories. Glycol chitin was purchased from Seikagaku Kogyo Co., and *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide and *Micrococcus lysodeikticus* were from Sigma Chemical Co.. All other reagents were of analytical grade.

Enzyme assay Chitinase activity was assayed at 37℃ for 30 min, as in the previous study [20], originally according to the method of Imoto and Yagishita [22] by measuring the amount of reducing sugar produced by the enzyme reaction with glycol chitin as substrate. Colloidal chitin hydrolyzing activity was assayed according to the method of Ohtakara [23] by measuring the amount of reducing sugar produced by an enzyme reaction. Measurement of hydrolytic activities toward *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and *Micrococcus lysodeikticus* were made by the method of Ohtakara [23], and Miura *et al.* [24], respectively. The enzymatic reaction for *N*-acetylchitooligosaccharides ($GlcNAc_n$) was made by the method of Koga *et al.* [25] with a slight modification. One hundred microliters of 50 mM sodium phosphate buffer (pH 6.7) containing 0.11 mM $GlcNAc_n$ (n=2 to 6) and 10 μl of the enzyme solution (0.4 μg of enzyme) were incubated for 5 min at 25℃. The reaction was stopped by boiling for 5 min. A 20 μl portion of the reaction mixture was injected into a Waters μ Bondasphere 5 μ NH_2 100Å column (3.9 x 150 mm) and eluted with 70% acetonitrile. The $GlcNAc_n$ eluted were monitored at 210 nm, and the concentration of each $GlcNAc_n$ was estimated from the peak height on the chromatogram compared with that of authentic $GlcNAc_n$.

Protein determination Protein concentration was determined by the method of Lowry *et al.* [26] with bovine serum albumin as a standard.

Purification of chitinase *Gigartina mikamii* (500 g) was homogenized with 2 volumes of 20 mM sodium acetate buffer (pH 6.0) followed by centrifugation at 7,000 x *g* for 20 min. The precipitate was further extracted with the same manner and the supernatant obtained was combined as a crude extract. The crude extract was saturated to 60% with ammonium sulfate, left for one day, and the produced precipitate was collected by centrifugation at 7,000 x *g* for 20 min. The precipitate was dissolved in a small volume of 20 mM sodium acetate buffer (pH 5.5) and dialyzed against the same buffer. The dialyzate was centrifuged at 10,000 x *g* for 20 min to remove insoluble material.

The supernatant was applied to a DEAE Sepharose Fast Flow column (2.6

x 29 cm) previously equilibrated with the same buffer. After the column was washed with the same buffer, and the absorbed protein was eluted by 0.3 M NaCl in the same buffer. Fractions containing chitinase activity were pooled and dialyzed against 20 mM phosphate buffer (pH 7.2) containing 20% saturation of ammonium sulfate. The dialyzate was applied to a Butyl-Toyopearl 650S column (1.6 x 17 cm) equilibrated with the same buffer, which was washed with the same buffer, and eluted with 20 mM phosphate buffer (pH 7.2). Fractions containing chitinase activity were pooled. After dialysis against 1 mM phosphate buffer (pH 6.8), the chitinase solution was put on a Macro-Prep Ceramic Hydroxyapatite column (1.6 x 10 cm) previously equilibrated with the same buffer. After the column was washed with the same buffer, the absorbed protein was eluted with 0.15 M phosphate buffer (pH 6.8). The main active fractions, eluted by 1 mM phosphate buffer (pH 6.8), were pooled and dialyzed against 20 mM phosphate buffer (pH 7.2) containing 20% saturation of ammonium sulfate. The dialyzate was applied to a Butyl-Toyopearl 650S column (1.6 x 14 cm) equilibrated with the same buffer, which was washed with the same buffer, and eluted by a linear gradient of ammonium sulfate from 20 to 0% saturation in the 20 mM phosphate buffer (pH 7.2). Chitinase fractions were pooled and dialyzed against 20 mM sodium acetate buffer (pH 4.5). The dialyzate was put on a DEAE-Toyopearl 650S column (1.0 x 12 cm) equilibrated with the same buffer and the enzyme was eluted with a linear gradient of NaCl from 0 to 0.2 M in the same buffer. The active fractions were rechromatographed by DEAE-Toyopearl 650S column in the same manner as described above.

Gel electrophoresis Polyacrylamide gel electrophoresis (PAGE) was done at pH 8.0 on 12.5% polyacrylamide gel by the procedure described by Williams and Reisfeld [27]. Sodium dodecyl sulfate (SDS)-PAGE was carried out in 12.5% polyacrylamide gel (Phast Gel, Pharmacia LKB) according to the manufacture's instructions (Phast Gel, Pharmacia LKB). The sample was previously heated for 5 min in 1 mM EDTA, 2.5% SDS, and 5% 2-mercaptoethanol. Low molecular weight calibration kit (Pharmacia LKB) was used as standard protein. Isoelectric focusing was performed on a flat-bed analytical electrofocusing apparatus in a thin layer polyacrylamide gel (Phast Gel, Pharmacia LKB) in the pH range 3.0 - 9.0. Isoelectric focusing calibration kit (pI 3.5 - 9.3, Pharmacia LKB) was used as standard protein. The proteins on the gels were stained with Coomassie brilliant blue R-250.

Results and discussion

Purification of chitinase A high content of mucilage and chromoprotein were observed in the crude extract of *Gigartina mikamii* . Therefore, selective removal of those were necessary for the purification of chitinase. The viscosity of the crude extract was reduced with ammonium sulfate fractionation and column chromatography of DEAE Sepharose Fast Flow. Most of chromoproteins were removed by column chromatographies (step elution) of Butyl-Toyopearl 650S and Macro-Prep Ceramic Hydroxyapatite. Resulting enzyme solution was added to Butyl-Toyopearl 650S column. As shown in Fig. 1A, two active peaks,

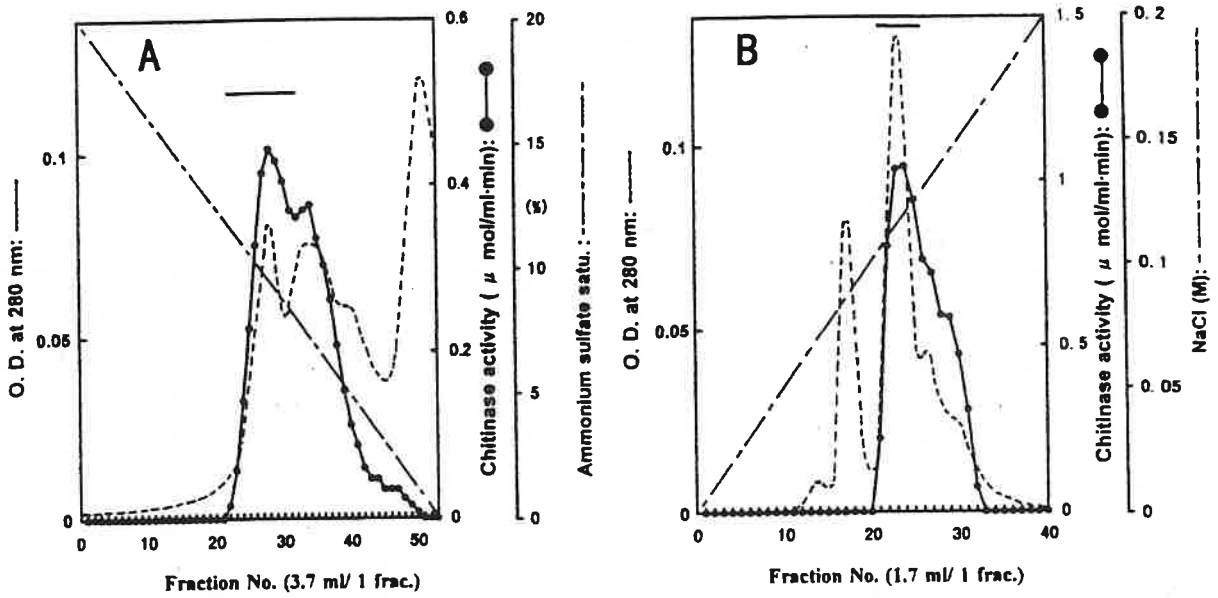

Fig. 1. Column chromatographies of *Gigartina mikamii* chitinase.
A, Butyl-Toyopearl 650S; B, DEAE-Toyopearl 650S.

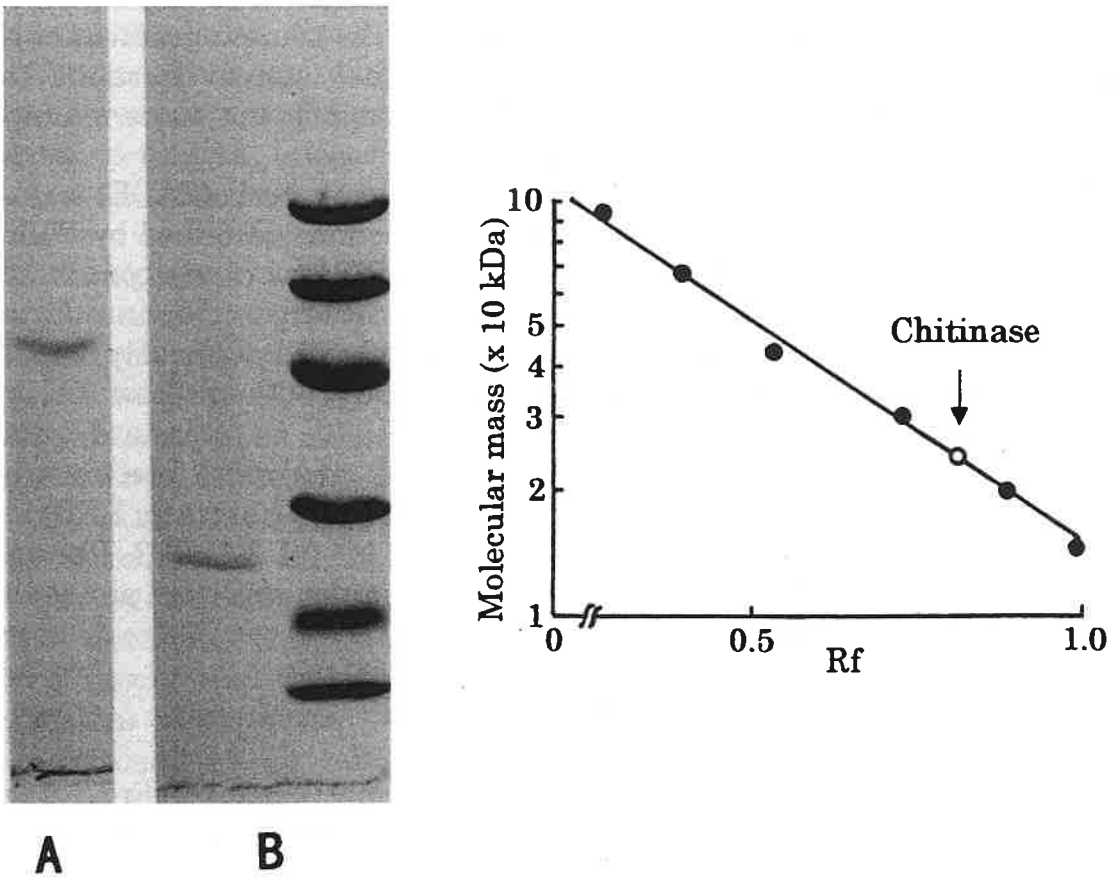

Fig. 2. Electrophoresis of *Gigartina mikamii* chitinase.
A, Native PAGE; B, SDS-PAGE.

around the position of 9% and 7% saturation of ammonium sulfate, were obtained. The active fractions around the position of 9% saturation, indicated by bar, were pooled. Active fraction was chromatographed on a DEAE-Toyopearl 650S column. As shown in Fig. 1B, main active fraction around the position of 0.12 M NaCl and small active fractions were obtained. The main active fraction, indicated by bar, were pooled and finally chromatographed on a DEAE-Toyopearl 650S column. In the present study, chitinase activity was fractionated into several active peaks by the column chromatographies. From those observations, it was considered that *Gigartina mikamii* has several isozymes of chitinase. We purified a main active peak of chitinase from those. The purified chitinase gave a single band on native-PAGE (Fig. 2A). Table 1 summarized the result of purification of *Gigartina mikamii* chitinase.

Table 1 Purification of chitinase from *Gigartina mikamii*

	Total Activity (μ mol/min)	Total protein (mg)	Specific activity (μ mol/mg·min)	Purification (-fold)	Recovery (%)
Crude extract	66.15	1,558	0.0425	.1	100
Ammonium sul. frac.	46.97	543.6	0.0864	2.0	71.0
DEAE Sepharose FF	41,63	237.8	0.1751	4.1	62.9
Butyl-Toyopearl 650S	39.50	113.3	0.3846	8.2	59.7
Macro-Prep C. H.	30.96	64.04	0.4834	11.4	46.8
Butyl-Toyopearl 650S	11.77	6.791	1.733	40.8	17.8
DEAE-Toyopearl 650S	7.94	0.682	11.64	273.9	12.0
DEAE-Toyopearl 650S	7.61	0.619	12.29	289.2	11.5

Properties of purified chitinase

Molecular mass and pI value The molecular mass of *Gigartina mikamii* chitinase was estimated to be 24 kDa by SDS-PAGE (Fig. 2B). The molucular masses of higher plant chitinase have been reported as follows: 23, 25 and 31 kDa for yam aeria tuber [28], 25 and 29 kDa for leaves of pokeweed [29], 26 and 33 kDa for seeds of rye [30], 28 kDa for cultured cells of *Wasabia japoica* [31], and 39.5 kDa for roots of carrot [32]. The value obtained in the present investigation was within the molecular mass range reported for higher plant chitinase. The isoelectric point of the chitinase, as determined by isoelectric focusing, was less than 3.5. Higher plant chitinase generally have very acidic or very basic isoelectric point. The isoelectric point of acidic chitinase so far reported are 3.4 for roots of carrot [32], 3.7 for leaves of pokeweed [29], and 3.8-4.05 for yam [33]. Therefore, the enzyme is a acidic chitinase, like that of acidic chitinases of higher plants.

Effect of pH and temperature The effect of pH on chitinase activity was examined using glycol chitin as substrate. As shown in Fig. 3A, the chitinase showed two optimum pHs at 3.5 and 9.0. This values were similar to the optimum pH values of 3.5 and 8.5 in yam [33], and 1.5 and 8.5 in the liver of

Japanese common squid [20]. The chitinase activity was measured at various temperatures. The chitinase was most active at temperature between 50 and 70℃, with an optimum temperature at 60℃ (Fig. 3B). The value was similar to that reported for cultured cell of *Wasabia japoica* between 50 and 60℃ [31]. With regard to thermal stability, on the other hand, the chitinase was stable after incubation at 60℃ for 60 min (pH 6.2) (Fig. 3C).

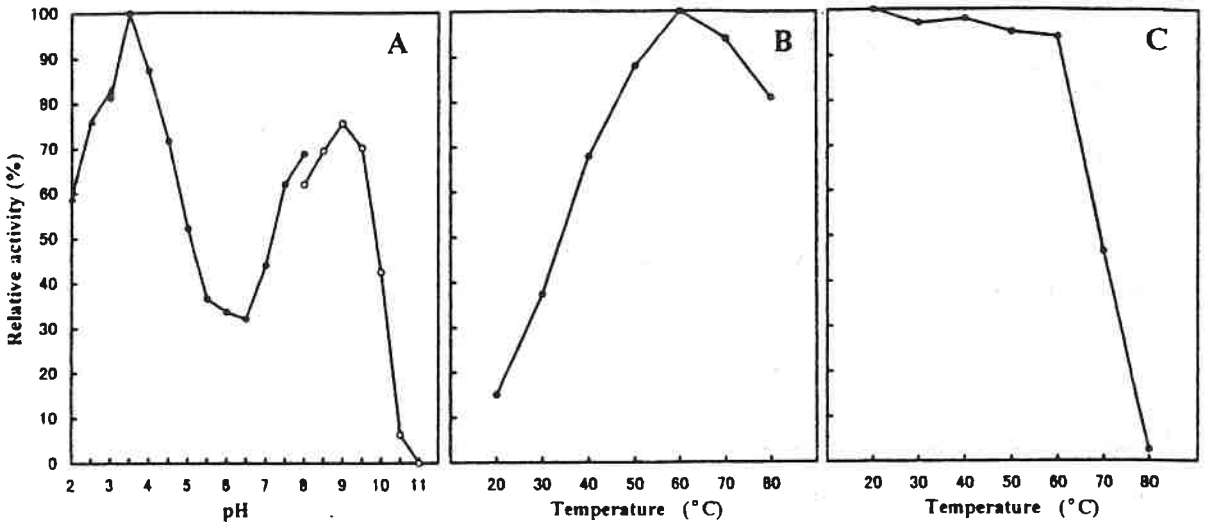

Fig. 3. Effect of pH and temperature on chitinase activities and stability. A, Effect of pH on chitinase activity was examined at 37℃ for 30 min incubation; B, Effect of temperature on chitinase activity was examined at various temperature for 30 min incubation at pH 4.0; C, Effect of temperature on chitinase stability. The enzyme solution was incubated at various temperature at pH 6.2 for 60 min. Remaining activity was measured.

Effect of metal ions The effect of metal ions on chitinase activity was shown in Table 2. The enzyme activity was inhibited by Mg^{2+} and Ag^{+}, and slightly activated by Na^{+}, Ca^{2+}, Hg^{2+}, Zn^{2+} and Al^{3+}, and strongly activated by Cu^{2+}, Ni^{2+} and Sr^{2+}. It was reported that chitinases in seeds of corn and *Coix lachryma-jobi* were inhibited by Cu^{2+}[34]. However, chitinases in marine organisms, such as common mackerel [17], red sea bream [15], and *Gigartina mikamii* were strongly activated by Cu^{2+}. According to the above comparison, activation of chitinase by Cu^{2+} seems to be peculiarity of chitinases of marine organisms.

Substrate specificity and cleavage pattern Substrate specificity of the chitinase was shown in Table 3. The enzyme showed activity against glycol chitin, which is a substrate of endo-type chitinase and lysozyme [35], and rapidly hydrolyzed colloidal chitin, which is a substrate of endo-type chitinase [23]. Neither *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and *Micrococcus lysodeikticus,* which are known to be the substrates of exo-type chitinase [36], nor lysozyme [24], were hydrolyzed by the enzyme obtained from *Gigartina mikamii.*

The enzymatic reaction on $GlcNAc_n$ (n=2 to 6) was shown in Table 4. The

Table 2. Effect of metal ions on chitinase activity

Ions	Relative activity (%)	Ions	Relative activity (%)
None	100	Pb^{2+}	96
Na^{+}	117	Cu^{2+}	151
Mg^{2+}	68	Zn^{2+}	119
Ba^{2+}	105	Ni^{2+}	143
Ca^{2+}	121	Sr^{2+}	140
Hg^{2+}	120	Al^{3+}	119
Cd^{2+}	99	Ag^{+}	51

Final concentration; 1 mM

Table 3. Substrate specificity of *Gigartina mikamii* chitinase

Substrate	Enzyme activity (μ mol/ml·min)		
	pH 4.0	pH 6.0	pH 8.0
Glycol chitin	0.284	0.109	—
Coloidal chitin	0.321	0.209	—
p-nitrophenyl *N*-acetyl-β-D-glucosaminide	ND	ND	ND
Micrococcus lysodeikticus (O. D. at 550 nm)	ND	ND	ND

Table 4. Rates and cleavage patterns of substrate hydrolysis by *Gigartina mikamii* chitinase

Substrate	Reaction	Initila velocity (μ M/min)	Cleavage pattern
$GlcNAc_2$ (II)	ND	0	
$GlcNAc_3$ (III)	ND	0	
$GlcNAc_4$ (IV)	ND	0	
$GlcNAc_5$ (V)	V ⟶ II + III	4.07	and/or G – G – G – G- G
$GlcNAc_6$ (VI)	VI ⟶	3.70	
	⟶ 2 III	2.59 (70%)	G – G – G – G – G - G
	⟶ II + IV	1.11 (30%)	and/or G – G – G – G – G - G

chitinase cleaved $GlcNAc_5$ to $GlcNAc_2$ plus $GlcNAc_3$, and $GlcNAc_6$ to two molecules of $GlcNAc_3$ (70%) and $GlcNAc_2$ plus $GlcNAc_4$ (30%). Hydrolysis of $GlcNAc_2$, $GlcNAc_3$, and $GlcNAc_4$ was not observed. It is reported that the prawn liver chitinase is a typical endo-type enzyme that hydrolyzes $GlcNAc_6$ either to $GlcNAc_2$ plus $GlcNAc_4$ (87%), or to two molecules of $GlcNAc_3$ (13%) [37]. The hydrolysis patterns of common squid liver chitinase against $GlcNAc_n$ (n=2 to 6) correspond almost perfectly to those of prawn liver chitinase [20]. On the other hand, yam chitinase, random-type enzyme, hydrolyzes $GlcNAc_6$ by three ways to GlcNAc plus $GlcNAc_5$ (32%), $GlcNAc_2$ plus $GlcNAc_4$ (42%) and two molecules of $GlcNAc_3$ (26%) [38]. These results clearly indicated that the chitinase obtained in the present investigation from *Gigartina mikamii* was an endo-type chitinase.

In conclusion, our present study strongly indicates that the properties of chitinase in red alga *Gigartina mikamii* are almost the same as those of the endo-type acidic chitinases in higher plants. Moreover, we observed the presence of several isozymes of chitinase in the alga. Higher plant chitinses are considered to protect plant against fungal pathogens by degrading chitin, a major constituent of the cell walls of many fungi [39]. Red alga lacks chitinous substance as architectural components. It is considered that the chitinase in the *Gigartina mikamii* may plays a role in self-defense same as higher plants.

Acknowledgment

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KINETICS OF HYDROLYSIS REACTION OF GLYCOL CHITIN WITH A NOVEL ENZYME IMMOBILIZED THROUGH NONIONIC SURFACTANT ADSORBED ON SILICA GEL

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Abstract

As a novel procedure to immobilize enzyme, lysozyme and α-chymotrypsin were dissolved into the molecular assembly of nonionic surfactant, Triton X-100 and Triton X-405, adsorbed on silica gel. The adsorption isotherms of the surfactants on silica gel and of enzymes into the molecular assembly of adsorbed surfactants on silica gel were obtained. The isotherms for the latter was significantly influenced by pH or buffering agents, and was affected by the ionic strength of buffering agents. The leakage of lysozyme immobilized was influenced by pH and temperature; it was found that much lysozyme leaked when pH and temperature was low. However, under a limited condition the immobilized enzyme was very stable. The immobilized lysozyme was used in the enzymatic hydrolysis reaction of a water-soluble chitin derivative, glycol chitin. The enzymatic reaction obeyed the Michaelis-Menten mechanism and the values of kinetic parameter, Michaelis constant K_m and maximum velocity V_{max}, were estimated. The apparent activation energy E_a was also obtained.

Keywords; Glycol chitin, hydrolysis, lysozyme, α-chymotrypsin, silica gel, nonionic surfactant, molecular assembly, adsorption

Materials and methods

Silica gel 60 as a support, whose diameter is 38-63 μ m (230-400 mesh), was used without any treatment. Nonionic surfactants, Triton® X-100 (polyoxyethylene octylphenyl ether, C_8H_{17}-C_6H_4-$(OCH_2CH_2)_{9\text{-}10}$-OH; TX-100), and Triton X-405 (C_8H_{17}-C_6H_4-$(OCH_2CH_2)_{40}$-OH; TX-405) of reagent grade were used without further purification. As the enzymes, lysozyme chloride from hen egg white and α-chymotrypsin from bovine pancreas of guaranteed reagent grade were used. As a raw material for synthesizing the substrate of enzymatic reaction, water-soluble glycol chitin, chitin from crab shells of reagent grade was used. Cellulose dialyzer tubing that has 8,000 M. W. cutoff, 32 mm diameter, and 0.02 mm thickness was also used. Phenol reagent(acidity 1.8N) for protein quantification of reagent grade and copper (Ⅱ) sulfate pentahydrate of guaranteed reagent grade were used. Citric acid monohydrate, di-sodium hydrogenphosphate 12-water, sodium hydroxide, and 2-chloroethanol, acetic acid, and acetic anhydride for preparing the glycol chitin were all of guaranteed reagent grade.

In the case of TX-100 as the surfactant, immobilization of lysozyme onto silica gel was carried out as follows. Silica gel (2.7g) and 0.05 mol/dm^3 TX-100 aqueous solution (20 ml) were mixed and shaken lightly in a tightly sealed glass vial at room temperature for 3 minutes. Then 15 ml of 10 kg/m^3 lysozyme aqueous solution was added into the vial, and shaken

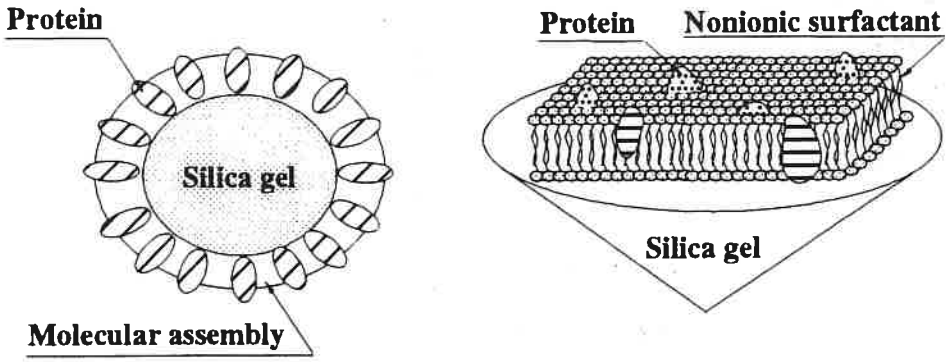
overnight at 303K. As a buffer solution, 0.1 mol/dm^3 citric acid and 0.1 mol/dm^3 di-sodium hydrogenphosphate were used to control pHs. The mixture was filtrated with a filter paper (ADVANTEC No. 3), and washed carefully with distilled water. The immobilized enzyme prepared was kept in the distilled water at 4℃.

Measurement of adsorption quantity of the surfactants onto silica gel was carried out using UV-VIS spectroscopy (Shimadzu UV-1200) at λ = 280 nm. Determination of adsorption quantity of the protein in the presence of TX-100 was practiced based on the manner proposed by Sugawara and Soejima [1] where Lowry method was modified as described below. Solutions were prepared by dissolving 2g of sodium carbonate into 100 ml of 0.36N NaOH aqueous solution (solution 1), and by adding 0.25g of $CuSO_4 \cdot 5H_2O$ to 50 ml of 10 kg/m^3 sodium tartrate aqueous solution (solution 2), and 50 ml of the solution 1 and 1 ml of the solution 2 were mixed (solution 3). Then 1 ml of the filtrate in the immobilization process and 5 ml of the solution 3 were mixed and stood for 10 minutes at room temperature. To which, then 1 ml of phenol reagent was added and the mixture solution stood for 25 minutes at room temperature. And this solution was analyzed using UV-VIS spectroscopy at λ = 760 nm. The protein content was determined by a calibration curve made by using lysozyme chloride preliminarily.

Enzymatic reactions were carried out according to our previous report [2]. As the substrate of enzymatic reaction, water-soluble chitin derivative, that is, glycol chitin was synthesized [3]. The substrate solution was prepared using a buffer solution composed of 0.1 mol/dm^3 citric acid and 0.1 mol/dm^3 di-sodium hydrogenphosphate, whose pH was adjusted. One g of the immobilized enzyme was added into the substrate solution (7 ml) in test tubes sealed tightly with a screw cap, then the reaction was started in a shaking water-bath at 310K. Here enzyme concentration in the reaction medium was 2.0 g-protein/dm^3. Enzyme assay was conducted by modified Schales method [4] using UV-VIS spectroscopy at λ = 418.5 nm, which determines the reducing sugar formed, where monomer of chitin (N-acetyl-D-glucosamine, GlcNAc) was used as the standard.

Results and discussion

The structure of the immobilized enzyme is assumed to be shown in **Scheme 1**, which is similar to the fluid mosaic model of biomembrane.



Scheme 1 Schematic representation of proposed system

Fig. 1 shows the adsorption isotherms of TX-100 and TX-405 onto silica gel at 303K. The curves of the adsorption isotherm showed the rapid increasing of amount of TX-100

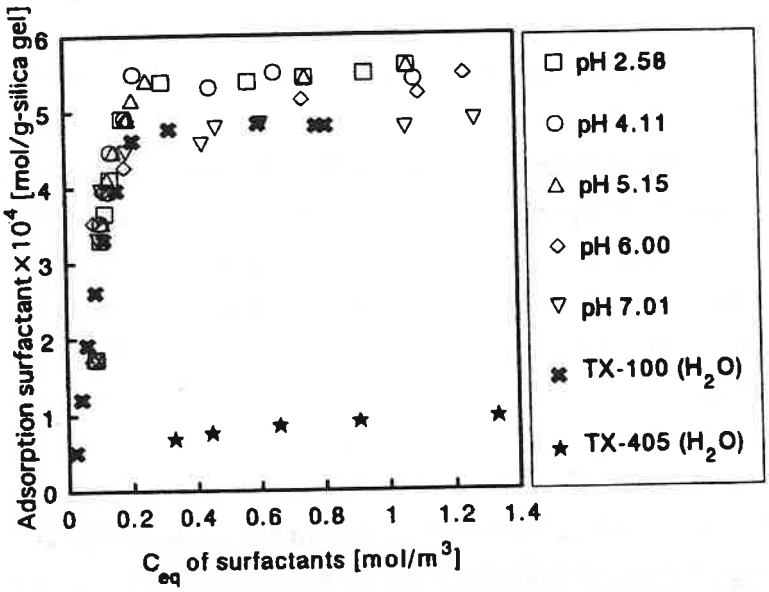


Fig. 1 Adsorption isotherms of TX-100 and TX-405 on silica gel at 303K

adsorbed at the low equilibrium concentration and reached plateaus when the equilibrium concentration was about 0.3 mol/m^3. This equilibrium concentration is due to the high affinity of TX-100 and silica gel. The maximum amount of TX-100 adsorbed on silica gel was scarcely affected by pH at the acidic region, however, at pH 7.01 the saturated amount of TX-100 on silica gel decreased to 88% the value of that at the low pH region. Generally the interaction between the hydrophilic group of TX-100 and silica gel surface is mainly due to hydrogen bonding. The isoelectric point of silanol groups on the silica gel is 3.0. Accordingly when the pH of bulk solution is high, the surface of silica gel would be a state of Si-O$^-$ shown in Eq. (1).

$$\text{Si-OH} + \text{OH}^- \rightleftharpoons \text{Si-O}^- + \text{H}_2\text{O} \qquad (1)$$

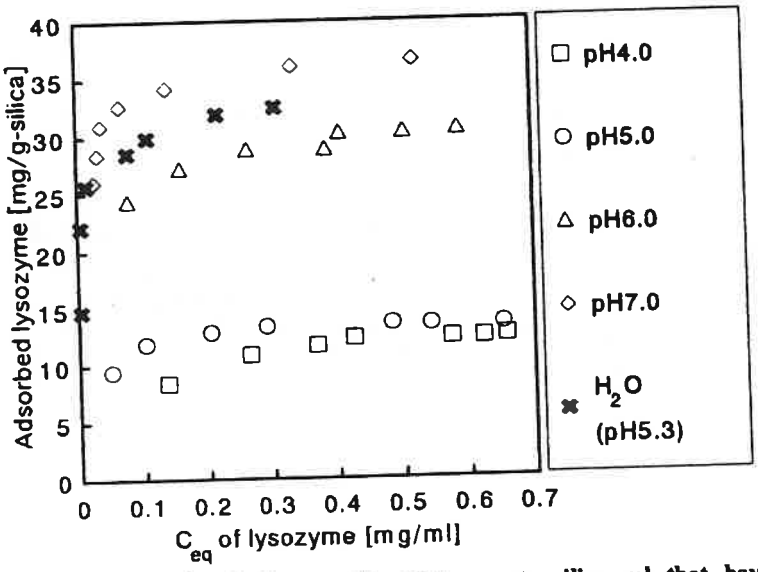


Fig. 2 Adsorption isotherms of lysozyme onto silica gel that have adsorbed TX-100 for various pHs at 303K

As a result, ability of the hydrogen bonding is low and the amount of TX-100 adsorbed was decreased when pH is high. In non pH-controlled medium, the amount of TX-405 adsorbed onto silica gel was 20% the value of that of TX-100. This is due to the molecular size of the surfactant, that is, TX-405 has a larger occupied area on the silica gel surface.

Fig. 2 shows the pH dependences of adsorption isotherm of lysozyme on the bilayer formed by TX-100, together with the case when the pH of bulk solution was not controlled. It was found that the adsorption of lysozyme was influenced significantly by pH, and it could be estimated roughly that the maximum amount of lysozyme adsorbed in the pH-controlled medium (symbol ○ in Fig. 2) was about 42% the amount of that in the non pH-controlled system (symbol × in Fig. 2). In contrast to the adsorption of TX-100 on silica gel, the amount of lysozyme adsorbed increased with increasing in pH. To study the influence of ionic strength on the adsorbed amount suggested in Fig. 2, the effect of ionic strength of the buffering agents on adsorption isotherms of lysozyme into the bilayer of TX-100 on silica gel surface was examined. The results are shown in **Fig. 3**. Here the pH of bulk solution was 5.0. The adsorption of lysozyme was slightly affected by the concentration of buffering agents. The maximum amount of lysozyme adsorbed had a tendency to increase with decreasing in the ionic strength. In the case of the pH-controlled system the maximum amount of lysozyme adsorbed was from 42 to 56% the amount adsorbed in the non pH-controlled medium as was expected. This is due to the decreasing in the ability of hydrophobic interaction by an electrolyte such as Na^+ ion. Accordingly, we prepared the immobilized enzyme without controls of pH and ionic strength. In the case of α-chymotrypsin, the influence of ionic strength was shown in **Fig. 4**. When we compare Fig. 3 with Fig. 4, it is found that the amounts of lysozyme adsorbed in the non pH-controlled environment is about 3.4 times as much as α-chymotrypsin.

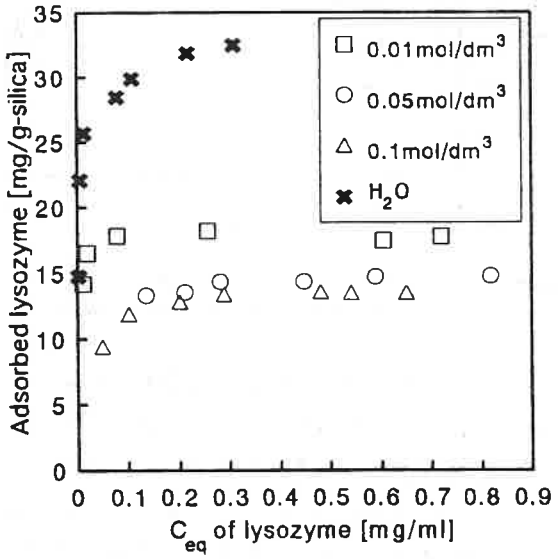


Fig. 3 Effect of ionic strength of buffering agents on adsorption isotherm of lysozyme at 303K

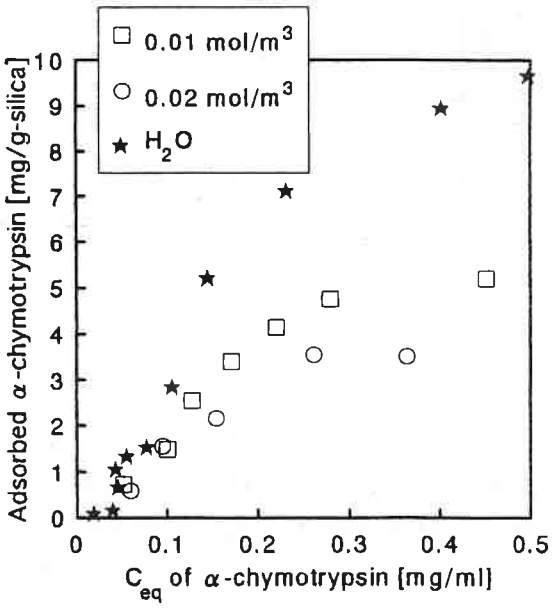


Fig. 4 Effect of ionic strength of buffering agents on adsorption isotherm of α-chymotrypsin at 303K

Fig. 5 shows the pH dependences of the leakage of lysozyme from the immobilized one. The leakage of protein was influenced remarkably by both temperature and pH. In regard to pH, almost no leakage of protein was observed in the region from neutral to basic pHs at all

temperatures investigated. On the other hand, more lysozyme leaked at the acidic pH. It was found that in the region from neutral to basic pHs, the leakage of protein did not occur for one week as far as we have investigated. It was also found that more lysozyme leaked at a lower temperature. These results are contrast to the general thought that adsorption is an exothermic process and is facilitated at a lower temperature. If a formation constant of the hydrophobic associate is defined as K_f, it can be expressed using free energy as Eq. (2).

$$R \ln K_f = -\frac{\Delta G^\circ}{T} = -\frac{\Delta H^\circ}{T} + \Delta S^\circ \qquad (2)$$

Because the hydrophobic interaction is based on a strong aggregation force of water molecules, both enthalpy effect and entropy effect are significant. When the hydrophobic association is strong, ΔH° must have much negative value or ΔS° must be enough large positive value. Accordingly even if ΔH° is a positive value, K_f is increased due to a large positive value of ΔS°, which leads to the increase of K_f with increasing in temperature. This manner is opposite to temperature dependence of K_f for general cases. The stabilization of the associated lysozyme at high temperature region affords a proof that lysozyme is firmly adsorbed by the hydrophobic association with the aliphatic area of nonionic surfactant bilayer. We are encouraged to conclude that lysozyme is not entrapped on the hydrophilic surface of bilayer but is embedded into it.

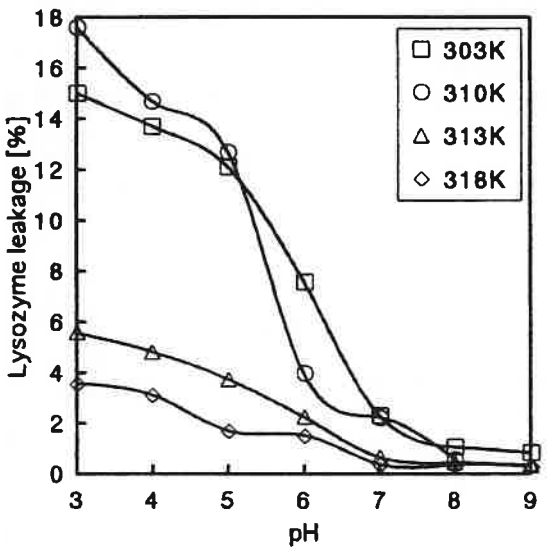


Fig. 5 pH Dependences of lysozyme leakage at various temperatures

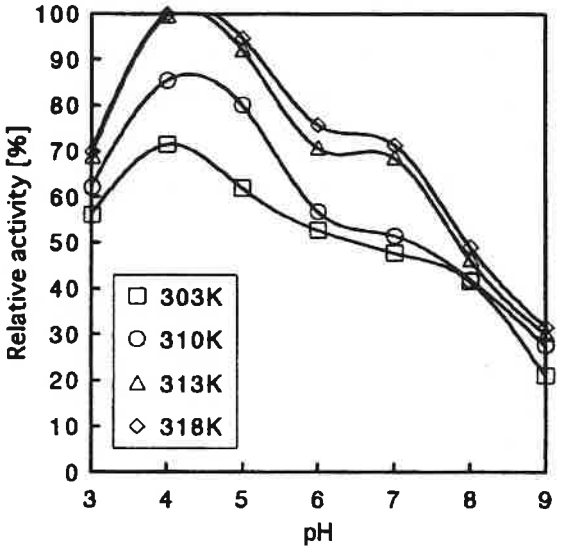


Fig. 6 pH Dependences of relative activity of immobilized lysozyme at various temperatures

Fig. 6 shows the pH dependences of activity of the immobilized lysozyme in the enzymatic reaction with glycol chitin as the substrate. Where the relative activity in the vertical axis was defined as the largest activity obtained in the optimum pH at 318K to be 100%. It is clear that the optimum pH of this enzymatic reaction is to be 4.0 regardless of temperature. This optimum pH is similar to that of free lysozyme, pH 4.2 [2]. **Fig. 7** shows the time course of the reactivity of the immobilized lysozyme prepared. The reactivity of the enzyme at pH 4.0 increased rapidly and arising of the curve was much steeper at about 3h. This is owing to the leakage of lysozyme. **Fig. 8** shows the relationship between the initial reaction rate of immobilized lysozyme and the substrate concentration for various temperatures. Lineweaver-Burk plot at various temperatures based on Eq. (3) is shown in **Fig. 9**.

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]_0} + \frac{1}{V_{max}} \qquad (3)$$

As a result of **Figs. 8** and **9**, it was found that the reaction kinetics of this enzymatic reaction

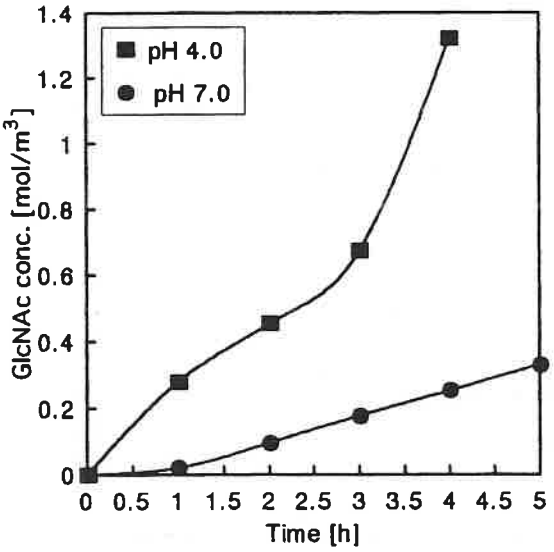


Fig. 7 Time courses of reactivity of immobilized lysozymes. Substrate; Glycol chtin, Reaction temperature; 310K

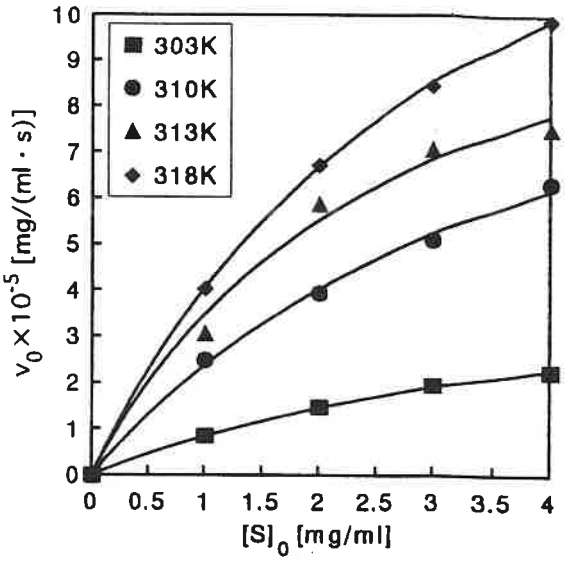


Fig. 8 Relationship between initial rate of enzymatic reaction and concentration of substrate at various temperatures

obeyed Michaelis-Menten mechanism. The kinetic parameters, the maximum velocity and Michaelis constant, were estimated from Lineweaver-Burk plot at various temperatures and shown in **Table 1**. There was not any temperature dependence of K_m, that is, the affinity between the substrate and enzyme was not affected by temperature. Solid lines in **Fig. 8** were the calculated ones by these constants.

Table 1 Values of maximum velocity and Michaelis constant

	K_m [kg/m^3]	V_{max} [kg/(m^3 · s)]
303K	5.29	5.29×10^{-5}
310K	3.69	1.15×10^{-4}
313K	4.85	1.83×10^{-4}
318K	3.76	1.92×10^{-4}

Fig. 10 shows the Arrhenius plots for the immobilized lysozyme and free lysozyme. The apparent activation energies of the enzymatic reaction were calculated to be 71.4 and 98.9 kJ/mol for the immobilized lysozyme and the free one [2], respectively. The reason why the activation energy of immobilized lysozyme is smaller than free lysozyme is considered to be the difference in circumstances where the enzyme exists. Briefly the free lysozyme exists in a complete aqueous medium, and to the contrary the lysozyme immobilized into TX-100 bilayer adsorbed on silica gel particle exists in hydrophobic area formed by the single molecular chain of hydrocarbon of nonionic surfactant. This difference does not contribute to the increase in the affinity between enzyme and substrate, but does to the lowering of the energy barrier over the transition state that is accompanied by the formation of enzyme-substrate complexes. It is

known that oligomeric protein and membrane protein are combined hydrophobically with TX-100 without change in the higher structure of proteins. Accordingly lysozyme is supposed to be immobilized keeping its very stable higher structure. Under these immobilization states, since the enzyme molecules exist in the hydrophobic region, the active site and/or the surface site of the protein were changed.

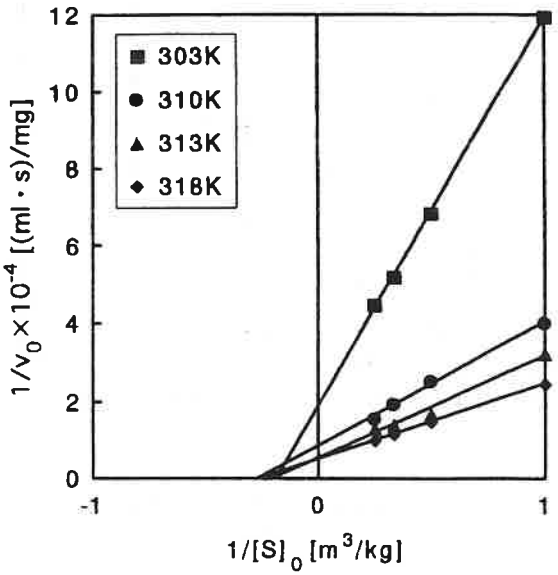


Fig. 9 Lineweaver-Burk plot

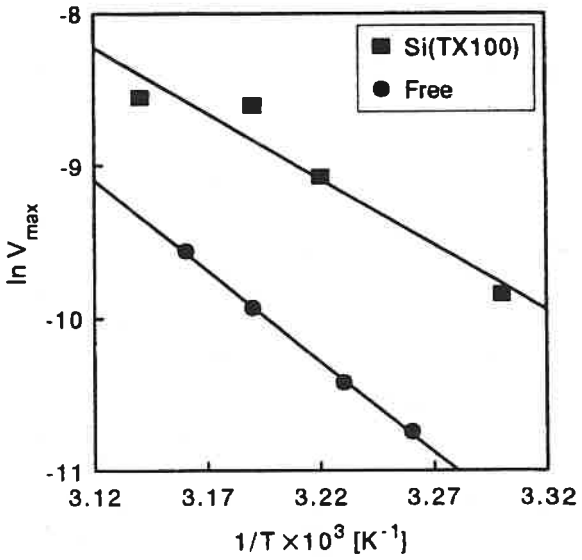


Fig. 10 Arrhenius plot

Conclusion

Lysozyme and α-chymotrypsin were immobilized into the bilayer of nonionic surfactant, Triton X-100 or Triton X-405, adsorbed on silica gel. The adsorption isotherms of the surfactants on silica gel and of lysozyme or α-chymotrypsin into the bilayer of adsorbed Tritons on silica gel were obtained. The isotherms investigated here were influenced by pH and ionic strength. Moreover, the leakage of lysozyme immobilized was influenced by pH and temperature, so lysozyme is considered to be embedded into the molecular assembly of the nonionic surfactants. The immobilized lysozyme prepared was used in the enzymatic hydrolysis reaction of the water-soluble chitin derivative, glycol chitin. The enzymatic reaction obeyed Michaelis-Menten mechanism and values of kinetic parameters, Michaelis constant K_m and maximum velocity V_{max} were estimated. Moreover, the apparent activation energy E_a was obtained to be 71.4 kJ/mol, which is lower than that of free lysozyme. It is expected that lysozyme can exist stably in the aliphatic region of the bilayer.

The studies on novel immobilization methods and mechanism of enzymatic reaction are still being developed.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF MICROBIAL CHITOSANASES

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ABSTRACT

Chitosanase from *Bacillus circulans* MH-K1 was overexpressed in *B. brevis* 47-5Q, purified and crystallized by vapor-diffusion procedure at 20°C using ammonium sulfate as a precipitant. Rod-shaped colorless crystals appeared after 24 h and grew to 0.1 x 0.3 x 1.2 mm within a week. They belong to the orthorhombic space group $P2_12_12$ with unit-cell dimensions of a =43.3Å, b =128.0Å, and c =57.7Å. X-ray diffraction data were collected at 1.7 Å resolution using synchrotron radiation. The crystal structure is now being solved by the multiwavelength anomalous diffraction (MAD) phasing method on the basis of a platinum derivative.

Actinomycetes chitosanases purified from *Nocardioides* sp. K-01 and *Amycolatopsis* sp. CsO-2 were also crystallized by vapor-diffusion at 20°C from a polyethyleneglycol solution (PEG 6,000 and $CaCl_2$ in Tris-HCl). Colorless crystals appeared within two weeks.

Keywords: microbial chitosanase, *Bacillus circulans* MH-K1, *Nocardioides* sp. K-01, *Amycolatopsis* sp. CsO-2, crystallization, X-ray crystallography

INTRODUCTION

Chitin is an abundant biopolymer of β-(1,4)-linked *N*-acetyl-D-glucosamine (GlcNAc) which is hydrolyzed by chitinase (EC 3.2.1. 14). Chitinase is one of the key enzymes in plant defense system against fungal infection [1, 2]. It is widely distributed in micro-organisms and plants, with many of their primary structures having been reported. They are classified into either families 18 or 19 in glycosyl hydrolases, which have 62 families [3-5]. The chitinases in family 19 are similar in three-dimensional structure

to the well studied glycosyl hydrolase, lysozyme, but differ in specificity. Lysozyme can split the linkage between GlcNAc and GlcNAc and between GlcNAc and *N*-acetyl-D-muramic acid.

On the other hand, chitosanase (EC 3.2.1.132) is the enzyme that hydrolyzes chitosan, a polymer of D-glucosamine (GlcN) which is produced by partial (over 60%) or full deacetylation of chitin. Most of chitosanases are found in microorganisms, and a few in plants [6-9]. Seven complete primary structures of chitosanases have been reported from *B. circulans* MH-K1 (MH-K1 chitosanase) [10], *Streptomyces* sp. N174 (N174 chitosanase) [11], *Nocardioides* sp. N106 (N106 chitosanase) [12], *Fusarium solani* f. sp. *phaseoli* SUF386 [13], chlorella virus PBCV-1 [14], chlorella virus CVK2 [15], and *Nocardioides* sp. K-01 (K-01 chitosanase) [unpublished results]. In addition, two partial sequences have been obtained from *Amycolatopsis* sp. CsO-2 (CsO-2 chitosanase) [unpublished results], *Pseudomonas* sp. A-01 [unpublished results]. No sequence similarities were found between chitosanases and chitinases, even though they hydrolyze substrates which are different only at the acetyl group on the sugar-C2 position. In order to understand differences in catalytic mechanisms among chitosanase, chitinase, and lysozyme, determination of their three-dimensional structures is indispensable. While the crystal structures of chitinases from *Hordeum vulgare* [16-18], *Hevea brasiliensis* [19] and *Serratia marcescens* [20] are now available, only that of N174 chitosanase has been reported [21].

Here, the crystallization and preliminary crystallographic study of three microbial (MH-K1, K-01, and CsO-2) chitosanases is reported along with the progress of the X-ray crystallographic analysis of the MH-K1 chitosanase structure determination.

CHARACTERISTICS OF MICROBIAL CHITOSANASES

Even though *Nocardioides* sp. K-01 and *Amycolatopsis* sp. CsO-2 belong to mesophile actinomycetes, K-01 and CsO-2 chitosanase exhibit thermal stability (Table 1) [22-24]. As for K-01 chitosanase, about 50% of the initial activity remains after heating at 60°C for 15 min, and as for CsO-2 chitosanase, about 50% of that remains after same treatment at 70°C.

Table 1. Enzymatic properties of MH-K1, K-01, and CsO-2 chitosanases

	MH-K1	K-01	CsO-2
Molecular Weight	29,024	27,000 (SDS-PAGE)	27,000 (SDS-PAGE)
Optimum pH	6.5	5.0	5.3
Optimum Temp.	50°C	60°C	55°C
Stable Range of pH	4.0 ~ 9.0	4.5 ~ 6.0	4.5 ~ 6.0
Stable Range of Temp.	0 ~ 50°C	0 ~ 60°C[#1]	0 ~ 70°C[#2]
pI	9.3	9.0	8.8

#1 Residual enzyme activity is 50% after incubating at 60°C for 15 min.

#2 Residual enzyme activity is 50% after incubating at 70°C for 15 min.

ACTION PATTERN OF CHITOSANASES ON CHITOSAN

An attempt to subdivide microbial chitosanases into three subclasses, based on the action pattern of chitosanases on partially acetylated chitosan has been reported [12]. Chitosanases, such as N174 chitosanase [25], which can split GlcN-GlcN and GlcNAc-GlcN linkages are in subclass I, those which can split only GlcN-GlcN linkage, such as *Bacillus* sp. No.7-M chitosanase [26], are in subclass II, and those, such as MH-K1 chitosanase [27], which can split GlcN-GlcN and GlcN-GlcNAc linkages are in subclass III.

The action pattern of K-01 and CsO-2 chitosanases have not been investigated yet.

PRIMARY STRUCTURE OF MICROBIAL CHITOSANASES

A highly conserved sequence segment is found in the N-terminal region of the prokaryotic chitosanase, as well as a significant similarity of the whole sequence among five chitosanases excluding MH-K1 chitosanase (Fig. 1). The analysis of N174 chitosanase by site-directed mutagenesis has revealed that Glu 22 and Asp 40, localized within the conservative N-terminal region, are essential for catalytic activity [28]. As for MH-K1 chitosanase, Asp 55 corresponding to Asp 40 in N174 chitosanase is thought to be one of the catalytic residues [unpublished results].

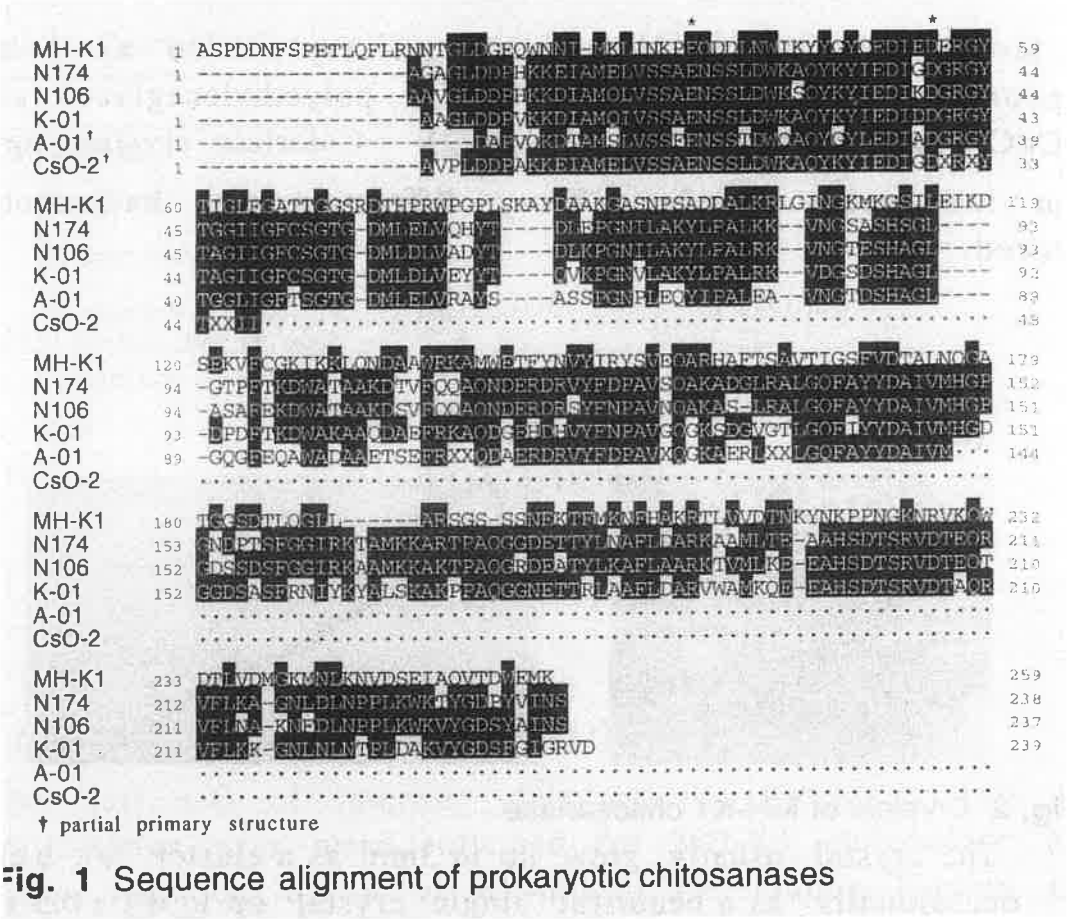

Fig. 1 Sequence alignment of prokaryotic chitosanases

PURIFICATION OF MICROBIAL CHITOSANASES

The hyper expression system was designed by inserting MH-K1 chitosanase gene into the plasmid pNU210 (named pNUE) by which was transformed *B. brevis* 47-5Q [29], a protein secreting bacterium [unpublished results]. Using this system, MH-K1 chitosanase is much more obtainable than using that of *Escherichia coli.* MH-K1 chitosanase produced by *B. brevis* 47-5Q/pNUE was purified in the usual manner [22].

K-01 and CsO-2 chitosanases were purified from native sources as previously described [23, 24]. Construction of hyper expression systems of these chitosanase genes is in progress.

CRYSTALLIZATION OF MICROBIAL CHITOSANASES

Purified MH-K1 chitosanase solution was concentrated to about 50 mg/ml. Crystallization was performed by the sitting drop vapor-diffusion method at 20°C using ammonium sulfate as precipitant [30]. Rod-shaped colorless crystals, which grew up to 0.1 x 0.3 x 1.2 mm within a week (Fig. 2), belong to the orthorhombic space group $P2_12_12$ with unit-cell dimensions of a =43.3Å, b =128.0Å, and c =57.7Å.

K-01 and CsO-2 chitosanases were crystallized by a similar procedure at 20°C from a 30% (w/v) polyethyleneglycol solution and $CaCl_2$ in 0.1 M Tris-HCl (Table 2). Colorless crystals appeared within two weeks (Fig.3). X-ray diffraction data have not been collected.

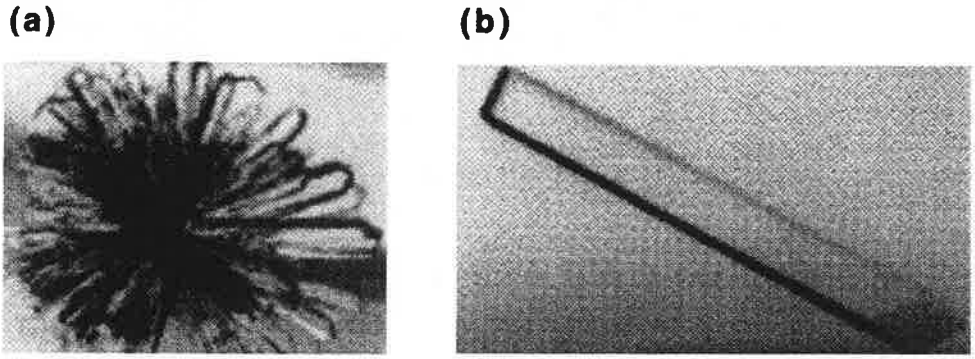

Fig. 2 Crystals of MH-K1 chitosanase
The crystal usually grew up to 3mm as a cluster (a), but occasionally as a beautiful single crystal up to 0.1 x 0.3 x 1.2 mm in average (b).

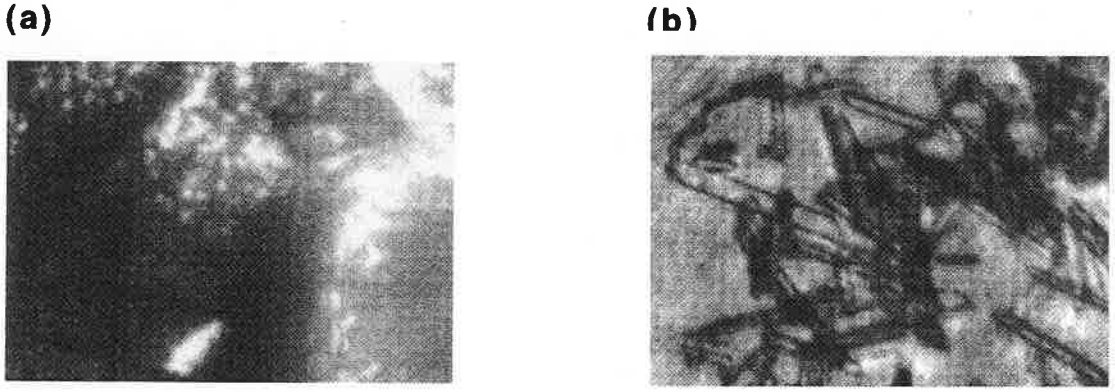

Fig. 3 Crystals of K-01 **(a)** and CsO-2 **(b)** chitosanases
Crystals of K-01 chitosanase (0.08 x 0.22 x 0.55 mm) were sufficient in size but insufficient in quality for the use in X-ray crystallographic works.
CsO-2 chitosanase affords only microcrystals with the length of 0.1mm.

Table 2. Crystallization conditions of K-01 and CsO-2 chitosanases

	K-01	CsO-2
Method	Vapor Diffusion	Vapor Diffusion
Temp. (°C)	20	20
Protein conc. (mg/ml)	30	10
Precipitant	PEG 6,000	PEG 6,000
protein drop (% w/v)	6	20
reservoir (% w/v)	12	30
Salt	0.1 M $CaCl_2$	0.15 M $CaCl_2$
Buffer	0.1 M Tris-HCl	0.1 M Tris-HCl
pH	9.0	7.2

DATA COLLECTION OF MH-K1 CHITOSANASE

Two full sets of intensity data on the native crystals with different rotation axes were collected for MH-K1 chitosanase using synchrotron radiation 1.0 Å at BL-6A of the Photon Factory (PF), the National Laboratory for High Energy Physics, Japan. A screenless Weissenberg camera for macromolecular crystals was used with a 0.1 mm aperture collimator and a cylindrical cassette of radius 286.5 mm [31]. The diffraction intensities were recorded on 200 mm x 400 mm imaging plates (Fuji Photo Film, Co., Ltd.), which were read out on a Fuji BAS 2000 scanner [32]. The intensity data up to 1.7 Å resolution were processed using the programs *DENZO* and *SCALEPACK* [33].

An extensive search for heavy atom derivatives was performed, but only K_2PtCl_4 derivative is found to be effective that was prepared by soaking crystals with 0.1 mM K_2PtCl_4 in 81% (w/v) saturated ammonium sulfate in Tris-HCl buffer for 24 h. The intensity data sets of this derivative were collected using one crystal at BL-18B of the PF with four different wavelengths for the MAD phasing method. The MAD phasing seemed to be successful, resulting in the electron density map where the initial molecular model could be constructed. The model building and refinement are in progress.

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Engineering of the chitosanase from *Streptomyces* sp N174 for an easy immobilization on cellulose

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Summary. The gene encoding the chitosanase from *Streptomyces* sp. N174 was fused to gene segments encoding protein domains binding to cellulose and originating from the endoglucanase *cex* gene from *Cellulomonas fimi* or the xylanase B gene *(xln*B) from *Streptomyces lividans.* The resulting multi-domain genes were expressed in *Streptomyces lividans.* Both fusion proteins were secreted extracellularly. Immobilization of the multi-domain proteins on cellulose consisted in a simple contact between the culture supernatants and the cellulose carrier. The cellulose with immobilized chitosanases was then packed into colum reactors and used for chitosan hydrolysis. The extent of chitosan hydrolysis could be controlled by varying the flow rate of the chitosan substrate through the column. The half life time of immobilized enzymes was of 7 and 36 days at 37°C and 2 days at 45°C. This is a promising system for the production of low and medium M.W. chitosans.

INTRODUCTION

Several biological activities of chitosan depend on the degree of polymerization. Medium and low molecular weight chitosan can be obtained by chemical or enzymatic hydrolysis of the high M.W. polymer. The chemical hydrolysis is usually achieved using strong acids and it is an unexpensive and rapid method. Its drawback is the necessity to purify extensively the low M.W. chitosan products for biological applications due to the toxicity of the reagents used for hydrolysis. Furthermore, the extent of hydrolysis is rather difficult to control.

The method of chitosan hydrolysis in an immobilized-enzyme reactor is an attractive alternative method for controlled decrease of the average M.W. of chitosan. Previous works on chitosanase immobilization involved covalent coupling

with glutaraldehyde (Davis and Eveleigh, 1984), ionic binding on an anion-exchange resin, adsorption on alginate beads or on Chitopearl (Yamasaki *et al.*, 1992). Chitosan hydrolysis was also studied with immobilized papain (Terbojevich *et al.*, 1996).

Here we present a new method of continuous chitosan hydrolysis with multi-domain chitosanases immobilized on cellulose. The chitosanases used here are said to be "multi-domain" enzymes because they result from the fusion (by genetic manipulation) of protein domains originating from different enzymes from various microorganisms. Briefly, the chitosanase from *Streptomyces* sp. N174 (see Fukamizo and Brzezinski, 1997 for a review) was fused to protein modules binding specifically (CBD) or unspecifically (XBD) to cellulose, originating, respectively, from the endoglucanase CEX from *Cellulomonas fimi* (O'Neill *et al.*, 1986) or the xylanase B (XLN-B) from *Streptomyces lividans* (Shareck *et al.*, 1995). The catalytic module is separated from the binding module by a linker, respectively a pro+thr-rich linker or a gly-rich linker. Such multi-domain chitosanases can be immobilized on cellulose by simple adsorption.

MATERIALS AND METHODS

Construction of the genes encoding the multi-domain chitosanases. Genetic manipulations have been achieved by standard methods. The plasmid pIJ680-PTCBD$_{cex}$ carrying the exoglucanase gene from *Cellulomonas fimi* was a gift of Dr. R.A.J. Warren (University of British Columbia, Vancouver, B.-C., Canada). The plasmid pIAF42, carrying the xylanase B gene from *Streptomyces lividans* was kindly offered by Drs D. Kluepfel, R. Morosoli and F. Shareck (Institut Armand-Frappier, Ville de Laval, Québec, Canada). The plasmid pALTER-csn harbouring the chitosanase gene from *Streptomyces* sp. N174 and the shuttle vector pFD666 replicating in *E. coli* and *Streptomyces* spp. have been described previously (Boucher *et al.*, 1995; Denis and Brzezinski, 1992). *Streptomyces lividans* strain TK24 has been used as host for extracellular enzyme production.

Details of fusion gene construction will be described elsewhere. Shortly, a *Bcl*I site was created by oligonucleotide-directed mutagenesis close to the end of the coding sequence of the *csn* gene, giving the plasmid pALTER-csnB. The *Bcl*I-digested pALTER-csnB was then ligated to a 820 bp *Msl*I+*Pst*I-digested fragment from pIJ680-PTCBD$_{cex}$ plasmid to give the plasmid pBP102 carrying the fusion gene *csn-pt-cbd* or, alternatively, it was ligated to a 765 bp *Sau*IIIA fragment from pIAF42 plasmid to give the plasmid pBP151 carrying the fusion gene *csn-g-xbd*.

The fusion genes were then excised from pBP102 and pBP151 plasmids by digestion with *Hin*dIII and *Xba*I and subcloned into the vector pFD666, giving the plasmids pBP202 and pBP251.

Production of the multi-domain chitosanases. The shuttle plasmids were introduced into *Streptomyces lividans* strain TK24 by protoplast transformation. Methods for chitosanase medium inoculation and chitosanase production have been as described previously (Boucher *et al.*, 1995).

Continuous chitosan hydrolysis. 50 ml of culture supernatant containing the multi-domain chitosanase were mixed with 3.5 g of microcrystalline cellulose (Avicell PH-101, 50μm) and incubated with moderate shaking for 1.5 hour at 4°C. The cellulose carrier was then recovered by centrifugation, suspended in Na-acetate buffer, pH 5.5 and packed into a column (26 mm diameter). The bed volume was 10 ml. The dead volume of the column was estimated at 2 ml. The chitosan substrate (Sigma, acetylation degree ≈ 0.2) was dissolved in the same buffer and fed continuously to the column. Extent of hydrolysis was estimated by measuring the difference in reducing sugar content (method of Lever, 1972) between the outlet and the inlet of the column. The leakage of the enzyme from the column was estimated by incubation of the column effluent samples (containing the chitosan substrate) for 24 hours at 37°C and by comparison of their reducing sugars content with non incubated control effluent samples.

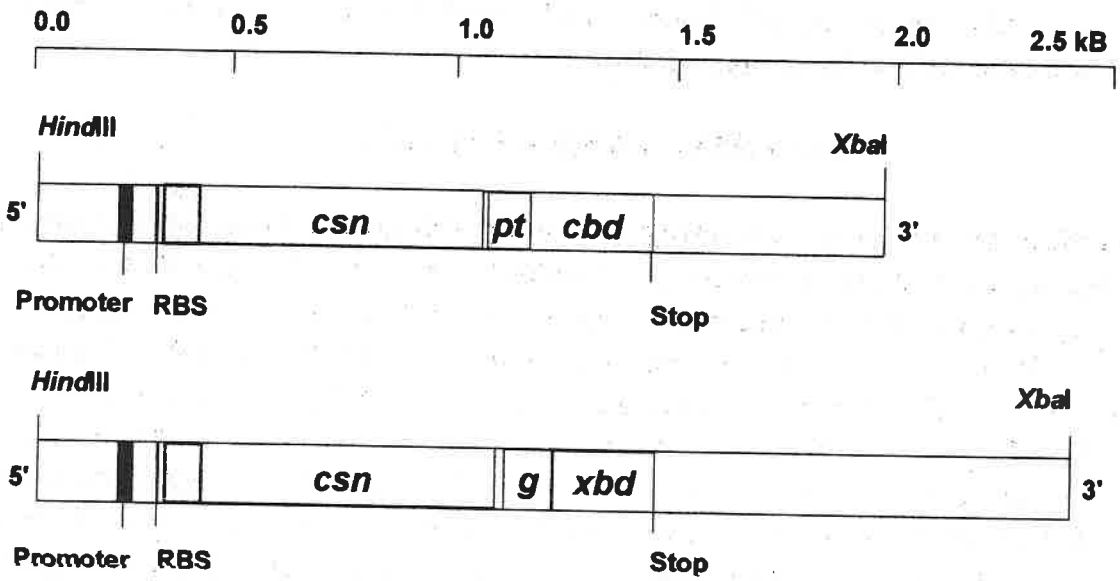

Figure 1: Schematic representation of fusion genes *csn-pt-cbd* and *csn-g-xbd*. Shaded box represent the signal peptide.

RESULTS

Structure of the fusion genes. The structures of the fusion genes encoding the multi-domain chitosanases are shown on Fig. 1. The coding sequence of the *csn-pt-cbd* gene consists of nucleotides 225 to 1050 from the *csn* gene (GenBank acc. nr. LO7779) fused to nucleotides 844 to 864 followed by nucleotides 1795 to 2195 from the *cex* gene (GenBank L11080). The sequence encodes a 415-residues long multi-domain chitosanase protein (CSN-PT-CBD) containing a signal peptide (40 a.a), the chitosanase catalytic domain (236 a.a), the pro+thr-rich linker (31 a.a) and the cellulose binding domain (108 a.a). The coding sequence of the *csn-g-xbd* gene consists of nucleotides 225 to 1052 from the *csn* gene (L07779) fused to nucleotides 2819 to 3187 from the *xln*B gene (M64552). The sequence encodes a 397-residues

long protein (CSN-G-XBD) containing a signal peptide (40 a.a), the chitosanase catalytic domain (236 a.a), a glycine-rich linker (35 residues) and the xylan-binding domain (86 residues).

Expression of the fusion genes. The plasmids pBP202 and pBP251 harbouring, respectively, the genes encoding the multi-domain chitosanases CSN-PT-CBD and CSN-G-XBD were introduced into *Streptomyces lividans* TK24. In our previous work, this host was used for efficient expression of the wild type chitosanase gene from *Streptomyces* sp. N174 (Masson *et al.*, 1993; A. Leroux and R. Brzezinski, unpublished data).

As an example of the time-course of multi-domain chitosanase production, Fig.2 shows the pattern of chitosanase activity and total extracellular protein accumulation obtained in a culture of *Streptomyces lividans* (pBP202) using our previously described culture conditions and media composition (Boucher *et al.*, 1995). As for the wild-type enzyme, we observed a sharp decrease of chitosanase activity at late culture stages due to proteolytic degradation. For enzyme immobilization, the cultures were usually harvested at 48 - 52h, when the protease activity was still very low.

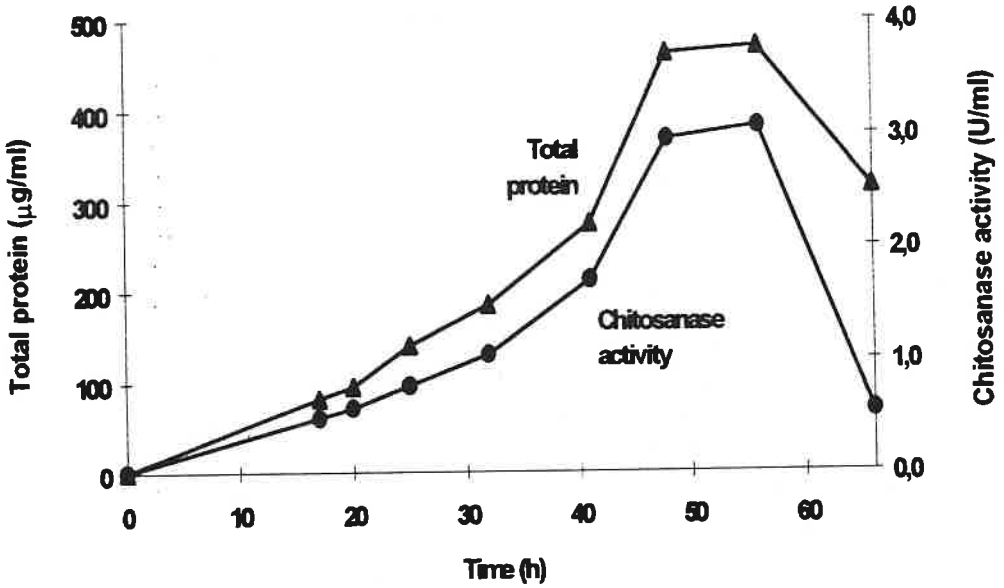

Figure 2: Time-course of CSN-PT-CBD enzyme production by S. lividans TK24 (pBP202). ▲ indicate total protein; ● indicate chitosanase activity.

Examination of the extracellular proteins by SDS-PAGE followed by Western blotting and probing with anti-chitosanase antibody (data not shown) revealed that various chitosanase forms constituted the vast majority (75 - 80%) of the proteins secreted into the medium. However, only a modest proportion of the chitosanase was in the complete multi-domain form (M.W. of about 45 kDa). The major signal revealed by anti-chitosanase antibody was related to a 29 kDa protein, i.e. the catalytic domain alone.

Chitosan hydrolysis in a plug-flow reactor with multi-domain chitosanase immobilized on cellulose. A simple contact between the culture supernatant and the cellulosic carrier was sufficient for the immobilization of the multi-domain chitosanase. Once adsorbed on cellulose, the enzyme could be desorbed only using a strong denaturation reagent such as 4M Guanidine-HCl. Interestingly, the chitosanase CSN-G-XBD harbouring the xylan-binding domain was adsorbed on cellulose as efficiently as the CSN-PT-CBD enzyme, even if the former was said to "adsorb on cellulose in an unspecific way" (D. Kluepfel, personal communication).

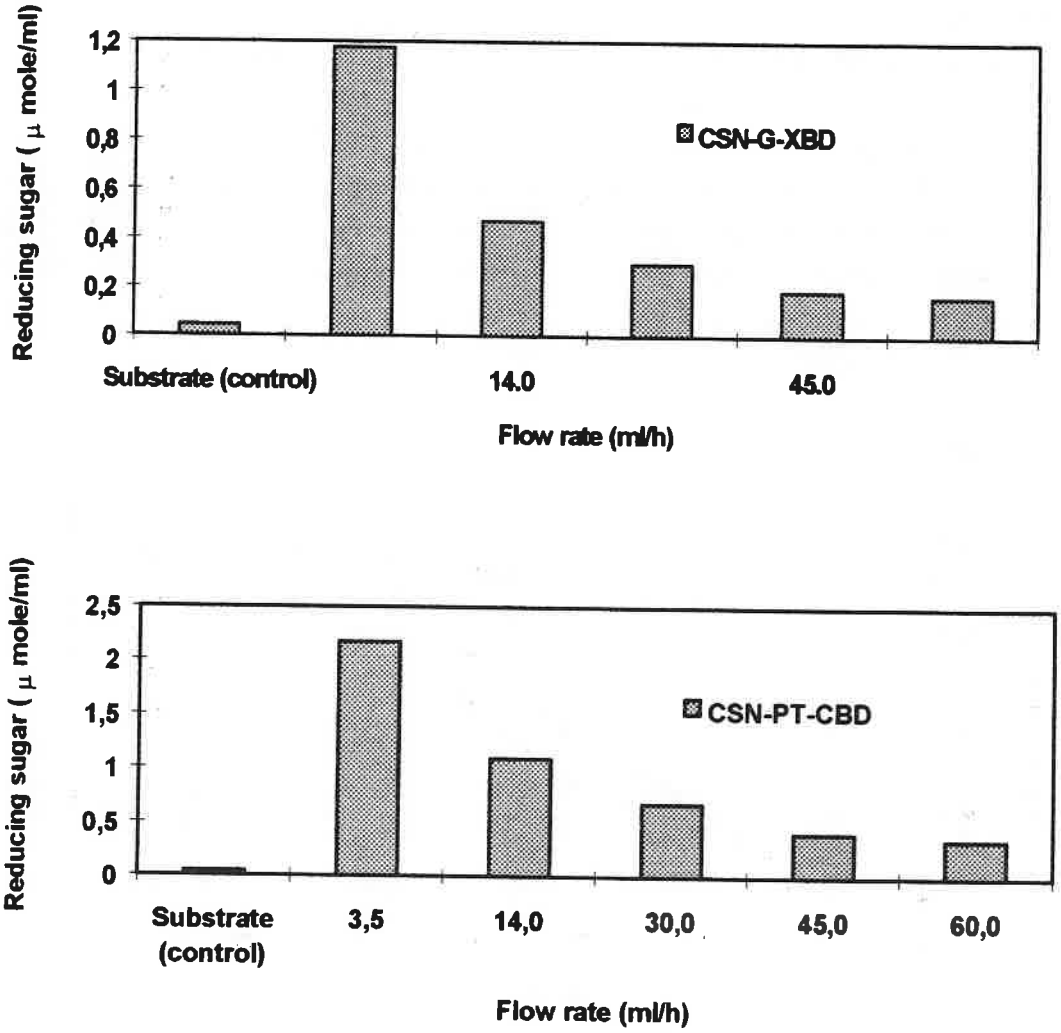

Figure 3: Reducing sugar content in the reactor effluent at various flow rates of chitosan solution.

The cellulose carriers with immobilized chitosanases were introduced in 10 ml columns and connected to peristaltic pumps delivering a 0.1% chitosan solution in 50 mM Na-acetate buffer pH 5.5. As anticipated, the extent of hydrolysis of chitosan could be controlled by simply varying the substrate flow rate through the column (Fig.3).

Taking into acount the linearity of the Lever assay for polymeric substrates (C. Dupont, personal communication), we can estimate that, for a 0.1% chitosan

solution, a reducing sugar concentration of 0.69 µmole/ml corresponds to an average DP of 8, while 0.11 µmole/ml correspond to an average DP of 50.

Chitosans of various average degrees of polymerization can thus be easily obtained by hydrolysis in the immobilized enzyme reactor. No leakage of enzymes from the columns could be detected.

Estimation of stability of the immobilized enzymes. The plug-flow reactors with immobilized multi-domain chitosanases were operated for many days at a constant substrate flow rate, either at 37°C or at 45°C. The enzyme activity was measured periodically in order to estimate the enzyme stability.

The half life of the CSN-PT-CBD enzyme was of 7 days at 37°C (Fig. 4-A) and of 2 days at 45°C (Fig. 4-C). The half life of the CSN-G-XBD enzyme was of 36 days at 37°C (Fig. 4-B). The stability was found to be much improved as compared with the native enzyme incubated in chitosan solution. Previously we estimated the stability of native chitosanase in the presence of the substrate to be in the range of 20 - 24 h at 37°C and 1.5 - 2h at 45°C (Boucher, 1992).

DISCUSSION

The first enzymes shown to be catalytically active when artificially fused to a cellulose-binding domain and immobilized on cellulose were the alkaline phosphatase from *E. coli* and the β-glucosidase from *Agrobacterium* sp. (Greenwood *et al.*, 1989; Ong *et al.*, 1989). Our preliminary work on the chitosanase from *Streptomyces* sp. N174 showed that this enzyme is relatively insensitive to additions of residues at its C-terminus (I. Boucher and R. Brzezinski, unpublished). This is why we constructed fusion chitosanases by modification of the *csn* gene at the 3'-end. Such chitosanases appear to be novel as no multi-domain chitosanases, neither of natural nor of unnatural origin have been described so far.

Both multi-domain chitosanases studied in this work kept their enzymatic activity in the immobilized state, allowing to design a reactor for continuous hydrolysis of chitosan with an easy control of the average degree of polymerization of the hydrolyzed product. Enzyme stability was also much improved as compared with the native chitosanase.

Several problems remain to be solved. Avicell microcrystalline cellulose was shown to be an excellent support for the immobilization of proteins (Ong *et al.*, 1989), however its chromatographic behaviour is not satisfactory, creating flow irregularities. Other supports must be investigated. It would be also useful to increase the thermal stability of the chitosanase. The possibility of reactor operation at much higher temperatures would allow to use chitosan solutions of higher concentration (what now is difficult because of viscosity problems).

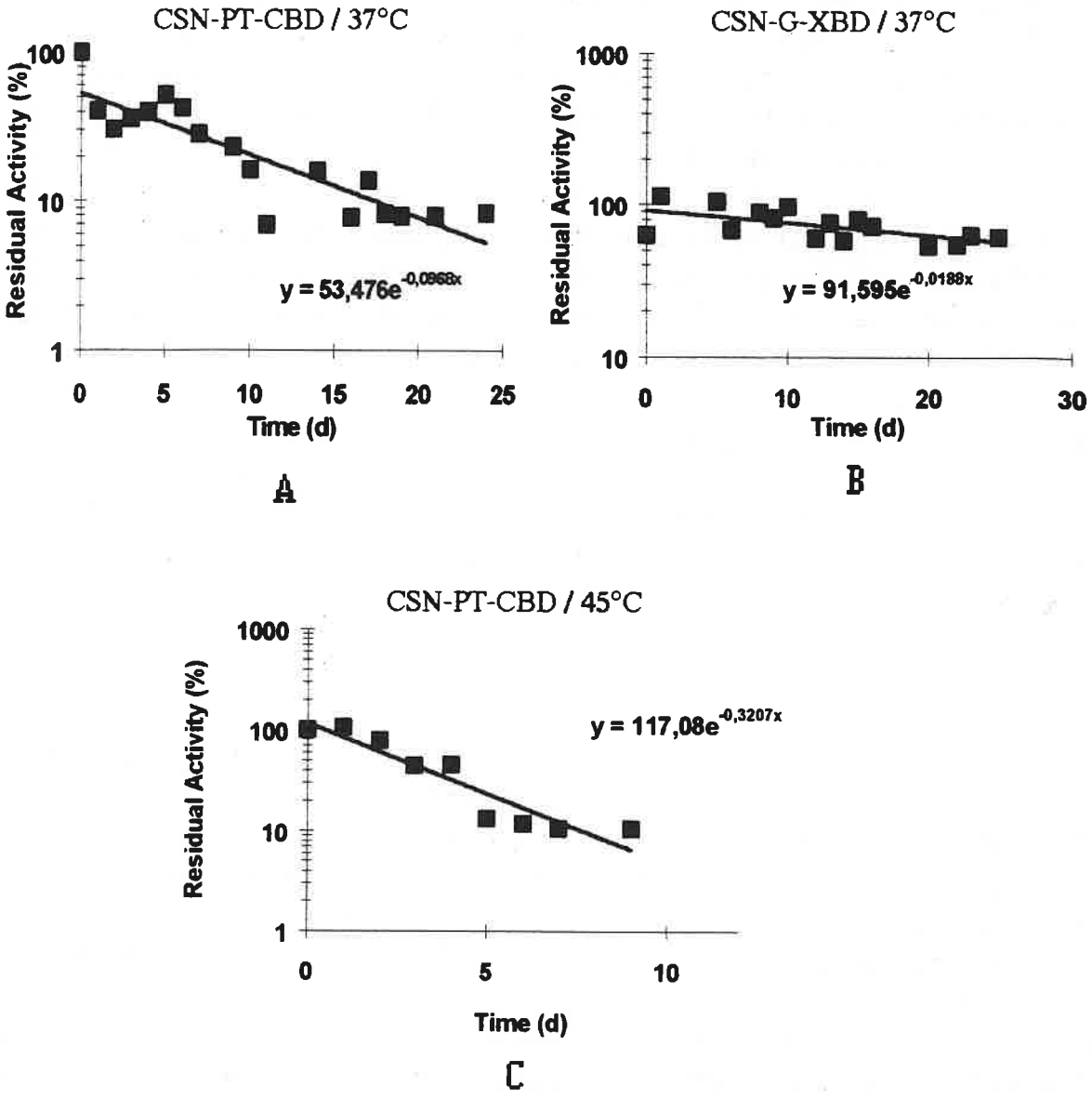

Figure 4: Semi-logarithmic plots of residual activity of immobilized multi-domain chitosanases versus time of reactor operation

The main challenge will be however to increase the ratio of uncleaved v/s cleaved multi-domain chitosanase in the supernatants of cultures of recombinant microorganisms used for multi-domain enzyme production. This is not a problem intrinsic to multi-domain chitosanase production, as the cleavage of cellulose-binding domains from the catalytic domains is a generalized, physiological mechanism used by cellulose-degrading microorganisms to optimize the use of the various glucanases. This mechanism, involving delicate equilibria between proteolytic cleavage and protection by glycosylation (Ong et al., 1994) has been little investigated so far. With future progress we can expect to develop expression systems more efficient in producing intact multi-domain proteins.

In conclusion, we presented here a promising system for continuous enzymatic hydrolysis of chitosan which allows to obtain chitosan forms whose molecular weight would be optimal for their various biological applications.

ACKNOWLEDGEMENTS

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PURIFICATION AND PROPERTIES OF EXO-β-D-GLUCOSAMINIDASE FROM *PENICILLIUM* SP. AND ITS APPLICATIONS

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Abstract
Exo-β-D-glucosaminidase from *Penicillium* sp. AF9-P-112 was purified from the culture filtrate by relative simple method with a good efficiency and yield. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis and the molecular weight was estimated to be about 110kDa by SDS-PAGE and 125kDa by gel filtration, and the isoelectric point was approximately at pH 7.4. The enzyme hydrolyzed chitosan and its derivatives but didn't act on chitin, its derivatives and carboxymethyl cellulose. The sole product produced in the whole course of hydrolysis was a glucosamine when chitosan and chitooligosaccharides were used as substrates. The enzyme therefore is typically an exo-typed chitosanase which can remove single glucosamine residues from β-1,4-linked glucosamine chain. The some interesting applications of exo-β-D-glucosaminidase are discussed.

Keywords : Chitosan, exo-typed chitosanase, exo-β-D-glucosaminidase, *Penicillium* sp. AF9-P-112, glucosamine, transglycosylation

Chitosanases (EC 3.2.1.99) are widely distributed in nature and have been isolated and purified from some microorganisms, including fungi, bacteria and actinomycetes. We had previously reported the purification and properties of endo-typed chitosanases from *Bacillus* sp. No.7-M[1,2] and *Bacillus licheniformis* UTK[3,4]. Lately we found that *Penicillium* sp. AF9-P-112 produced only exo-typed chitosanase and *Penicillium* sp. AF9-P-128 also did produce two typed(endo- and exo-typed) chitosanases when the cells were grown on the medium containing fine powdered chitosan.

In this paper, we describe the procedure of purification of exo-typed chitosanase (exo-β-D-glucosaminidase) from *Penicillium* sp. AF9-P-112 and the properties of the enzyme. Moreover, as the applications of the enzyme, we discuss a new method for the determination of deacetylation of chitosan and the enzymatic formation of chitooligosaccharides by transglycosylation reaction.

Materials and Methods

Microorganism and Cultivation

Penicillium sp. AF9-P-112, obtained from Biological Engineering Laboratory, Asahi Industries Co.Ltd., Japan, was used as the source of the enzyme production. The medium for enzyme production was composed of 1.0% powdered chitosan(DAC 89%), 0.2% peptone, 0.05% yeast extract, 0.4% lactose, 0.1% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, and 0.4% $(NH_4)_2SO_4$, pH 6.5. The seed culture(50 ml) cultivated at 25℃ for 4 days was tranferred to a 5 liter conical flask containing 1 liter of the enzyme production medium. The cultivation for enzyme production was performed at 25℃ for 4 days on a circular shaker.

Exo-β-D-glucosaminidase Assay

A reaction mixture(1ml) containing 0.5ml of 4mM chitotriitol, 0.4ml of 0.1M acetate buffer(pH5.0), and 0.1ml of an enzyme solution was incubated for 10min at 37℃. The reaction was stopped by the addition of 3ml of Schales reagent, and the amount of reducing sugars liberated was determined by the method of Imoto et al[5], with glucosamine as standard. One unit of exo-β-D-glucosaminidase was defined as the amount of enzyme which liberated 1μ mol of reducing sugar as glucosamine per min.

Thin Layer Chromatography

The products of the enzymatic hydrolysis of substrates were analyzed by thin layer chromatography on Merk Silica Gel 60 aluminum plates using iso-propanol, pyridine, acetic acid and water (10:6:6:9 v/v) as the developing soluvent. Glucosamine and chitooligosaccharides were visualized by ninhidrin spray.

Results and Discussion

Purification of Exo-β-D-glucosaminidase

All oerations during the purification of enzyme were performed at 0～5℃.

Step 1. Preparation of culture filtrate.

The culture filtrate(950ml) was obtained by centrifugation from the culture broth added NaCl to final concentration to 0.5M.

Step 2. Precipitation with ammonium sulfate.

Solid ammonium sulfate was added to the culture filtrate to 30% saturation left for 24 hr, centrifuged, and the precipitate discarded. The process was repeated after taking the supernatant to 90% ammonium sulfate saturation. After standing overnight, the precipitate produced was collected by centrifugation and dissolved in 0.05M phosphate buffer, pH6.5(P-buffer). The solution was desalted and equilibrated by dialyzation against P-buffer.

Step 3. Chromatyography on CM-Sephadex C-50.

The enzyme solution from step 2 was applied to a CM-Sephadex C-50 column(2.6 × 25cm) previously equilibrated with P-buffer. The column was completely washed with same buffer, and then eluted with 600ml linear

gradient of 0～0.4M NaCl in P-buffer, at a flow rate 30ml/hr. As shown in Fig.1, the peak for exo-β-D-glucosaminidase activity appeared at the narrow range of NaCl concentration of 0.2M.

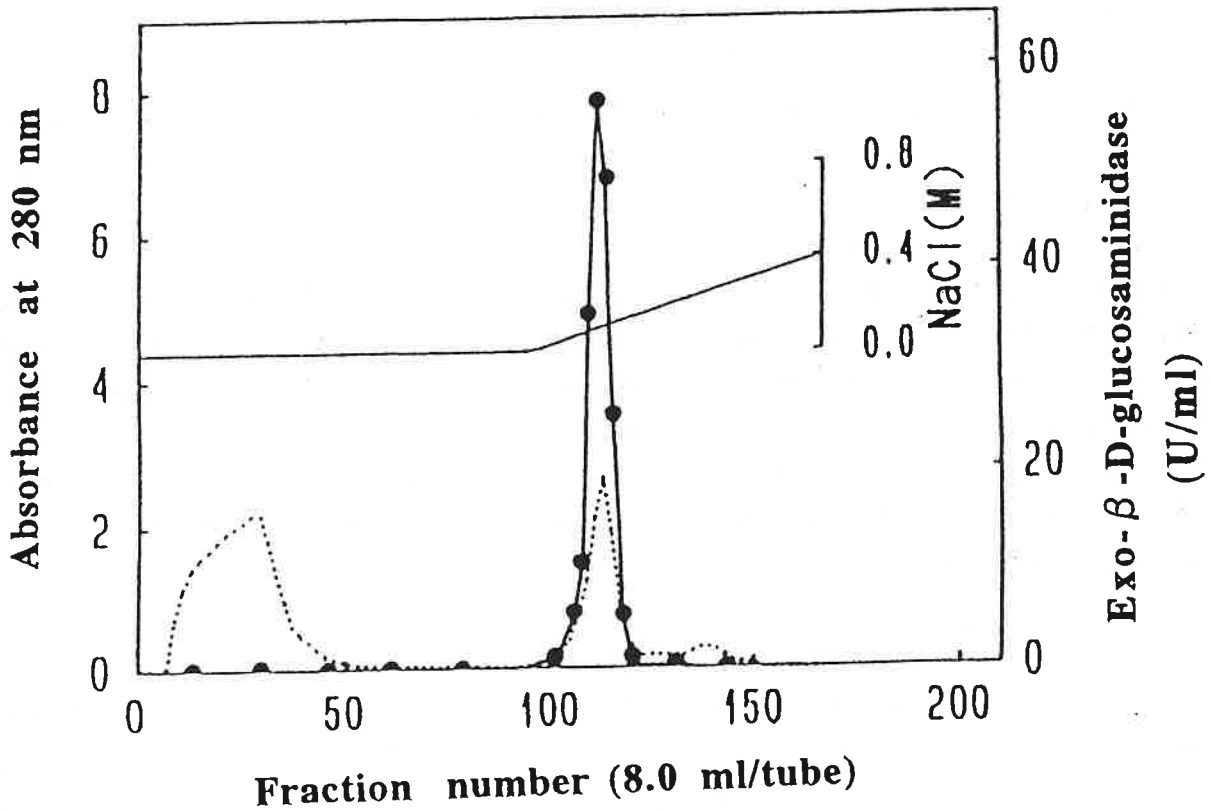

Figure 1. Column chromatography of exo-β-D-glucosaminidase on CM-Sephadez C-50

····· , absorbace at 280nm
● , exo-β-D-glucosaminidase
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Step 4. Gel filtration of Sephacryl S-200.

The active fraction was pooled and concentrated by ultrafiltration with Amicon membrane PM-10, and applied to Sephacryl S-200 column(2.6 × 94cm) previously equilibrated with P-buffer. The column was eluted with the same buffer at a flow rate of 24ml/hr, and 3ml fractions were collected. The fractions(No.82-97) containing enzyme activity, as not indicated,were pooled and used as the purified enzyme. The specific activity, yield and recovery of exo-β-D-glucosaminidase at each purification step are summarized in Table 1. The enzyme was purified about 19-fold with a yield of 73%. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis.

Table 1. Purification of exo- β -D-glucosaminidase

Step	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	1,036	2,156	2.08	1.0	100
Amonium sulfate(30-9C	225	1,873	8.33	4.0	87
CM-Sephadex C-50	48	1,736	36.11	17.4	80
Sephacryl S-200	40	1,578	39.66	19.1	73

Enzymatic Properties of Exo- β -D-glucosaminidase

The molecular weight of the enzyme was estimated to be about 110kDa by SDS-PAGE and 125kDa by gel filtration on Sephacryl S-200. The isoelectric point of this enzyme was about pH7.4. The enzyme had the optimum pH at 4.0 and the optimum temperature at 60℃ and was stable between pH3.0 and 7.0, and below 50℃.

Mode of Action of Exo- β -D-glucosaminidase

The purified enzyme acted specifically on chitosan and chitooligosaccharides. The products of hydrolysis of the substrates by the enzyme were analyzed by thin layer chromatography. As the results are shown in Fig.2, the only product produced over the whole course of hydrolysis was glucosamine when chitosan was used as the substrate. The enzyme produced glucosamine and smaller chitooligosaccharides than the oligosaccharides used as the initial substrate in the course of hydrolysis.

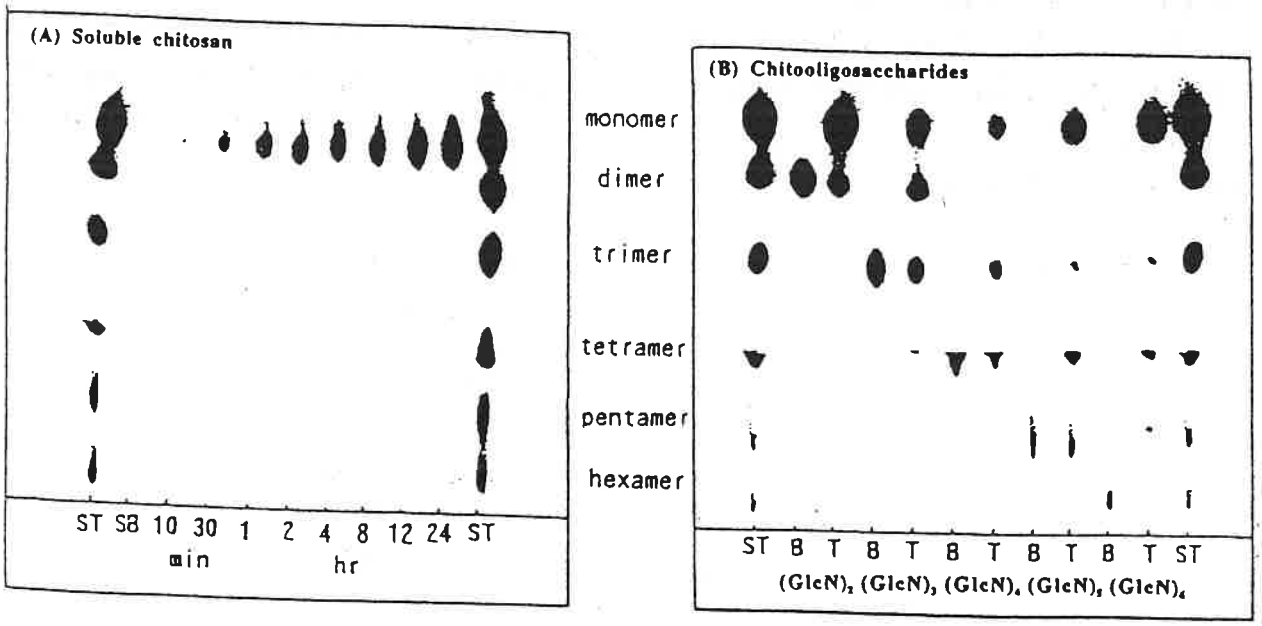

Figure 2. TlC of hydrolyzates of chitosan(A) and chitooligosaccharides(B) with exo- β -D-glucosaminidase.

Finally, only glucosamine was produced after prolonged hydrolysis. The enzyme therefore is typically an exo-chitosanase which is able to remove single glucosamine residues from the nonreducing terminal and characterized as an exo-β-D-glucosaminidase, another one of which had been reported by Nanjo et al[6].

Substrate Specificity of Exo-β-D-glucosaminidase

The purified enzyme hydrolyzed soluble chitosan, powdered chitosan, colloidal chitosan and glycol chitosan, but did not cleave powdered chitin, colloidal chitin, glycol chitin and carboxymethyl cellulose as indicated in Table 2.

Table 2. Substrate specificity of exo-β-D-glucosaminidase against different kinds of β-1,4-glucan

Substrate	Concentration (%)	Reducing sugar (μg/mg protein/hr)
Soluble chitosan	1.0	1,788
Powdered chitosan	1.0	1,324
Colloidal chitosan	1.0	890
Glycol chitosan	1.0	198
Powdered chitin	1.0	0
Colloidal chitin	0.6	0
Glycol chitin	0.2	0
CM-cellulose	0.2	0

Next, the substrate specificity of exo-β-D-glucosaminidase was examined based on the initial velocity measurement with reduced chitooligosaccharides. The results are listed in Table 3. The enzyme showed maximum activity on chitopentaitol, but hydrolysis rate of the enzyme toward chitibiitol decreased drastically. Therefore, exo-β-D-glucosaminidase appears to require more than 3 glucosamine residures for full activity.

Table 3. Relative rate of hydrolysis of reduced chitooligosaccharides by exo-β-D-glucosaminidase

Substrate	Relative rate (%)
Chitobiitol	0.4
Chitotriitol	85
Chitotetraitol	97
Chitopentaitol	100
Chitohexaitol	89
Chitosan	90

Applications of Exo- β -D-glucosaminidase

As the applications of exo- β -D-glucosaminidase, we at first describe a new method for the accurate and simple determinatin of the degree of deacetylation of chitosans. It was expected that exo- β -D-glucosaminidase completely hydrolyzes partially acetylated chitosans to glucosamine and *N*-acetylglucosamine in combination with β -*N*-acetylglucosaminidase and endo-typed chitosanase[7]. The amounts of glucosamine and *N*-acetylglucosamine liberated were determined by the specific colorimetric assays. The degree of deacetylation of several chitosan samples was mesured by enzymatic method. The results, as not shown, were compared with those obtained from colloidal titration method and the degree of deacetylation obtained both methods is in good agreement.

In the second place, we found that exo- β -D-glucosaminidase, which is essentially a hydrolase, also catalyzed a transglycosylation reaction on chitobiose and chitotriose. For exsample, the enzyme converted the chitotriose into chitotetraose and glucosamineas as the major products. The ratio of the transglycosylation depends on the temperature, the concentration and kind of substrate, and the pH.

Conclusion

The exo-typed chitosanase was purified to homogeneity from the culture filtrate of *Penicillium* sp. AF9-P-112 by the relative simple method with a good efficiency and yield. The enzyme hydrolyzed chitosan and chitooligosaccharides to glucosamine in a exo-splitting manner from non-reducing terminal. From these results, the enzyme was characterized as an exo- β -D-glucosaminidase. As the applications of the enzyme, we discussed a new method for the determination of deacetylation of chitosan and the enzymatic formation of chitooligosaccharides by transglycosylation reaction.

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MODE OF ACTION OF FAMILY 19 CHITINASES

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Abstract

Class I, II, and IV chitinases from plant and chitinase C from *Streptomyces griseus* HUT 6037 belong to family 19 of glycosyl hydrolases. In order to study the mode of action of family 19 chitinases, the hydrolysis of partially *N*-acetylated chitosan by pokeweed chitinase PLC-A (classII) and *S. griseus* HUT 6037 chitinase C was examined. Oligosaccharides from their digests were separated using CM-Sephadex C-25 and μBondapak NH_2. The products were structurally analyzed to elucidate the specificity of the chitinases. The oligosaccharides isolated had GlcNAc at the non-reducing end and GlcNAc or GlcN at the reducing end residues. The results indicate that the enzymes cleave both the *N*-acetyl-β-D-glucosaminidic and the β-glucosaminidic linkages as long as the residue at the reducing end side of the linkage is a GlcNAc. Thus, the position of the GlcNAc residue required for hydrolysis was different between the family 18 and family 19 chitinases.

Keywords: chitinase, pokeweed, *Phytolacca americana*, *Streptomyces griseus*, sequence specificity

Partially *N*-acetylated chitosan is a soluble substrate common to chitinase and chitosanase in elucidating their mode of action. Oligosaccharides produced during the hydrolysis of partially *N*-acetylated chitosan by microbial chitinases have been previously studied in an attempt to clarify their specificity for the hydrolysis of β-1,4-glycosidic linkages in chitosan molecules[1-5].

On the other hand, chitinases are classified into two different

families(families 18 and 19) of the classification of glycosyl hydrolases based on amino acid sequence similarities[6,7]. Family 18 chitinases are found in a wide range of organisms including bacteria, fungi, viruses, plants, and animals. On the other hand, class I, II, and IV plant chitinases belong to family 19. Family 18 microbial chitinases[3,5] hydrolyzed GlcNAc-GlcNAc and GlcNAc-GlcN linkages whereas chitinase C-1 from *Streptomyces griseus* HUT 6037[4] hydrolyzed GlcN-GlcNAc and GlcNAc-GlcNAc linkages in partially *N*-acetylated chitosan. Recently, we found that chitinase C-1 from *S. griseus* HUT 6037 belongs to family 19 of the glycosyl hydrolases[8]. Chitinase C-1 is the first family 19 chitinase identified in an organism other than higher plants. In this study, we attempted to isolate and identify oligosaccharides produced during the digestion of partially *N*-acetylated chitosan by pokeweed class II chitinase PLC-A(family 19)[9], in order to clarify the difference in the mode of action between family 18 chitinase and family 19 chitinase. This paper deals with the specificity of pokeweed chitinase PLC-A for the hydrolysis of the β-1,4-glycosidic linkages in partially *N*-acetylated chitosan and comparison with those of the family 18 chitinases.

Materials and methods

Materials. 54% *N*-acetylated chitosan was the product of Ajinomoto Co., Ltd. GlcNAc was purchased from Sigma Chemical Co. Chitinase PLC-A was purified from pokeweed leaves by the procedure described in a previous paper[9]. β-*N*-Acetylglucosaminidase (β-GlcNAcase) was prepared from *Pycnoporus cinnabarinus* IFO 6139 as described previously[10].

Reducing sugar measurement. The amount of reducing sugar during column chromatography was measured by the Schales' method with modification[11], using GlcNAc as a standard.

High performance liquid chromatography (HPLC). The HPLC system consisted of an 880-PU pump, an 875-UV/VIS detector (Japan Spectroscopic Co., Ltd), and a D-2500 Chromato-Integrator (Hitachi Ltd.). Sugars were separated on a Radial-PAK μBondapak NH_2 column (8.0 x 100 mm, Millipore Co.) using acetonitrile-water mixtures in various ratios as the mobile phase, at a flow rate of 2.0 ml per min. *N*-Acetylchitooligosaccharides were detected by monitoring the absorbance at 210 nm.

Enzymatic hydrolysis of oligosaccharides. A reaction mixture

consisting of 100 µl of 0.4% of each oligosaccharide, 50 µl of 0.04 M phosphate buffer (pH 5.5), and 50 µl of β-GlcNAcase (0.1 units) was incubated for 20hr at 37° C. The reaction was stopped by boiling for 3 min. The hydrolysis products were analyzed by HPLC.

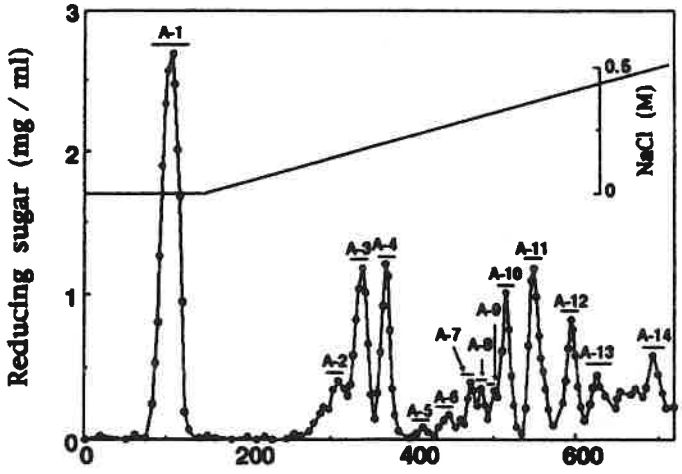
Mass spectrometry. The molecular weights of oligosaccharides were measured on a Voyager matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (PerSeptive Biosystems). 2,5-Dihydroxybenzoic acid was used as the matrix.

Hydrolysis of partially N-acetylated chitosan by chitinase. A reaction mixture consisting of 1000 ml of 1.2% chitosan (54% *N*-acetylated chitosan) in 0.1 M acetate buffer (pH 5.0), 10 ml of 2% NaN_3, and 2 ml of chitinase (10 units) was incubated for 120hr at 37° C. The reaction was stopped by boiling for 5 min. The reaction mixture was adjusted to pH 9.5 with 0.1 N NaOH to precipitate products with high molecular weights, and then the precipitate was removed by centrifugation. The supernatant was acidified to pH 5.0 by addition of 1 N acetic acid, concentrated in a rotary evaporator under reduced pressure at a temperature below 35° C, and dialyzed through an electric dialyzer (Micro Acilyzer G1, Asahikasei Kogyo Co., Ltd.).

Results and discussion

Separation of oligosaccharides. To identify the oligosaccharides produced during the hydrolysis of 54% *N*-acetylated chitosan by chitinase PLC-A from pokeweed, the hydrolyzate was separated by CM-Sephadex C-25 column chromatography. The dialyzed hydrolyzate was loaded onto a column (5.0 x 30 cm) of CM-Sephadex C-25 previously equilibrated with 0.02 M acetate buffer (pH 5.0). After the column was washed with the same buffer, oligosaccharides were eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer. Figure 1 shows the elution pattern of the oligosaccharides. Fractions from A-1 to A-6 indicated by a bar were combined, dialyzed through an electric dialyzer, and lyophilized. A-1 contained GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$, while A-2, A-3, and A-4 were homogeneous based on the HPLC. The A-5 and A-6 fractions were homogeneous after further purification by HPLC on Radial-PAK µBondapak NH_2.

Characterization of oligosaccharides. In the MALDI-TOF MS analyses of A-2, A-3, A-4, A-5, and A-6, the $[M+Na]^+$ ions were detected at m/z 1217.19, 1014.27, 811.22, 608.16, and 405.12, indicating that these are pentaacetylchitohexaose, tetraacetylchitopentaose, tri-



Fraction number (5.0 ml / tube)

Fig. 1. CM-Sephadex C-25 column chromatography of oligosaccharides produced in the hydrolysis of partially *N*-acetylated chitosan with Pokeweed chitinase PLC-A.

●, Reducing sugar; ——, NaCl

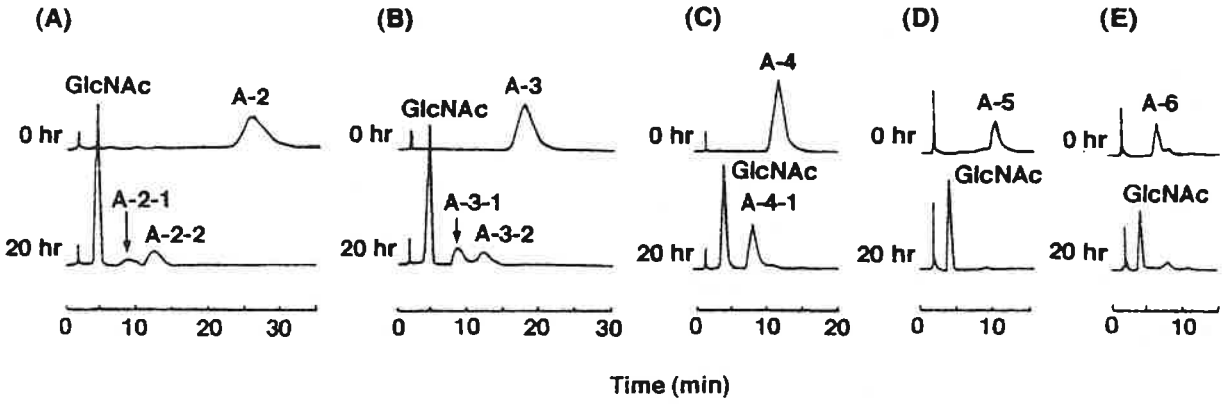
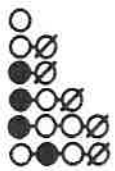
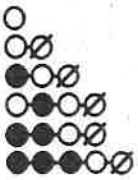
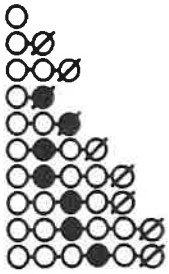


Fig. 2. HPLC of the hydrolyzates of A-2 (A), A-3 (B), A-4 (C), A-5 (D), and A-6 (E) by β-GlcNAcase.

acetylchitotetraose, diacetylchitotriose, and monoacetylchitobiose, respectively. To clarify the structures of these hetero-chitooligosaccharides, the samples were hydrolyzed by β-GlcNAcase. Figure 2 shows the HPLC pattern of the hydrolyzates of each sample. As the absorbance at 210 nm was monitored during the HPLC's, GlcNAc and GlcNAc-containing oligosaccharides were detected as the hydrolysis products. A-2, A-3, A-4, A-5, and A-6 were hydrolyzed by β-GlcNAcase to release GlcNAc, indicating that the non-reducing end residues in the oligosaccharides are GlcNAc. After the hydrolysis of A-5 and A-6 by β-GlcNAcase, only GlcNAc was detected as the product. From these

Table 1. Oligosaccharides obtained in the digestion of partially *N*-acetylated chitosan by chitinases

Family 18			Family 19	
Bacillus circulans WL-12 chitinase A1 (3)	*Bacillus circulans* WL-12 chitinase D (3)	*Aeromonas* sp. No.10S-24 (5)	*Streptomyces griseus* HUT 6037 (4)	Pokeweed PLC-A
				

○, GlcNAc ; ●, GlcN ; Ø, ⌀, reducing end residue

results, A-5 and A-6 were identified to be $(GlcNAc)_2$-GlcN and GlcNAc-GlcN, respectively. The products from A-2 consisted of three peaks, GlcNAc, A-2-1, and A-2-2, and those from A-3 were three peaks, GlcNAc, A-3-1, and A-3-2. A-4 was hydrolyzed to GlcNAc and A-4-1. In the MALDI-TOF MS spectra of A-2-1, A-2-2, A-3-1, A-3-2, A-4-1, the peaks corresponding to the mass numbers of $[M+Na]^+$ ion of tri-, tetra-, tri-, tetra-, and trisaccharides containing one molecule of GlcN were recognized at m/z 608.36, 811.29, 608.18, 811.32, and 608.21, respectively. These results indicate that the structures of A-2-1, A-2-2, A-3-1, A-3-2, and A-4-1 are GlcN-$(GlcNAc)_2$, GlcN-$(GlcNAc)_3$, GlcN-$(GlcNAc)_2$, GlcN-$(GlcNAc)_3$, and GlcN-$(GlcNAc)_2$, respectively. Thus, it can be presumed that A-2 is a mixture of $(GlcNAc)_2$-GlcN-$(GlcNAc)_3$ and $(GlcNAc)_3$-GlcN-$(GlcNAc)_2$, and A-3 is a mixture of GlcNAc-GlcN-$(GlcNAc)_3$ and $(GlcNAc)_2$-GlcN-$(GlcNAc)_2$, and A-4 is GlcNAc-GlcN-$(GlcNAc)_2$.

As summarized in Table 1, hetero-chitooligosaccharides with GlcNAc at the non-reducing end and GlcNAc or GlcN at the reducing end residues together with GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$ were isolated from the hydrolyzate of partially *N*-acetylated chitosan by pokeweed chitinase PLC-A, indicating that the chitinase hydrolyzes GlcNAc-GlcNAc and GlcN-GlcNAc linkages but not GlcNAc-GlcN or GlcN-GlcN linkages in chitosan molecules. The specificity of the enzyme for various linkages in partially *N*-acetylated chitosan was similar to that of chitinase C-1 from *S. griseus* HUT 6037[4]. On the other

hand, as presented in Table 1, chitinases from *Bacillus circulans* WL-12[3] and *Aeromonas* sp. No. 10S-24[5], belong to family 18, and hydrolyze only GlcNAc-GlcNAc and GlcNAc-GlcN linkages.

Thus, chitinases can be classified into two groups according to their mode of action toward partially *N*-acetylated chitosan; the enzymes that cleave only the *N*-acetyl-β-D-glucosaminidic linkages and the enzymes that cleave both the *N*-acetyl-β-D-glucosaminidic and the β-glucosaminidic linkages in chitosan molecules. Family 19 chitinases hydrolyze both the *N*-acetyl-β-D-glucosaminidic and the β-glucosaminidic bonds in partially *N*-acetylated chitosan as long as the residue at the reducing end side of the linkage is a GlcNAc. Chitinase requires at least one GlcNAc residue at one side of the linkage undergoing hydrolysis and the position of the GlcNAc residue required for hydrolysis is different between family 18 and family 19 chitinases.

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