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Purification and characterization of a 54 kDa chitinase from *Bombyx mori*

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Abstract

The 54 kDa protein that was suggested to be processed from the 65 kDa and 88 kDa chitinases of *Bombyx mori* [Koga et al., Insect Biochem. Mol. Biol. 27, 757–767 (1997)] was purified and proved to be a third chitinase (EC 3.2.1.14). This chitinase was purified from the fifth larval instar of *B. mori* by chromatography on DEAE-Cellulofine A–500, hydroxylapatite, Butyl-Toyopearl 650M, and Fractogel EMD DEAE 650(M) columns. The apparent molecular mass was confirmed to be 54 kDa by SDS–PAGE. Its optimum pH was 6.0 toward a short substrate, N-acetylchitopentaose ($GlcNAc_5$), while in its reaction with a longer substrate, glycolchitin, the enzyme showed a wide pH-range between 4.0 and 10. Kinetic parameters for the chitinase could be obtained in the hydrolysis of glycolchitin but not in that of N-acetylchitooligosaccharides ($GlcNAc_n$, n=2–6) because of substrate inhibition. The chitinase hydrolyzed N-acetylchitooligosaccharides except for dimer as follows: trimer to monomer plus dimer, tetramer to two molecules of dimer, pentamer to dimer plus trimer, and hexamer to dimer plus tetramer as well as two molecules of trimer. These results suggest that the 54 kDa chitinase is an endo-type hydrolase and preferred the longer-chain N-acetylchitooligosaccharides. Moreover, the anomeric forms of N-acetylchitooligosaccharides were analyzed in the reaction with the 54-kDa chitinase. It was revealed that this enzyme cleaves the substrate to produce the β anomeric product. With respect to inhibition of the 54 kDa chitinase, it was specifically inhibited by allosamidin in a competitive way with K_i values depending on the pH of the reaction mixture (K_i=0.013−0.746 μM). Comparing the properties and kinetic behavior of this chitinase with those of the 88 and 65 kDa chitinases from *B. mori*, regarding the specific activity of the three enzymes, the 65-kDa chitinase was 2.15 and 2.8 times more active than the 88 and 54-kDa chitinases, respectively. However, in the overall reaction of glycolchitin (k_{cat}/K_m), the 88-kDa enzyme was 4 and 40 times more active than the 65-kDa and the 54-kDa enzymes, respectively. Concerning the affinity ($1/K_m$) to glycolchitin, the 88 kDa chitinase affinity (at pH 6.5) was 5.8 times higher than that of the 65 kDa chitinase (at pH 5.5) and 4.0 times higher than that of the 54 kDa chitinase (at pH 6.0). These kinetic results suggest that *B. mori* chitinases are processed during ecdysis from the larger chitinase to smaller ones that leads to changes in their kinetic properties such as K_m, k_{cat} and k_{cat}/K_m successively.

Keywords: *Bombyx mori*; 54 kDa chitinase; Insect ecdysis; N-acetylchitooligosaccharide

1. Introduction

Insects shed their exoskeleton periodically to achieve growth and development. Chitin, poly-β-(1–4)-N-acetyl-D-glucosamine, is a major component of the skeleton and alimentary canal of insects (Kramer et al., 1985). The endo-type enzymes, chitinases, and exo-splitting β-N-acetylglucosaminidases of the insect molting fluid are involved in degradation of the cuticular chitin during the process of ecdysis. Several studies have been conducted to clarify the mechanism of insect ecdysis mainly using the tobacco hornworm, *Manduca sexta*, and the silkworm, *Bombyx mori* (Dziadik-Turner et al., 1981; Koga et al., 1982, 1986, 1989, 1991, 1992, 1997; Koga et al., 1983a,b; Kramer and Koga, 1986; Fukamizo and Kramer, 1985, 1987; Koga et al., 1987b). Furthermore, Koga et al. (1997) reported that *B. mori* 65 kDa chitinase

Abbreviations: DEAE, diethylaminoethyl; GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranoside; $GlcNAc_n$, β(1–4) linked oligosaccharide of GlcNAc (N-acetylchitooligosaccharide); II to VI, β (1–4) linked di to hexasaccharides of GlcNAc; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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degraded to a 54 kDa protein, and the 88 kDa chitinase degraded to a trace of the 65 and 54 kDa protein during storage. Regarding the inhibition of *B. mori* chitinases, the specific inhibition of this enzyme and its kinetics of inhibition with allosamidin were investigated using partially purified chitinases from the alimentary canal and the integument. Both enzymes were inhibited competitively with a K_i of 0.072 and 0.106 μM, respectively (Koga et al., 1987a).

The partial amino acid and nucleotide sequences analyzed from *B. mori* chitinases and the 54 kDa protein (Koga et al., 1997) revealed that they include the two conserved regions of family 18 glycosyl hydrolases (Henrissat and Bairoch, 1993; Terwisscha van Scheltinga et al., 1994). The stereochemistry of chitin hydrolysis has been determined for several chitinases. Family 18 bacterial chitinases from *Bacillus circulans* WL–12 and *Streptomyces griseus* were shown to retain the configuration at C1 (Armand et al., 1994). This mechanism was also proposed for *S. marcescens* chitinase A (Perrakis et al., 1994). HPLC analysis of the products of the hydrolysis of chitopentaose showed that hevamine, a plant chitinase, acts with retention of the configuration (Terwisscha van Scheltinga et al., 1995). Human and hen lysozymes catalyze cleavage of the disaccharide with a retention mechanism (Dahlquist et al., 1969). X-ray structures of chitinase A, hevamine (glycosyl hydrolases family 18) and chitobiase (family 20) complexed with substrates or inhibitors identify a retaining mechanism involving a single glutamic acid as the catalytic acid and the carbonyl oxygen atom of the substrate's C2 N-acetyl group as the nucleophile (Tews et al., 1997). On the other hand, yam chitinase E (family 19 of the glycosyl hydrolases), which produces the α anomer (Fukamizo et al., 1995), may not form the oxazoline ring as the intermediate in the enzymatic reaction and acts in the inverting mechanism (Koga et al., 1998). Therefore, it is interesting to analyze the hydrolytic mechanism of an insect chitinase and compare its mechanism of action with those of families 18, 19, 20 of the glycosyl hydrolases.

In this study, we purified a 54 kDa protein from *B. mori* with chitinase activity and investigated its physicochemical properties, kinetic behavior, and the catalytic mechanism of its action on substrates as well as the cleavage patterns of N-acetylchitooligosaccharides.

2. Materials and methods

2.1. Chemicals

The following items were purchased as follows: DEAE-Cellulofine A–500 (Chisso Kogyo Co., Ltd), hydroxylapatite (Bio-Rad Laboratories), Butyl-Toyopearl 650 (M) (Tosoh Co., Ltd) and Fractogel EMD DEAE 650 (M) (Merck AG. Ltd, Germany). Glycolchitin was prepared by the method of Yamada and Imoto (1981) with reacetylation using acetic anhydride. *N*-Acetylchitooligosaccharides ($GlcNAc_n$, n=1–6) and allosamidin were generous gifts from Yaizu Suisankagaku Industry Co., Ltd, Shizuoka, Japan and Dr Sakuda, University of Tokyo, Tokyo, respectively. All of the reagents were of highest purity available.

2.2. Insect rearing and tissue collection

The silkworm, *Bombyx mori* (Kinshu×Showa), was reared on mulberry leaves at room temperature (about 27°C) with a photoperiod of 13:11 h light/dark. Integument was collected by dissection from fifth-instar larvae that were late in the third day after the beginning of spinning behavior. The integument was rinsed twice with 50 mM sodium phosphate buffer (pH 6.8) containing 20% sucrose, 1.0 mM diisopropylphosphofluoridate and a trace of N-phenylthiourea in an ice bath, immediately frozen on dry ice and stored at −80°C until used.

2.3. Purification of chitinase from B. mori

Unless otherwise noted, all operations were done at 4°C. Frozen integument from 100 insects was broken into pieces together with dry ice using a mortar and pestle, and homogenised in 3 volumes of 0.01 M sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 10 000***g*** for 20 min. The supernatant was mixed with DEAE-Cellulofine A–500 resin (500 ml) equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The mixture was gently stirred for 1 day, and then poured into a column (3×70 cm). After washing off the unadsorbed proteins with 0.01 M sodium phosphate buffer (pH 7.5) containing 0.10 M NaCl, chitinase was eluted with a linear gradient of NaCl from 0.10 to 0.70 M in 0.01 M sodium phosphate buffer (pH 7.5) at a flow rate of 50 ml/h. The active fraction was dialyzed against 0.01 M sodium phosphate buffer (pH 6.8) and applied to a hydroxylapatite column (2×20 cm) equilibrated with the same buffer. After washing off the unadsorbed proteins with 0.01 M sodium phosphate buffer (pH 6.8), chitinase was eluted with a linear gradient of sodium phosphate buffer (pH 6.8), from 0.01 to 0.30 M, followed by 0.50 M sodium phosphate buffer (pH 6.8), at a flow rate of 20 ml/h. The active fraction was dialyzed against 0.02 M sodium phosphate buffer (pH 7.5) containing 20% saturated ammonium sulfate, and applied to a Butyl-Toyopearl 650M column (2×20 cm) equilibrated with the same buffer. Proteins were successively eluted with 0.02 M sodium phosphate buffers (pH 7.5) containing 20, 10, 5 and 0% saturated ammonium sulfate. The active fraction eluted by the buffer containing 0% ammonium sulfate was dialyzed against 0.05 M sodium phosphate buffer (pH 7.0) and applied to a Fractogel

EMD DEAE 650(M) column (2×20 cm) equilibrated with the same buffer. After washing off the unadsorbed proteins with 0.05 M sodium phosphate buffer (pH 7.0), chitinase was eluted with a linear gradient of NaCl from 0.0 to 0.60 M in 0.05 M sodium phosphate (pH 7.0) at a flow rate of 20 ml/h.

2.4. Enzyme assay

Chitinase activity was measured using glycolchitin and *N*-acetylchitooligosaccharides as the substrates. For characterization of chitinase, 0.01–0.03 ml of the enzyme solution was mixed with 0.5 ml of 0.05% (w/v) glycolchitin dissolved in 50 mM sodium phosphate buffer (pH 8.0) and incubated at 32°C for 2–3 h. The reducing end group produced was measured colorimetrically at 420 nm with ferri-ferrocyanide reagent by the method of Imoto and Yagishita (1971). The kinetic experiments were performed in the reaction of the enzyme with glycolchitin (0.066–0.33 mg/ml) dissolved in Britton–Robinson buffer (Britton and Robinson, 1931) (pH 3.0–11.0) in the presence of allosamidin (0–1.0 μM) and the kinetic parameters were estimated from the difference absorbance at 420 nm (ΔA_{420}) between the sample and control experiments. When N-acetylchitooligosaccharides were used, 10 μl of the enzyme solution was mixed with 100 μl of $GlcNAc_n$ (n=2–6) dissolved in 50 mM sodium acetate buffer (pH 3.5–6.5), 50 mM sodium phosphate buffer (pH 6.0) or Britton–Robinson buffer (pH 3.0–11.0), and then incubated at 25°C. To measure the kinetic parameters, the enzymatic reaction proceeded linearly with the time for up to 20% completion of the reaction and then was stopped by boiling for 15 min. The reaction mixture was analyzed by HPLC (Shimadzu LC-10) with a Tosoh TSK–Gel G2000 PW column (0.75×60.0 cm) by the method of Koga et al. (1998).

2.5. Anomers formation by B. mori chitinase

To identify the anomeric forms produced due to enzymatic reaction with N-acetylchitooligosaccharides, 100 μl of 0.11 mM N-acetylchitooligosacharide dissolved in 4.4 mM sodium acetate buffer (pH 6.0) was reacted with 10 μl of the enzyme for 30 min at 25°C. The reaction mixture was immediately cooled in an ice bath, and 10 μl portion was analyzed by HPLC on a Tosoh TSK-Gel amide-80 column (0.46 ID×25 cm) at 28°C. The elusion of N-acetylchitooligosaccharides was performed with 70% acetonitrile at a flow rate of 0.70 ml/min, and monitored at 210 nm. The molar concentration of the oligosaccharides eluted was calculated using the relationship between the concentration of each oligosaccharide and its total peak-area of both anomers. Moreover, the calculation of the anomer ratio was performed based on the method of Koga et al. (1998) by comparing the peak-areas of both anomers in each N-acetylchitooligosaccharide.

2.6. Protein measurement

The absorbance was measured at 280 nm to monitor the proteins during chromatographic separation. The protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

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

SDS-PAGE was done with a 10% polyacrylamide slab gel containing 0.1% SDS (sodium dodecyl sulfate) by the method of Laemmli (1970). Protein sample (10 μl) was mixed with the same volume of 0.2 M Tris–HCl buffer (pH 6.8), containing 2% SDS, 2% 2-mercaptoethanol, 20% glycerol and 2×10^{-3}% bromophenol blue, and boiled for 1 min. The standard proteins used were myosin (200 kDa), MBP-β-galactosidase (158 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), glutamic dehydrogenase (55.6 kDa), MBP2 (maltose-binding protein) (42.7 kDa), lactate debydrogenase M (36.5 kDa), and triosephosphate isomerase (26.6 kDa) (BioLabs Inc., New England). After electrophoresis, proteins were stained with Coomassie Brilliant Blue R250.

3. Results

3.1. Purification of chitinase

Integuments from the fifth larval instar were used to prepare the enzyme. The supernatant of the homogenate was chromatographed on DEAE-Cellulofine A–500. Chitinase was eluted at a conductivity of 20 mmho (0.25 M NaCl) [Fig. 1(A)]. The active fraction was then chromatographed on hydroxylapatite, and eluted at a conductivity of 10 mmho (88 mM sodium phosphate) [Fig. 1(B)]. When Butyl-Toyopearl 650 M was used for further separation, the enzyme was mainly eluted at 0% ammonium sulfate [Fig. 1(C)]. This active fraction then applied onto a Fractogel EMD DEAE 650(M) column. Chitinase was eluted at a conductivity of about 4.0 mmho (0.04 M NaCl) [Fig. 1(D)]. The purity of these fractions was analyzed by SDS-PAGE. The results revealed that the enzyme was homogeneous and that the apparent molecular mass was 54 kDa (Fig. 2). The results of the purification are summarized in Table 1. At the final step, the 54 kDa chitinase was purified approximately 160-fold with a recovery of 7.2%.

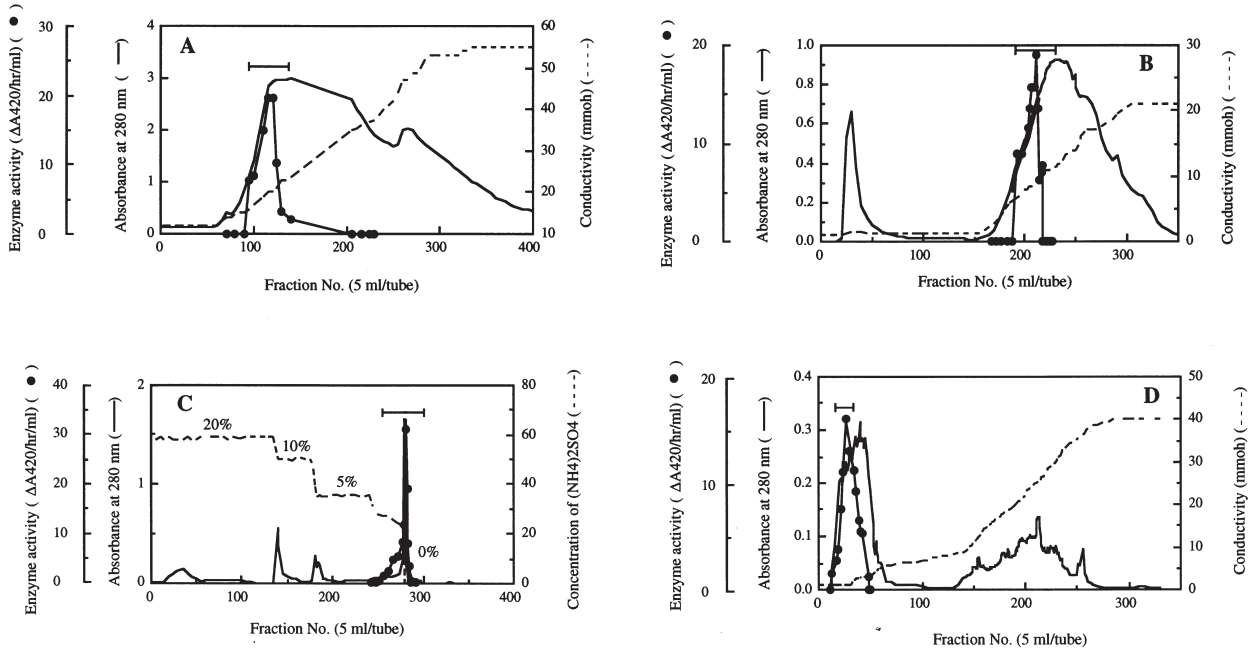


Fig. 1. Liquid chromatography of 54 kDa chitinase from *Bombyx mori*. (A) The homogenate obtained from the fifth larval instar of *B. mori* was chromatographed on a DEAE-Cellulofine A 500 column. (B) The active fractions indicated by the horizontal bar in (A) were pooled and chromatographed on a hydroxylapatite column. (C) The active fraction from (B) that indicated by the horizontal bar were collected and chromatographed on a Butyl-Toyopearl 560M column. (D) The active fractions indicated by the horizontal bar in (C) were collected and chromatographed on a Fractogel EMD DEAE 650 (M) column. The 54 kDa chitinase was eluted as indicated by the horizontal bar. For details, see Section 2.

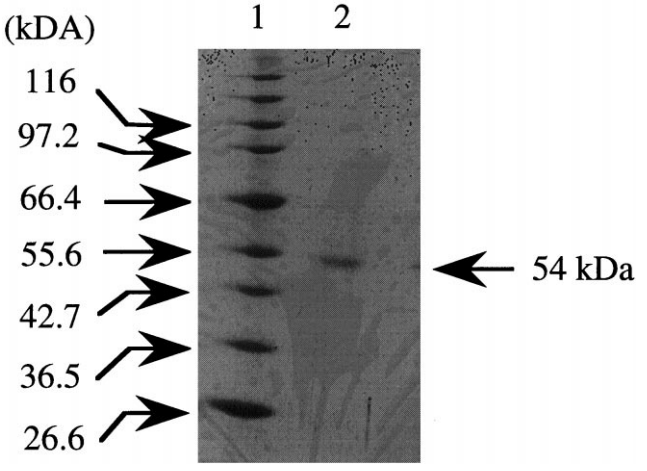


Fig. 2. SDS-PAGE of purified *B. mori* 54 kDa chitinase. The proteins were stained with Coomassie Brilliant Blue R 250. Lane 2, the 54 kDa chitinase (2.3 μg) obtained by Fractogel EMD-DEAE 650(M) column chromatography. Lane 1, represents broad range protein molecular mass markers, *from the top*, myosin (200 kDa), MBP-β-galactosidase (158 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), glutamic dehydrogenase (55.6 kDa), MBP2 (Maltose-binding protein) (42.7 kDa), lactate dehydrogenase M (36.5 kDa), triosephosphate isomerase (26.6 kDa).

3.2. Optimum pH and pH stability of B. mori 54 kDa chitinase

For investigation of the optimum pHs of this enzyme, the enzymatic reactions were conducted in Britton–Robinson buffer and sodium acetate buffer using a short substrate, $GlcNAc_5$, and a long substrate, glycolchitin, incubated at 25 and 32°C, respectively. In its reaction with $GlcNAc_5$, the enzyme showed the highest activities at pH 6.0 [Fig. 3(A)]. However, with glycolchitin, the pH activity profile indicated that this enzyme has a high activity between pH 9.5 and 10.0 and a low activity at pH 4.0 [Fig. 3(B)]. The pH stability of the 54 kDa chitinase was investigated in Britton–Robinson buffer. The results revealed that this enzyme was stable from pH 5.0 up to pH 11.0 [Fig. 3(C)].

3.3. Optimum temperature and thermal stability of B. mori 54 kDa chitinase

The optimum temperature of the 54 kDa chitinase was investigated by enzymatic reactions conducted at 10–70°C in 0.05 M sodium phosphate buffer (pH 6.0) using $GlcNAc_5$ as the substrate. This chitinase showed its highest activity at 60°C [Fig. 4(A)]. For the thermal stability, the 54 kDa chitinase was treated at 0–70°C for 15 min and the remaining activity was measured with 0.1 mM $GlcNAc_5$ at the optimum pH. As shown in Fig. 4(B), this enzyme was stable only up to 20°C. It was not thermostable.

3.4. Kinetic analysis

A detailed kinetic analysis of the enzymatic hydrolysis was conducted using a long substrate, glycolchitin, and

Table 1
Purification of *Bombyx mori* 54 kDa chitinase

Step	Total activity (ΔA420/h)	Total protein (mg)	Specific activity (ΔA420/h/mg)	Overall yield (%)	Overall purification
Crude extract	1890	15 100.0	0.13	100.0	1.0
DEAE-Cellulofine A-500	1330	604.0	2.20	70.2	17.6
Hydroxylapatite	551	121.0	4.56	29.2	35.5
Butyl-Toyopearl	166	11.8	14.10	8.80	113.0
Fractogel EMD-DEAE	136	6.72	20.2	7.18	162.0

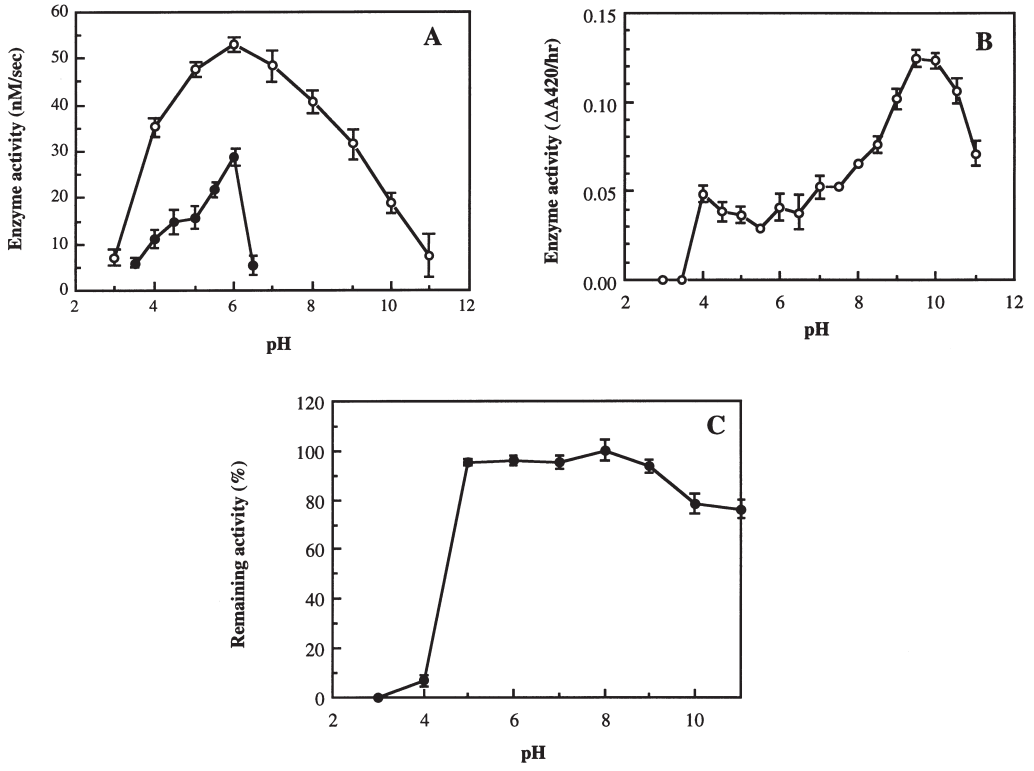


Fig. 3. Optimum pH and pH stability of 54 kDa chitinase from *B. mori*. (A) Optimum pH towards a short substrate. Activity of the 54 kDa chitinase (final concentration of 20 nM) was measured with $GlcNAc_5$ (final concentration of 0.1 mM) in sodium acetate buffer (pH 3.0–6.5) (solid circles) and Britton–Robinson buffer (pH 3.0–11.0) (open circles) at 25°C for 30 min. (B) pH-optimum towards a long substrate. Activity of the 54 kDa chitinase (final concentration of 12 nM) was measured with 0.05% (w/v) glycolchitin in Britton–Robinson buffer (pH 3.0–11.0) at 32°C. (C) pH stability. The 54 kDa chitinase (final concentration of 37 nM) was incubated in Britton–Robinson buffer, (pH 3.0–11.0) at 4°C for 5 h. The remaining activity was measured with 0.1 mM $GlcNAc_5$, and presented as a percentage against the activity of untreated enzyme. The points represent the mean values of two independent measurements. The ranges are shown by the vertical bars.

a shorter substrate, $GlcNAc_5$. The result of the reaction with glycolchitin is represented as a double reciprocal plots [Fig. 5(A)] and the values of kinetic parameters obtained are listed in Table 2. However, when the short substrate, $GlcNAc_5$ was used, a slightly negative slope was obtained from the double reciprocal plot at high substrate concentrations [Fig. 5(B)]. This may indicate that substrate inhibition occurred in the enzymatic reaction of N-acetylchitooligosaccharides, and therefore the kinetic parameters could not be obtained.

3.5. Kinetic analysis of inhibition of the 54 kDa chitinase

Inhibition kinetic analysis was performed using glycolchitin as a substrate incorporated with or without allosamidin. The enzyme was inhibited competitively as shown by the double reciprocal plots [Fig. 5(A)] and the K_i values are presented in Table 2. The results revealed that the degree of enzymatic inhibition was dependent on the pH of the reaction mixture. The inhibitor exerts

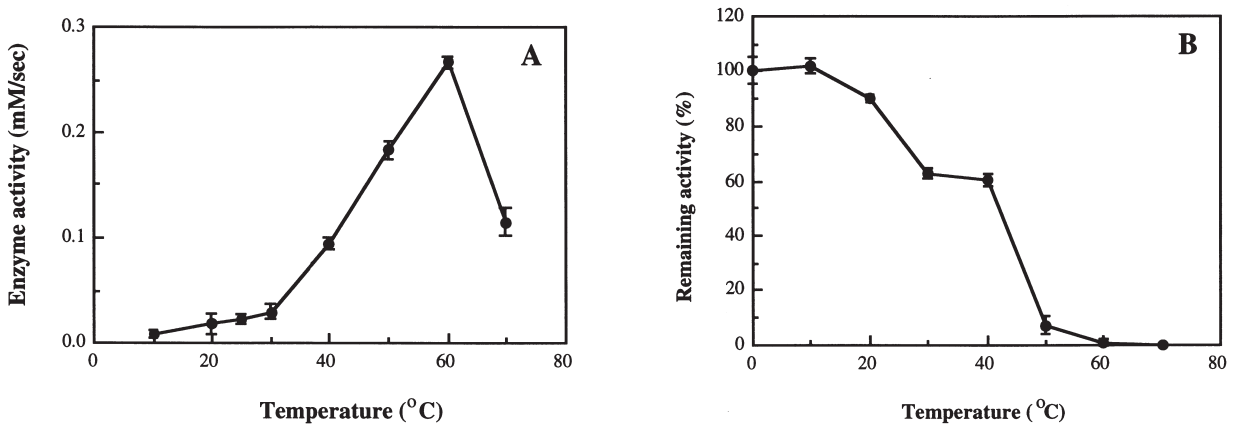


Fig. 4. Optimum temperature and thermal stability of *B. mori* 54 kDa chitinase. (A) Optimum temperature: activity of the chitinase (final concentration of 42.7 nM) was measured with 0.1 mM $GlcNAc_5$ in 0.05 M sodium phosphate buffer (pH 6.0) at temperatures of 10–70°C for 2–60 min. (B) Thermal stability: The 54 kDa chitinase (final concentration of 78.25 nM) was incubated with 0.05 M sodium phosphate buffer (pH 6.0) at the indicated temperature for 15 min. The remaining activity was measured with 0.1 mM $GlcNAc_5$, and indicated as a percentage relative to the activity of untreated enzyme. The points represent the mean values of two independent measurements. The ranges are shown by the vertical bars.

its strong inhibition toward the optimum pHs of the enzyme.

3.6. Cleavage pattern and substrate specificity of the 54 kDa chitinase

To investigate the cleavage patterns and substrate specificity of this chitinase, the initial velocities of its reactions with N-acetylchitooligosaccharides ($GlcNAc_n$, n=2–6) were measured using 0.1 mM N-acetylchitooligosaccharides in 0.05 M sodium phosphate buffer (pH 6.0). The results are shown in Table 3. It could be concluded that this chitinase cleave the N-acetylchitooligosaccharides except for the dimer as follows: trimer to dimer plus monomer, tetramer to two molecules of dimer, pentamer to dimer plus trimer, and hexamer to dimer plus tetramer as well as two molecules of trimer. The reactivities of the substrates by the 54 kDa chitinase was in the order of: $GlcNAc_6$>$GlcNAc_5$>$GlcNAc_4$>$GlcNAc_3$, demonstrating that this chitinase prefers the longer substrates. To estimate the cleavage patterns and cleavage rates by the 54 kDa chitinase, a Tosoh TSK–Gel amide-80 column was used for elusion of the reaction products. The results are presented in Table 3 and Fig. 7. N-Acetylchitotrisaccharide is cleaved into two patterns, OO|O (64.5%) and O|OO (35.5%) (the left side is the non-reducing end and (O), represents a GlcNAc moiety), N-acetylchitotetrasaccharide into three patterns, O|OOO (2.0%), OO|OO (93.4%) and OOO|O (4.6%), N-acetylchitohexasaccharide into two patterns, OO|OOOO (48.2%) and OOO|OOO (51.8%), while N-acetylchitopentasaccharide is cleaved into a pattern of OO|OOO (100%).

3.7. Identification of α and β anomeric forms

The N-acetylchitooligosaccharides (from monomer to hexamer) that were eluted from the Tosoh TSK-Gel amide–80 column showed two elusion peaks representing α anomer (earlier peaks) and β anomer (later peaks) (Fig. 6). The ratios of (α:β) anomeric forms in naturally occurring oligosaccharides were calculated from their respective peak areas according to Koga et al. (1998) and are presented in Fig. 6. The ratios of (α:β) anomers were almost similar (about 1:0.6) among the oligosaccharides. The products of the enzymatic reaction of 54 kDa chitinase with $GlcNAc_n$, (n=3–6) were analyzed and the results are shown in Fig. 7. The anomeric forms were determined by comparing the peaks of N-acetylchitooligosaccharides produced due to the enzyme hydrolysis. In the reaction with N-acetylchitotrisaccharide, the enzyme mainly produced the β anomer of the monomer and the dimer [Fig. 7(A)]. With N-acetylchitotetrasaccharide, the main product was the β anomer of the dimer [Fig. 7(B)]. With N-acetylchitopentasaccharide, the β anomer of the dimer was produced [Fig. 7(C)], and with N-acetylchitohexasaccharide, the enzyme produced β anomers of both the dimer and the trimer. These results indicate that *B. mori* 54 kDa chitinase hydrolyzes the substrates via a retaining mechanism and produces β anomers of the corresponding reaction products.

4. Discussion

Previously in our laboratory two isozymes of chitinase were purified from *B. mori* larval integument three days after the start of spinning behavior (Koga et al., 1997). It was also reported that the 88 kDa chitinase may be

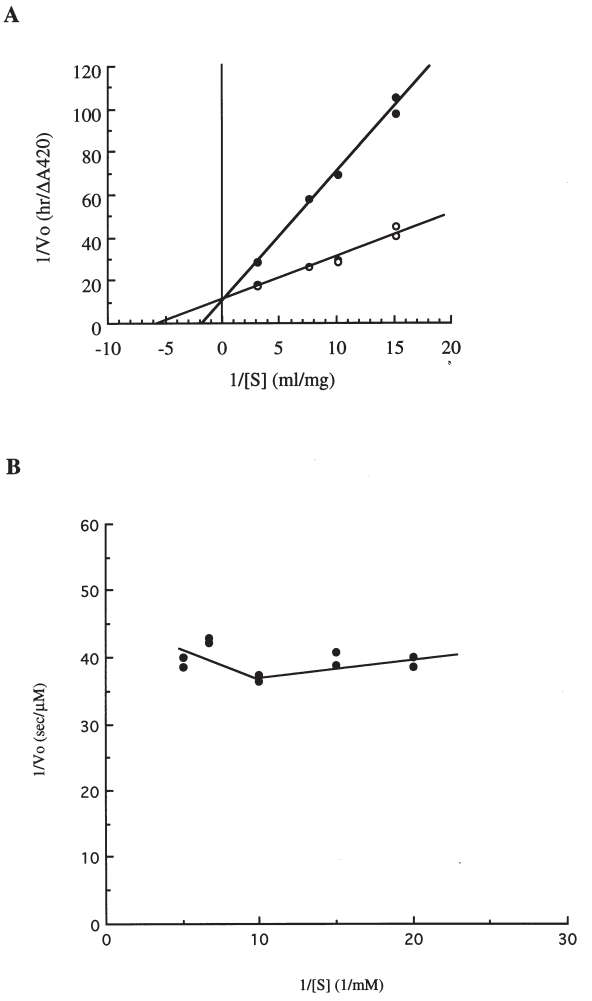


Fig. 5. Double reciprocal plots of *B. mori* 54 kDa chitinase using glycolchitin and N-acetylchitopentasaccharide as the substrates. (A) The 54 kDa chitinase (final concentration of 27 nM) was incubated with glycolchitin (0.066–0.33 mg/ml) in Britton–Robinson buffer (pH 9.5) in the absence (open circles) and presence of 0.1 μM allosamidin (solid circles) at 32°C for 3 h. For kinetic constants related to these plots, see Table 2. (B) Initial velocity of the 54 kDa chitinase (final concentration of 97.8 nM) was measured with $GlcNAc_5$ (final concentration 0.05–0.20 mM) in 0.05 M sodium phosphate buffer (pH 6.0) at 25°C.

successively processed to a 65 kDa chitinase and a 54 kDa protein. In this study, we conducted further investigations to test for the chitinase activity of the 54 kDa protein. Moreover, Koga et al. (1997) observed that the 88 kDa chitinase mainly appears at the early stage of one day before ecdysis, whereas the 65 kDa chitinase appears at a later stage. Therefore, we tried to purify the chitinase from the larval integuments later on the third day after the beginning of the spinning behavior. In the first step of the liquid chromatography, DEAE-Cellulofine A-500 was useful for separation of chitinase from a large amount of sample [Fig. 1(A)]. Hydroxylapatite resolved chitinase from the N-acetylhexosarninidase in the next chromatography [Fig. 1(B)]. The 54 kDa chitinase was eluted in the same pattern as the 65 kDa and 88 kDa chitinases by DEAE-Cellulofine A-500 and hydroxylapatite column chromatography. At the third step, Butyl-Toyopearl 650M column chromatography purified the chitinase from other proteins and the elusion of the 54 kDa chitinase in this step is unlike that of the 65 and 88 kDa chitinases which are eluted at 5% saturated ammonium sulfate (Koga et al., 1997). The 54 kDa protein eluted at 0% saturated ammonium sulfate. Fractogel EMD DEAE 650 (M) chromatography separated the 54 kDa chitinase in an earlier fractions (0.04 M NaCl) [Fig. 1(D)]. This chromatographic pattern is also different from those of the 65 and 88 kDa chitinases. The 65 kDa chitinase is eluted at 0.25 M NaCl and the 88 kDa chitinase is eluted at 0.39 M NaCl. The 54 kDa chitinase is only weakly adsorbed to the Fractogel EMD DEAE 650 (M) resin.

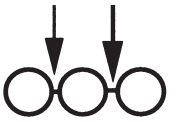
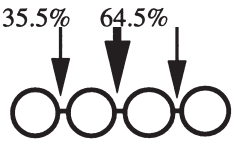
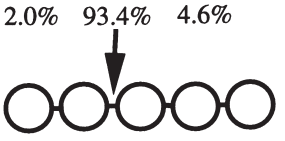
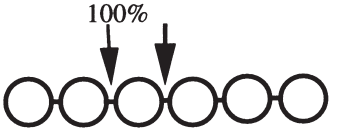
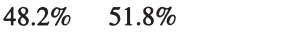
In comparison with the properties of other insect chitinases reviewed by Koga et al. (1997), *B. mori* 54 kDa chitinase has similar properties to other insect chitinases with respect to the molecular mass and the optimum pH. The optimum pHs of the 54 kDa chitinase depend on the substrate used, about pH 6.0 towards a short substrate, $GlcNAc_5$, and pH 10.0 (high activity) together with pH 4.0 (low activity) towards a long substrate, glycolchitin. This pH activity profile is similar to that of the 65 and 88 kDa chitinases from *B. mori* (Koga et al., 1997) and

Table 2
Kinetic parameters of *B. mori* 54 kDa chitinase in the reaction with glycolchitin[a]

	pH						
	4	5	6	7	8	9.5	10
K_m (mg/ml)	0.307	0.118	0.092	0.138	0.219	0.192	0.503
k_{cat} (l/s)	0.011	0.010	0.006	0.008	0.019	0.023	0.049
k_{cat}/K_m (ml/mg/s)	0.036	0.085	0.065	0.058	0.087	0.120	0.100
K_i (μM)	0.356	0.746	0.552	0.124	0.052	0.038	0.013

[a] Enzymatic reactions were conducted as described in [Fig. 5(A).]

Table 3
Substrate specificity of *B. mori* 54 kDa chitinase[a]

Substrate	Reaction	Initial velocity (nM/s±SE)	Cleavage patterns and relative destribution
$GlcNAc_2$ (II)	No reaction		
$GlcNAc_3$ (III)	III→II+I[b]	11.0±0.5 (1)	
$GlcNAc_4$ (IV)	IV→2 II	22.6±0.6 (2.1)	
$GlcNAc_5$ (V)	V→II+III	30.5±0.1 (2.8)	
$GlcNAc_6$ (VI)	VI→	31.4±1.3 (2.9)	
	→II+IV	23.1±0.8	
	→2 III	8.3±0.6	

[a] Initial velocities were measured in the reaction of 0.10 mM N-acetylchitooligosaccharides with 20 nM *B. mori* 54 kDa chitinase in 0.05 M sodium phosphate buffer (pH 6.0) at 25°C. The ratios of initial velocities to that for $GlcNAc_3$ are shown in parentheses.

[b] I and ○ represent GlcNAc. The left side in cleavage patterns and relative distribution is the non-reducing end. The arrows show the cleavage sites, while the percentages of the cleavage sites were shown at the bottom.

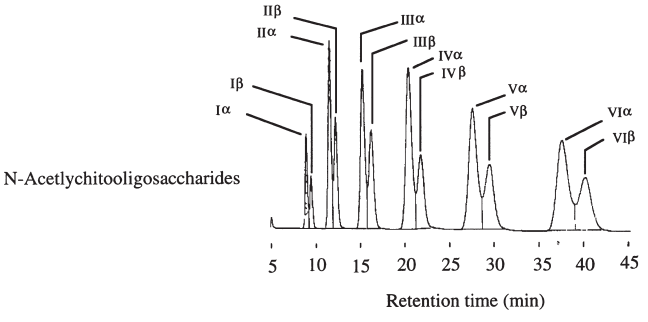


Fig. 6. HPLC analysis of anomeric forms of N-acetylchitooligosaccharides. As the standards, N-acetylchitooligosaccharides (final concentration of each 0.1 mM) from the monosaccharide to hexasaccharide were analyzed by HPLC. Their anomeric forms were identified as represented above the elusion peaks, and the ratio of the anomers are calculated and represented at the bottom of the panel.

that of *Manduca sexta* chitinases (Koga et al., 1983b). However, when comparing the specific activity of the 54 kDa chitinase (Table 1) with those of *B. mori* 65 and 88 kDa chitinases (Koga et al., 1997), the 54 kDa chitinase is less active than the other chitinases. As for temperature stability of the 54 kDa chitinase, this chitinase was not thermostable [Fig. 4(B)] relative to the other chitinases from *B. mori*, because the enzyme retains its activity only up to a temperature of 20°C, while the 65 kDa and the 88 kDa chitinases conserve a high activity up to a temperature of 30 and 40°C, respectively (Koga et al., 1997).

To investigate the enzymatic action of this chitinase, kinetic analysis was performed using a long substrate, glycolchitin, and a number of short substrates, N-acetylchitooligosaccharides ($GlcNAc_n$, n=2–6). The results of reaction with the short substrates indicate that the 54 kDa chitinase is an endo-type, and prefers the longer substrates (Table 3). Kinetic parameters (k_{cat} and K_m) could not be obtained in the reaction of $GlcNAc_5$, because at high substrate concentrations, a slightly negative slope was obtained from the double reciprocal plot. This kinetic behavior is the same as that of the 65 and 88 kDa chitinases and is probably due to substrate inhibition, suggesting that such a short substrate, $GlcNAc_5$, is not substrate in vivo but rather a product for chitinases (Koga et al., 1997). However, the only exception is that, the level of the enzyme inhibition is weak relative to that of the 88 and 65 kDa chitinases and this difference may be attributed to the assumption that the 54 kDa chitinase lacks the chitin binding domain that might be

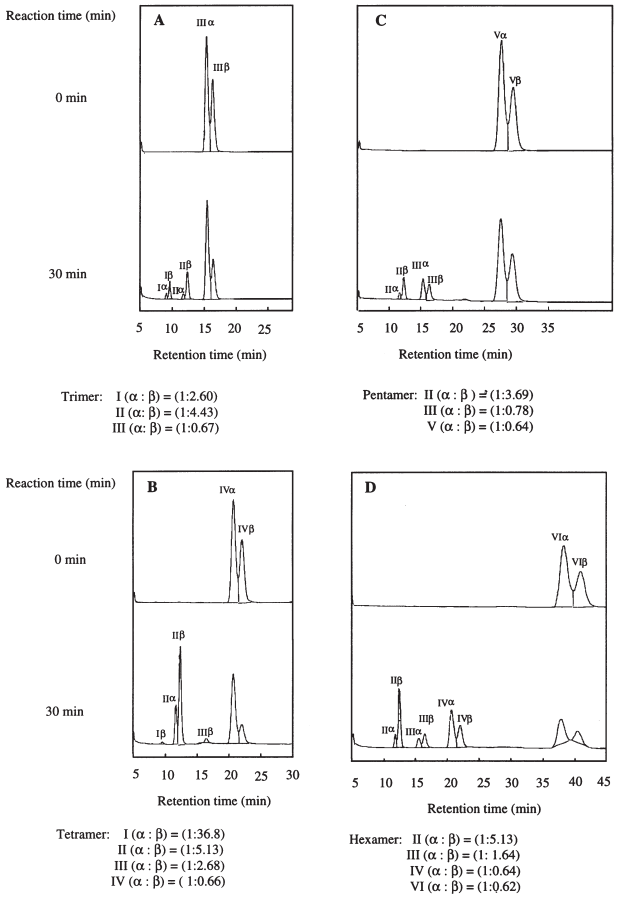


Fig. 7. HPLC analysis of the reaction of N-acetylchitooligosaccharides by *B. mori* 54 kDa chitinase. N-Acetylchitooligosaccharides (trisaccharide to hexasaccharide) (final concentration of 0.1 mM) were reacted with *B. mori* 54 kDa chitinase (final concentration 195 nM) for 30 min in 4.4 mM sodium acetate buffer (pH 6.0), at 25°C. The reaction mixtures were analyzed by HPLC as described in Section 2. (A), (B), (C), and (D) show the reactions of the trisaccharide, tetrasaccharide, pentasaccharide, and hexasaccharide of N-acetylchitooligosaccharides, respectively. The ratios of α and β anomers of N-acetylchitooligosaccharides after a 30 min reaction are represented below the panels.

located at the C-terminal side of the larger enzyme from which it is processed. Furthermore, the 54 kDa chitinase is similar to *B. mori* 65 and 88 kDa chitinases with respect to the cleavage pattern of the N-acetylchitooligosaccharides. Regarding to the substrate specificity, however, the 54 kDa chitinase is rather less specific to the longer chitooligosaccharides (Table 3) compared to the 65 kDa and 88 kDa chitinases (specificities to $GlcNAc_n$, n=4–6, for both chitinases ranged among 4.3–7.4) but it is more active towards the $GlcNAc_3$. On the other hand, when comparing the kinetic parameters obtained with glycolchitin (Table 2) such as $1/K_m$, k_{cat} and k_{cat}/K_m (at pH 6.0) with those of the 65 and 88 kDa chitinases from *B. mori* (k_{cat}=0.084 s^{-1}; K_m=0.134 mg/ml; k_{cat}/K_m=0.63 ml/mg/s for the 65 kDa chitinase at pH 5.5 and k_{cat}=0.059 s^{-1}; K_m=0.023 mg/ml; k_{cat}/K_m=2.60 ml/mg/s for the 88 kDa chitinase at pH 6.5), the 88 kDa chitinase has a 5.8-fold and a 4-fold higher affinity for glycolchitin than the 65 and 54 kDa chitinases, respectively. The 65 kDa chitinase catalyzes the reaction from the enzyme-substrate complex to the products 1.4 times faster than the 88 kDa chitinase and 14 times faster than the 54 kDa chitinase. The 88 kDa chitinase is approximately 4-fold and 40-fold more active in the overall reaction than the 65 kDa and 54 kDa chitinases, respectively. The partial N-terminal amino acid sequences of a mixture of the three chitinases were identical (Koga et al., 1997). However, these isozymes showed some differences in their activities and kinetic behaviors. These results suggest that the *B. mori* 88 kDa chitinase has a strong chitin binding domain located at the C-terminal side, and that for the process of ecdysis to be terminated, this enzyme may undergo a limited proteolysis from the C-terminal amino acid side releasing the smaller chitinases which are less active against the long substrates but comparatively more active towards the short chitooligosaccharides. Accordingly, we can conclude that a smaller chitinase with a lower activity against the natural substrate and a relatively higher activity against the products of chitin was released at a later stage during the process of ecdysis leading to a termination of the process.

Terwisscha van Scheltinga et al. (1995) reviewed the mechanism of glycoside hydrolysis with retention of configuration that involves two carboxylates. One donates a proton to the β-(1–4)-glycosidic oxygen, and aglycon departure is assisted by the second residue, which is thought either to make a covalent glycosyl intermediate or to provide an ion-pair stabilisation to the oxocarbonium ion intermediate. After the aglycon has diffused away, a water molecule enters the active site and performs a second nucleophilic substitution at the carbohydrate C1. This results in an overall retention of the anomeric configuration. Stabilisation is proposed to occur via a covalent oxazolinium ion intermediate for the retaining chitinolytic enzymes (Tews et al., 1997). To investigate the catalytic action of the hydrolysis of substrates with *B. mori* chitinases, the 54 kDa chitinase was reacted with N-acetylchitooligosaccharides. HPLC analysis of the reaction products revealed that the 54 kDa chitinase produces β anomers [Fig. 7(A–D)]. These data were identical to preliminary data obtained from a mixture of *B. mori* chitinases reacted with the same substrates (data not shown). This may suggest that *B. mori* chitinases hydrolyze the substrates, N-acetylchitooligosaccharides, and produce β anomer of the products. Furthermore, it also suggests that, similar to other family 18 chitinases (Terwisscha van Scheltinga et al., 1995; Tews et al., 1997), *B. mori* 54 kDa chitinase may form the oxazoline ring as the intermediate in the enzymatic reaction and acts in the retaining mechanism, since the newly produced oligosaccharides are all β anomers. This mechanism also relates to the inhibition of *B. mori* chitinases by the dead end analog inhibitor, allosamidin.

The compound consists of two β-(1–4) linked N-acetyl-allosamine units and a unique oxaxoline derivative, allosamizoline, in which the methly group is substituted by dimethylamine that stabilises the C1–O7 bond. Therefore, allosamidin may exert its inhibitory effect by acting as a nonhydrolysable analog of the oxazolinium ion intermediate (Tews et al., 1997). The 54 kDa chitinase was competitively inhibited by allosamidin with K_i values depending on the pH of the reaction mixture (Table 2). This result is in a general agreement with the findings of Koga et al. (1987a). Interestingly, yam chitinase E (family 19 glycosyl hydrolases) is not inhibited by allosamidin (Koga, 1996), it produces α anomers of the reaction products (Fukamizo et al., 1995) and the enzyme hydrolyzes the substrates in an inverting mechanism without forming an oxazoline ring as an intermediate for the reaction (Koga et al., 1998). This inhibition mechanism may be useful to clarify the inhibitory behavior of allosamidin to insect chitinases.

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