Kinetics of a Chitinase from a Prawn, *Penaeus japonicus*

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Kinetic analysis was done on a chitinase (EC 3.2.1.14) purified from the liver of a prawn, *Penaeus japonicus*, using N-acetylchitooligosaccharides (GlcNAcₙ, n = 2 to 6), p-nitrophenyl N-acetylchitooligosaccharides (pNP-GlcNAcₙ, n = 1 to 5), and colloidal chitin as the substrates. The enzyme hydrolyzed GlcNAc₄ to two molecules of GlcNAc₃, GlcNAc₂ to GlcNAc₁ plus GlcNAc₃, and GlcNAc₂ by two ways to GlcNAc₄ plus GlcNAc₂ (87%), and two molecules of GlcNAc₃ (13%). Neither GlcNAc₁ nor GlcNAc₂ was hydrolyzed. The *Kₘ* and *kₐ* were 0.249 mM and 3.38 sec⁻¹ for GlcNAc₃, 0.018 mM and 2.67 sec⁻¹ for GlcNAc₄, and 0.005 mM and 2.72 sec⁻¹ for GlcNAc₅, respectively. The cleavage patterns of p-nitrophenyl N-acetylchitooligosaccharides were different from those of the corresponding N-acetylchitooligosaccharides. The enzyme hydrolyzed colloidal chitin to produce mainly GlcNAc₂ and a trace of GlcNAc₃. Allosamidin inhibited prawn chitinase in a competitive way with a *Kᵢ* of 0.1 μM and *IC₅₀* of 0.14 μM. These results suggest that prawn chitinase is an endo-type chitinolytic enzyme, but different from insect chitinase or yam chitinase in the substrate specificity and cleavage pattern.

Chitinase is widely distributed in nature, but the physiological roles are different among various sources. Insect chitinase acts in ecdysis,¹ and plant chitinase does in self-defense against pathogens.²⁻³ Prawn chitinase seems to act in the molting process or the digestion of chitin-containing foods. As their physiological roles are different, their physicochemical properties and enzymatic reactions are expected to be different. The relative molecular mass of prawn chitinase (37 kDa)⁴ is similar to that of yam chitinase (33.5 kDa),⁵ but not to the insect chitinases (50 to 88 kDa)⁶⁻⁷ and Japanese eel chitinase (50 kDa).⁸ Some differences have also been observed in the cleavage pattern of N-acetylchitooligosaccharides and inhibition by allosamidin between yam chitinase⁹,¹⁰ and insect chitinases.⁶⁻⁷ With respect to the enzyme type, several enzymes are known. Two types of chitinolytic enzymes involved in insect ecdysis have been purified from the tobacco hornworm.¹¹ One is an endo-type enzyme, chitinase,⁶ and the other is an exo-type enzyme, β-N-acetylglucosaminidase (EC 3.2.1.30).¹¹⁻¹³ This exo-type enzyme releases GlcNAc from the non-reducing end of N-acetylchitooligosaccharides.¹³ However, another exo-type enzyme that releases a GlcNAc₂ unit from the terminus of the substrate has been reported in some microorganisms, and it is proposed to call it exochitinase.¹⁴,¹⁵

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Abbreviations: GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranoside; GlcNAcₙ, N-acetylchitooligosaccharides; HPLC, high performance liquid chromatography; pNP-GlcNAcₙ, p-nitrophenyl N-acetylchitooligosaccharides.
a chitinase which catalyzes a transglycosylation reaction has been found.\textsuperscript{16} It has also been clarified that yam chitinase is a random-type enzyme that hydrolyzes GlcNAc\(_6\) in all ways to GlcNAc plus GlcNAc\(_5\), GlcNAc\(_2\) plus GlcNAc\(_4\) and two molecules of GlcNAc\(_3\).\textsuperscript{9} Now we know several types of chitinase such as endo-type, random-type, transglycosylation-catalyzing type, and two different exo-type enzymes. Therefore, our interest is focused on the enzyme type of prawn chitinase.

In this study, we did steady-state kinetics to investigate the substrate specificity and hydrolysis fashion of prawn chitinase, and the inhibition by allosamidin.

**Materials and Methods**

**Materials.** N-Acetylchitooligosaccharides (GlcNAc\(_n\), \(n = 1\) to 6) were prepared from crab chitin (a generous gift from Katakurachikkarin Co., Ltd., Tokyo) by the method of Raftery et al.\textsuperscript{17} p-Nitrophenyl N-acetylc chitooligosaccharides (pNp-GlcNAc\(_n\), \(n = 1\) to 5) were generous gifts from Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan. Colloidal chitin was prepared from chitin by the method of Tracey.\textsuperscript{18} Allosamidin was a generous gift from Dr. A. Suzuki and Dr. A. Isogai (The University of Tokyo). Other reagents were all of analytical grade.

**Enzyme.** Chitinase was purified from the liver of a prawn, *Panaceus japonicus*, by the method of Kono et al.\textsuperscript{8} The concentration of enzyme was calculated from the absorbance at 280 nm using a molar extinction coefficient of 64,700.\textsuperscript{41}

**Enzyme assay.** Chitinase activity was assayed with various substrates. When N-acetylcchitooligosaccharides or their p-nitrophenyl derivatives were used as the substrate, the enzymatic reaction was done in a 10-ml glass tube (cone-type with a screw cap, Pyrex, Iwaki Glassware Corp., Japan) as follows:\textsuperscript{9,11} 10 \(\mu\)l of the enzyme solution (final concentration, 10 or 15 nm) was added to 100 \(\mu\)l of GlcNAc\(_n\) (\(n = 2\) to 6) (final concentration, 0.05 to 0.2 mm) or pNp-GlcNAc\(_n\) (\(n = 1\) to 5) (final concentration, 0.1 mm) dissolved in 50 mm Na–phosphate buffer, pH 6.7, in the absence or presence of allosamidin (0 to 8.33 \(\mu\)m), and incubated at 25°C. When the reaction had proceeded to less than 20% (8 to 40 min), it was stopped by boiling for 30 min. A 10-\(\mu\)l portion of the reaction mixture was injected into a Tosoh TSK Gel G2000 PW column (0.75 \times 60 cm) with a Tosoh TSK PWL Guard column (0.75 \times 7.5 cm) as a first column, and eluted with distilled water at a flow rate of 0.5 ml/min. The N-acetylchitooligosaccharides eluted were monitored at 210 nm, and the concentration of each N-acetylcchitooligosaccharide was estimated from the height on the chromatogram compared with that of authentic GlcNAc\(_n\). The initial velocity was calculated from both concentrations of the unreacted substrate and products. Kinetic parameters (\(K_m\) and \(k_{cat}\)) were calculated by the non-linear optimization method described elsewhere\textsuperscript{13} with a NEC ACOS-850 computer (Yamaguchi University), using an initial velocity equation, \(v = V_{\text{max}} \times S/(K_m + S)\).

When colloidal chitin was used as the substrate, the enzymatic reaction was done as follows. The reaction mixture consisting of 0.5 ml of 2.5 mg/ml colloidal chitin and 0.2 ml of 100 mm Na–phosphate buffer, pH 6.7, 2.5 ml of distilled water, and 0.5 ml of 1 \(\mu\)m prawn chitinase (final concentration, 143 nm) was incubated at 37°C with agitation for the given time. After measurement of the turbidity at 430 nm, a 100-\(\mu\)l portion of the reaction mixture was boiled for 30 min, and then centrifuged at 3,000 rpm for 10 min. After filtration of the supernatant through a membrane filter (0.45 \(\mu m\), HV type, Nippon Millipore Co., Ltd.), 10 \(\mu\)l of the filtrate was analyzed with a Tosoh TSK Gel G2000 PW column as mentioned above.

**Results**

**Substrate specificity and cleavage pattern**

To investigate the substrate specificity and cleavage pattern, the enzymatic reactions were done with GlcNAc\(_n\) (\(n = 2\) to 6) as the substrates. The results are shown in Table I. Prawn chitinase hydrolyzed GlcNAc\(_4\) to two molecules of GlcNAc\(_2\), GlcNAc\(_3\) to GlcNAc\(_2\) plus GlcNAc\(_3\), and GlcNAc\(_5\) to GlcNAc\(_2\) plus GlcNAc\(_4\) and two molecules of GlcNAc\(_3\). Neither GlcNAc\(_2\) nor GlcNAc\(_3\) was cleaved. The release of GlcNAc and the products with longer chains than the substrate were not observed in any case under these conditions. The reactivity of the substrates was in the following order, GlcNAc\(_6\) \(>\) GlcNAc\(_5\) \(>\) GlcNAc\(_4\).

pNp-GlcNAc\(_n\) (\(n = 1\) to 5) were also used as the substrates. The results are shown in Table II. The reactivity of pNp-GlcNAc\(_4\) was greater than that of pNp-GlcNAc\(_5\). Neither pNp-GlcNAc nor pNp-GlcNAc\(_2\) was cleaved, and pNp-GlcNAc\(_3\) was little cleaved. The cleavage sites of pNp-GlcNAc\(_4\) were the second (80%) and fourth (20%) \(\beta\)-1,4-linkage from the non-reducing end, and those of pNp-GlcNAc\(_5\) were the first (33%), second (50%), and third
Table I. SUBSTRATE SPECIFICITY OF PRAWN CHITINASE

Prawn chitinase (10 nM) was incubated with 0.1 mM substrate in 50 mM Na-phosphate buffer, pH 6.7, at 25°C. After an appropriate time, the reaction mixture was boiled for 30 min, and a 10-μl portion was analyzed by HPLC as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>Initial velocity (μM/min)</th>
<th>Cleavage pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc₂ (II)</td>
<td>Not detected</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>GlcNAc₃ (III)</td>
<td>Not detected</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>GlcNAc₄ (IV)</td>
<td>IV→II</td>
<td>0.635±0.082</td>
<td>G-G-G-G and/or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAc₅ (V)</td>
<td>V→II+III</td>
<td>1.40±0.14</td>
<td>G-G-G-G-G and/or</td>
</tr>
<tr>
<td>GlcNAc₆ (VI)</td>
<td>VI→II</td>
<td>1.52±0.15 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>→II+IV</td>
<td>1.33±0.12 (87%)</td>
<td>G-G-G-G-G-G</td>
</tr>
<tr>
<td></td>
<td>→2II</td>
<td>0.193±0.033 (13%)</td>
<td>G-G-G-G-G-G</td>
</tr>
</tbody>
</table>

Mean values ± SE, n=3.

Table II. SUBSTRATE SPECIFICITY OF PRAWN CHITINASE

The reactions were done as described in Table I, but the substrates were pNP-N-acetyltchitosaccharides.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>Initial velocity (μM/min)</th>
<th>Cleavage pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNP-GlcNAc (P-I)</td>
<td>Not detected</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pNP-GlcNAc₂ (P-II)</td>
<td>Not detected</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pNP-GlcNAc₃ (P-III)</td>
<td>A trace</td>
<td>1.01±0.12 (100%)</td>
<td>G-G-G-G-G-P</td>
</tr>
<tr>
<td>pNP-GlcNAc₄ (P-IV)</td>
<td>P-IV→</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→II+P-II</td>
<td>0.803±0.119 (80%)</td>
<td>G-G-G-G-G-P</td>
</tr>
<tr>
<td></td>
<td>→IV+P</td>
<td>0.205±0.026 (20%)</td>
<td>G-G-G-G²P</td>
</tr>
<tr>
<td></td>
<td>→I+P-IV</td>
<td>0.309±0.189 (33%)</td>
<td>G-G-G-G-G-G-P</td>
</tr>
<tr>
<td></td>
<td>→II+P-III</td>
<td>0.465±0.067 (50%)</td>
<td>G-G-G-G-G-P</td>
</tr>
<tr>
<td></td>
<td>→III+P-II</td>
<td>0.163±0.052 (17%)</td>
<td>G-G-G-G-G-P</td>
</tr>
</tbody>
</table>

Mean values ± SE, n=3. 1, GlcNAc.

(17%). In the reactions of p-nitrophenyl derivatives, monomeric residues such as p-nitrophenol and GlcNAc were released from pNP-GlcNAc₄ and pNP-GlcNAc₅, respectively.

Kinetic analysis

A detailed kinetic analysis was done on the enzymatic hydrolysis of a series of N-acetyltchitosaccharides ranging in chain length from 2 to 6. The results are represented as double reciprocal plots in Fig. 1. The kinetic parameters calculated by the non-linear optimization method are shown in Table III. The Km decreased and the k-cat/Km increased as the substrate chain length increased.

Reaction of colloidal chitin by prawn chitinase

A course of the reaction of colloidal chitin is shown in Fig. 2. Prawn chitinase decreased the turbidity of colloidal chitin solution and produced mainly GlcNAc₂ with a small amount of GlcNAc₃ during the course of the reaction. The production ratio of GlcNAc₃ to
The observations that GlcNAc\(_3\) was not detected. No time lag was observed in the production of \(N\)-acetylchitooligosaccharides, and their production proceeded proportionally to the decrease of turbidity at 430 nm. The overall reactions were calculated both from the decrease of turbidity and the production of \(N\)-acetylchitooligosaccharides, and the ratio of the former to the latter was almost constant, about 1:0.73, during the reaction.

**Inhibition of prawn chitinase by allosamidin**

The effects of allosamidin on prawn chitinase were investigated using GlcNAc\(_5\) as the substrate. Prawn chitinase was inhibited by allosamidin with an IC\(_{50}\) of 0.14 \(\mu\)M with a correlation factor of 0.93. (Fig. 3) As shown in the double reciprocal plots (Fig. 4), the enzyme was inhibited in a competitive way. The inhibition constant, \(K_i\), was calculated to be 0.1 \(\mu\)M. (Inset of Fig. 4)

**Discussion**

As shown in Table I, the hydrolysis patterns of GlcNAc\(_n\) (\(n=2\) to 6) suggest that prawn chitinase is a typical endo-type hydrolytic enzyme. With respect to the hydrolysis patterns, prawn chitinase is different from tobacco hornworm chitinase\(^{6}\) and yam chitinase.\(^{9}\) That is, prawn chitinase did not degrade GlcNAc\(_3\), while yam chitinase and the insect chitinases did. The enzyme did not release GlcNAc from GlcNAc\(_6\), but yam chitinase did. As the substrate chain length increased, the reactivity increased in the following order, GlcNAc\(_6\) > GlcNAc\(_5\) > GlcNAc\(_4\). (Tables I and III) The insect chitinase has a substrate specificity similar to prawn chitinase, although such a large difference in the reactivity among the substrates was not observed in the insect chitinase.\(^6\) However, yam chitinase showed a different substrate specificity both from prawn chitinase and insect chitinase, and the reactivity of \(N\)-acetylchitooligosaccharides by yam chitinase is the following order, GlcNAc\(_4\) > GlcNAc\(_5\) > GlcNAc\(_6\) > > GlcNAc\(_3\).\(^9\) Prawn chitinase hydrolyzed colloidal chitin to produce mainly GlcNAc\(_2\) with a small amount of GlcNAc\(_3\). The observation that GlcNAc\(_3\) was
Fig. 2. Reaction of Colloidal Chitin by Prawn Chitinase.
The enzyme (143 nM) was incubated with 0.36% (w/v) colloidal chitin in 5.7 mM Na-phosphate buffer, pH 6.7, at 37°C. At the given times, the turbidity of reaction mixture was measured at 430 nm, and the reaction product of soluble N-acetylchitooligosaccharides was analyzed by HPLC as described in Materials and Methods. Panel A: Changes in the turbidity of colloidal chitin solution (●) and N-acetylchitooligosaccharides produced, GlcNAc_2 (○) and GlcNAc_3 (△). Panel B: Top, HPLC pattern of N-acetylchitooligosaccharides produced from colloidal chitin after 57 hr; bottom, authentic GlcNAc(I), GlcNAc_2(II) and GlcNAc_3(III).

Fig. 3. Estimation of IC_{50} of Allosamidin for Prawn Chitinase.
The enzyme (10 nM) was incubated with 0.1 mM GlcNAc_3 in the presence of allosamidin (0 to 8.33 μM) in 50 mM Na-phosphate buffer, pH 6.7, at 25°C for 10 min. The reaction mixture was analyzed by HPLC as described in Materials and Methods. The arrow indicates IC_{50}. 
produced in addition to GlcNAc₂ from colloidal chitin supports the idea that prawn chitinase is an endo-type chitinase, but not an exo-type chitinase, which releases GlcNAc₆ unit from chitin. In the reaction of colloidal chitin by chitinases from yam, Japanese eel, and the silkworm, we have not observed the production of N-acetylchitooligosaccharides with larger chains than GlcNAc₆. We considered that since colloidal chitin is not soluble in an aqueous solution, only solution-contactable residues might be hydrolyzed by the enzyme. This reaction is probably a rate-limiting step. The N-acetylchitooligosaccharides released might be immediately hydrolyzed to the final products. With respect to the hydrolytic products from colloidal chitin, prawn chitinase is similar to silkworm chitinase, but different from yam chitinase, which releases only GlcNAc₂ and Japanese eel chitinase, which releases GlcNAc and GlcNAc₂.

To estimate the cleavage sites of N-acetylchitooligosaccharides by prawn chitinase, the corresponding chromogenic oligosaccharide analogues, pNP-GlcNAc₆, were used as the substrates. Comparing Tables I and II, however, they differed in the cleavage patterns. GlcNAc₄ was cleaved with the initial velocity of 0.635 μM/min, while pNP-GlcNAc三点 was little cleaved. GlcNAc₃ and GlcNAc₆ were cleaved in an endo-fashion, while monomeric residues such as p-nitrophenol and GlcNAc were released from pNP-GlcNAc₄ and pNP-GlcNAc₆, respectively. These differences suggest that GlcNAc and p-nitrophenyl residues were recognized differently by prawn chitinase. However, such a difference between GlcNAc and p-nitrophenyl residues was not observed in yam.
chitinase. \(^9\) Comparing prawn chitinase with yam chitinase, \(^9\) bacterial chitinases such as *Pseudomonas stutzeri*, *Serratia marcescens* and *Streptomyces griseus* \(^14\) on the hydrolysis of pNp-GlcNAc\(_2\), a large difference is found in these hydrolytic properties. Yam chitinase cleaved the first \(\beta\)-1,4-linkage from the non-reducing end, and bacterial chitinases did the second linkage and released \(p\)-nitrophenol. However, prawn chitinase did not hydrolyze it. Although the cleavage sites of GlcNAc\(_2\) and GlcNAc\(_6\) could not be identified, binding models are proposed as shown in Fig. 5, based on the data represented in Tables I and II. The binding of GlcNAc residues of the substrate to the subsites at both sides of the non-reducing and reducing ends in the enzyme seems to be important for the enzymatic reaction. The bindings of GlcNAc\(_2\), GlcNAc\(_3\), and GlcNAc\(_4\) residues to the binding subsites at the non-reducing end side may be productive (Types 1, 2, and 3 in Fig. 5). The binding of a GlcNAc residue to Subsite D (Type 4) and Subsite E (Type 10) may be non-productive for any \(N\)-acytchitoooligosaccharide. However, pNp-GlcNAc\(_5\) may be bound to the subsites over both the non-reducing and reducing end sides in a combination of Types 4 and 7, and GlcNAc may be released. \(p\)-Nitrophenyl residue may be hardly bound to Subsite F (Type 9). The binding of \(p\)-nitrophenyl residue to Subsite E (Type 8) seems to be dependent on the binding state of the other residues at the non-reducing end side. For example, pNp-GlcNAc\(_4\) could be bound to the enzyme in a combination of Types 2 and 8. With respect to allosamidin inhibition, prawn chitinase is inhibited in a competitive way with a \(K_i\) of \(0.1 \mu M\) similarly to silkworm chitinase \((K_i = 0.1 \mu M)\), while yam chitinase is not. \(^10\) This seems to be related to the difference in the binding of substrates between prawn chitinase and yam chitinase. Allosamidin is a pseudotrisccharide with the \(\beta\)-1,4-linked dimer of \(\beta\)-\(N\)-acetylallosamine bound with an aminocyclitl derivative. \(^10\)

On the basis of these results, it is suggested that prawn chitinase is a new endo-type chitinase.

**References**