Kinetic Analysis of a Chitinase from Red Sea Bream, *Pagrus major*

Shuji KARASUDA,1 Kosuke YAMAMOTO,1 Michiko KONO,2 Shohei SAKUDA,3 and Daizo KOGA1,

1Laboratory of Biochemistry, Department of Biological Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan
2Fisheries Research Laboratory, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Matsaka, Shizuoka 431-0211, Japan
3Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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Kinetic analysis was done on the 46-kDa chitinase (EC 3.2.1.14) purified from the stomach of red sea bream, *Pagrus major*, using glycolchitin and *N*-acetylchitooligosaccharides (GlcNAcn, n = 2–6) as substrates. High activity was observed at two pHs, such as 2.5 and 9.0, toward glycolchitin as seen in other insect chitinas, and also at both pH 2.5 and 5.0 even toward a short substrate, *N*-acetylchitopentasaccharide. Allosamidin competitively inhibited chitinase with *K*ᵢ value of 0.0214 μM at pH 2.5 and 0.0024 μM at pH 9.0 in the reaction of glycolchitin. Substrate inhibition was observed in the reaction of *N*-acetylchitopentasaccharide. The anomeric forms of the products from *N*-acetylchitooligosaccharides were analyzed to be *β* anomer by the high pressure liquid chromatography (HPLC) method. The data for both *β*-anomer formation and allosamidin inhibition suggest that red sea bream chitinase belongs to family 18 of glycosyl hydrolases. This suggestion is also supported by the results for the N-terminal amino acid sequence.

Key words: chitinase; red sea bream (*Pagrus major*); anomer formation; allosamidin; kinetics

Chitinases are widely distributed in nature, but their physiological roles differ among various organisms. Plant chitinase acts for self-defense against pathogens,1,2 while insect chitinase does for ecdysis.3,4 Chitinase also takes part in digestive action in fishes5,6 and mollusks.9,10 Prawn chitinase plays a role in ecdysis and digestion.11 Red sea bream chitinase appears to be involved in the digestion of chitin-containing foods. Since the physiological role of chitinase differs among various organisms, its physicochemical properties and enzymatic behaviors must also differ. In fact, some differences have been observed in the cleavage pattern of *N*-acetylchitooligosaccharides and in the effect of allosamidin.12-14 On the other hand, chitinases are classified into family 18 or 19 of glycosyl hydrolases on the basis of amino acid sequence.15 Moreover, there are two mechanisms in the hydrolytic action: one is a retaining mechanism to produce the *β* anomers of *N*-acetylchitooligosaccharides, and the other is an inverting one to produce the *α* anomers. It has been reported that yam chitinase H14 and *Bombyx mori* chitinases,13,16 all of which belong to family 18, produce *β* anomers and are inhibited by allosamidin, whereas yam chitinase E,17 which belongs to family 19, produces *α* anomers and is not inhibited by allosamidin. Furthermore, other family 18 chitinases from *Streptomyces metabolite*18 and *Coccidioides immitis*19 are inhibited by allosamidin. These results strongly suggest that there is a close relationship among anomer formation, allosamidin-inhibition, and family classification.

In this study, we investigated the kinetic behavior of 46-kDa chitinase purified from the stomach of red sea bream, the anomeric forms of the products, and the inhibition of this enzyme by allosamidin. We also analyzed the N-terminal amino acid sequence.

Materials and Methods

**Chemicals.** Glycolchitin was prepared by the method of Yamada and Imoto20 with reacetylation using acetic anhydride.

*N*-Acetylchitooligosaccharides were a generous gift from Yaizu Suisankagaku Industry Co., Ltd., of Shizuoka, Japan. All reagents were of the highest grade available.

**Enzyme.** Chitinase (46 kDa) was purified from the stomach of red sea bream, *Pagrus major*, by the method of Kono et al.8

**Enzyme assay.** Chitinase activity was measured using glycolchitin as the substrate. A reaction mixture con-
sisting of 10 µl of enzyme solution and 0.5 ml of 0.05% (w/v) glycolchitin in 0.1 M acetate buffer, pH 4.0, was incubated at 32 °C for 20 min. The reducing end group produced was measured colorimetrically at 420 nm with ferri-ferrocyanide reagent by the method of Imoto and Yagishita. For optimum pH, Britton–Robinson buffer (pH 2.0 to 12.0) was used. The kinetic experiments were performed in the reaction of glycolchitin (0.05 to 0.5% (w/v)) dissolved in Britton-Robinson buffer, pH 2.5 and pH 9.0, in the absence and the presence of allosamidin (final concentration, 5 nM). N-Acetylchito-oligosaccharides were also used for kinetics. A reaction mixture consisting of 10 µl of enzyme solution and 100 µl of pentasaccharide dissolved in Britton–Robinson buffer, pH 9.0, was incubated at 25 °C. This reaction mixture was analyzed by HPLC with a Tosoh TSK-Gel G2000 PW column (0.75 × 60 cm) by the method of Koga et al.

Protein measurement. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.

Anomer formation. To identify the anomer form of the product in the enzymatic reaction of N-acetylchito-oligosaccharides, 100 µl of 0.11 mM N-acetylchito-oligosaccharide (final concentration, 0.1 mM) dissolved in Britton-Robinson buffer, pH 9.0, was reacted with 10 µl of the enzyme at 25 °C. The reaction mixture was immediately cooled in an ice bath in order to delay the equilibrium reaction between α and β anomers, and a 10-µl portion was analyzed by HPLC using a Tosoh TSK-Gel amide-80 column (0.46 ID × 25 cm) by the method of Koga et al.

Amino acid sequence analysis. The N-terminal amino acid sequence of the red sea bream chitinase was analyzed with a protein sequencer (PPSQ21, Shimadzu Corporation, Kyoto, Japan).

Results

Optimum pH of red sea bream chitinase
To investigate the optimum pH of red sea bream chitinase, enzymatic reactions were done in Britton–Robinson buffer (pH 2.0 to 12.0) using a short substrate, N-acetylchitopentasaccharide, and a long substrate, glycolchitin, at 25 and 32 °C respectively. High activity toward GlcNAc₅ was observed at pH 2.5 and 9.0, while high activity toward glycolchitin was observed at pH 2.5 and 9.0 (Fig. 1). Although a double optimum pH toward a long substrate, glycolchitin, has been reported in insect chitinases, one toward a short substrate, N-acetylchitopentasaccharide, has not yet been reported.

Substrate specificity of red sea bream chitinase
To investigate the substrate specificity of this chitinase, the initial velocities toward N-acetylchito-oligosaccharides (disaccharide to hexasaccharide, 0.1 mM) were measured in Britton–Robinson buffer, pH 9.0, by HPLC with a Tosoh TSK-Gel G2000 PW column. The results are summarized in Table 1. The reactivity of the substrate was in the order hexasaccharide > pentasaccharide > tetrasaccharide, suggesting that this chitinase prefers the longer substrates. But it did not hydrolyze disaccharide and trisaccharide.

Anomeric formation and cleavage pattern in the hydrolytic reaction
In order to confirm the relationship between the anomer formation and family classification of chitinase, the anomic forms of the product in the enzymatic reaction were also measured by HPLC with a TSK-Gel amide-80 column. As shown in Fig. 2, each N-acetylchito-oligosaccharide was separated into two peaks, such as α anomer (the earlier peak) and β anomer (the later peak) of N-acetylglucosamin moiety at the reducing end. The ratios of α anomer to β anomer of naturally occurring monosaccharide to hexasaccharide of N-acetylchito-oligosaccharide were 1:0.84, 1:0.75, 1:0.70, 1:0.69, 1:0.65, and 1:0.62 respectively. After the en-
zymatic reaction, the products were separated by this HPLC system. In the case of pentasaccharide, after a 7-min reaction, disaccharide and trisaccharide appeared while the substrate of pentasaccharide was reduced. The ratios of α/C11 anomer to β/C12 anomer for disaccharide and trisaccharide were 1:1.51 and 1:1.15 respectively. In view of the ratios of naturally occurring disaccharide and trisaccharide (1:0.75 and 1:0.70 respectively), it is obvious that the disaccharide and trisaccharide were produced in α/C11 anomer by cleaving at the second and third linkages from the non-reducing end, respectively. Hence, the cleavage sites and their frequencies were calculated by the method of Koga et al. The result is shown in Table 1. The cleavage patterns for the other substrates were also analyzed by the same method. They are shown in Table 1. These results strongly suggest that red sea bream chitinase hydrolyzes N-acetylchitooligosaccharide in an endo-type fashion by the retaining mechanism to produce β anomers.

**Kinetic analysis**

To investigate the enzymatic action, kinetic analysis was carried out using a long substrate, glycolchitin, and a short substrate, N-acetylchitopentasaccharide. When glycolchitin was used as the substrate, positive slopes were obtained in the double reciprocal plots (Fig. 3), and

### Table 1. Hydrolytic Patterns of N-Acetylchitooligosaccharides by Red Sea Bream Chitinase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>Initial velocity (µM/sec ± SE)</th>
<th>Cleavage pattern (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc2 (II)</td>
<td>No reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAc3 (III)</td>
<td>No reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAc4 (IV)</td>
<td>IV→2II</td>
<td>0.0218 ± 0.0081</td>
<td>(100)</td>
</tr>
<tr>
<td>GlcNAc5 (V)</td>
<td>V→II+III</td>
<td>0.0452 ± 0.0073</td>
<td>(62.4) (37.6)</td>
</tr>
<tr>
<td>GlcNAc6 (VI)</td>
<td>VI→ II+IV</td>
<td>0.0505 ± 0.0118</td>
<td>(50.0)(18.2)(31.8)</td>
</tr>
</tbody>
</table>

The initial velocities were measured in the reaction of N-acetylchitooligosaccharides (0.1 mM) with red sea bream chitinase (5.2 nM) in Britton–Robinson buffer, pH 9.0, at 25°C. Represents the GlcNAc moiety, and the left side of the whole molecule figure is the non-reducing end. The downward arrows show the cleavage sites, while the relative distributions of the cleavage sites were shown as percentages on the arrows.

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Fig. 2. HPLC Analysis of the Hydrolytic Reaction of N-Acetylchitooligosaccharides by Red Sea Bream Chitinase.

Panels A, B, and C: Analysis of the enzymatic reaction with tetrasaccharides, pentasaccharides, and hexasaccharides of N-acetylchitooligosaccharides, respectively. The final concentration of each substrate was 0.1 mM, and that of the chitinase was 5.2 nM. The reaction was done in Britton-Robinson buffer, pH 9.0, at 25°C for 7 min, and 10 µl of reaction mixture was analyzed by HPLC. The ratios of α and β anomic forms of N-acetylchitooligosaccharides after 7 min of reaction are represented below the panels.
The kinetic values were calculated (Table 2). The $K_m$ value was almost the same at pH 9.0 and 2.5, meaning that red sea bream chitinase has the same affinity at both pHs. The $k_{cat}$ value at pH 9.0 was larger than that at pH 2.5. With respect to the overall reaction based on the $k_{cat}/K_m$, red sea bream chitinase had 1.42 fold-higher activity at pH 9.0 than at pH 2.5. Such a pH-dependence on the overall reaction was also observed in reactions by 54 kDa- $Bombyx mori$ chitinase (family 18) and yam chitinase E (family 19), but not in reactions by yam chitinase H (family 18) (Table 2). The values of the overall reaction are not remarkable for red sea bream chitinase compared with other chitinases, except for 38 kDa- $Todarodes pacificus$ chitinase, which has very high activity.

On the other hand, when pentasaccharide was used as the substrate, a straight line with positive slope was not obtained in the double reciprocal plot (Fig. 4), suggesting that substrate inhibition occurred in the enzymatic reaction. Table 2. Comparison of Kinetic Parameters of Red Sea Bream Chitinase with Other Chitinases toward Glycolchitin

<table>
<thead>
<tr>
<th>Chitinase Family</th>
<th>Optimum pH</th>
<th>$K_m$ (mg/ml)</th>
<th>$k_{cat}$ (1/s)</th>
<th>$k_{cat}/K_m$ (ml/mg/s)</th>
<th>$K_i$ for allosamidin ($\mu$m)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pagrus major</td>
<td>pH 2.5</td>
<td>0.261</td>
<td>0.262</td>
<td>1.00</td>
<td>0.0214</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pH 9.0</td>
<td>0.232</td>
<td>0.329</td>
<td>1.42</td>
<td>0.0024</td>
<td></td>
</tr>
<tr>
<td>Todarodes pacificus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 kDa</td>
<td>pH 4.0</td>
<td>0.074</td>
<td>0.196</td>
<td>2.65</td>
<td></td>
<td>[27]</td>
</tr>
<tr>
<td>38 kDa</td>
<td>pH 4.0</td>
<td>0.071</td>
<td>1.22</td>
<td>17.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bombyx mori</td>
<td>pH 6.5</td>
<td>0.023</td>
<td>0.059</td>
<td>2.60</td>
<td>1.0 (ID$_{50}$)</td>
<td>[24]</td>
</tr>
<tr>
<td>65 kDa</td>
<td>pH 5.5</td>
<td>0.136</td>
<td>0.084</td>
<td>0.63</td>
<td>3.5 (ID$_{50}$)</td>
<td>[16]</td>
</tr>
<tr>
<td>54 kDa</td>
<td>pH 4.0</td>
<td>0.307</td>
<td>0.011</td>
<td>0.036</td>
<td>0.356</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>pH 9.5</td>
<td>0.192</td>
<td>0.023</td>
<td>0.120</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>Dioscorea opposita</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>pH 4.0</td>
<td>0.381</td>
<td>1.069</td>
<td>2.803</td>
<td>1260 (ID$_{50}$)</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>pH 8.0</td>
<td>0.323</td>
<td>0.591</td>
<td>1.827</td>
<td>44.4 (ID$_{50}$)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>pH 4.0</td>
<td>0.639</td>
<td>0.629</td>
<td>0.984</td>
<td>—</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>pH 8.0</td>
<td>0.518</td>
<td>0.645</td>
<td>1.250</td>
<td></td>
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</tbody>
</table>

Kinetic parameters were measured in the reaction of glycolchitin (0.05 to 0.5%) with red sea bream chitinase (5.2 nM) in Britton–Robinson buffer, pH 2.5 and pH 9.0, at 25°C. The enzyme concentrations were 28 nM and 5.9 nM for $T. pacificus$ 42 kDa and 38 kDa chitinase respectively; 21 nM, 10 nM, and 20 nM for $B. mori$ 88 kDa, 65 kDa, and 54 kDa chitinase respectively; and 18.4 nM for both $D. opposita$ chitinase H and E.

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reaction of pentasaccharide. Such substrate inhibition was observed in B. mori chitinases,\textsuperscript{13,20} but not in prawn chitinase.\textsuperscript{11} In the case of yam chitinase, substrate inhibition was observed at a low enzyme concentration (40 nm), but not at a high one (100 nm).\textsuperscript{28} This might mean that such N-acetyltchitooligosaccharides are not true substrates, but products for red sea bream chitinase. Hence we did not obtain the values of kinetic parameters toward N-acetyltchitooligosaccharides.

**Inhibition by allosaminid**

In order to confirm the relationship between allosaminid inhibition and family classification, kinetic analysis was done using glycolchitin as the substrate in the absence and the presence of allosaminid at pH 2.5 and 9.0. The double reciprocal plots are shown in Fig. 3. The two lines for the reactions with and without allosaminid crossed on the vertical axis, indicating that allosaminid inhibited red sea bream chitinase competitively. The $K_i$ values were calculated and are shown in Table 2 for comparison with other chitinases. Allosaminid inhibited family 18 chitinases but not family 19 chitinases, even though yam chitinase H was not strongly inhibited.\textsuperscript{29} Therefore it is suggested that red sea bream chitinase belongs to family 18 of glycosyl hydrolases. The $K_i$ value at pH 9.0 was 8.9-fold smaller than that at pH 2.5, meaning that red sea bream chitinase was 8.9-fold more strongly inhibited by allosaminid at pH 9.5 than at pH 2.5. Similarly to other family 18 chitinases, such as B. mori 54 kDa chitinase and yam (Dioscorea opposita) chitinase H, allosaminid inhibition was stronger at the alkaline optimum pH than at the acidic optimum pH.

**N-terminal amino acid sequence**

In order to confirm that red sea bream chitinase belongs to family 18 18 glycosyl hydrolases, the N-terminal amino acid sequence was analyzed up to the 15th position. The results are shown in Table 3 for comparison with other family 18 and 19 chitinases. The N-terminal sequences of red sea bream chitinase showed high homology to family 18 chitinases but not to family 19 chitinases, suggesting that red sea bream chitinase belongs to family 18 glycosyl hydrolases.

**Discussion**

**Classification of red sea bream chitinase**

The N-terminal amino acid sequence analyzed suggests that red sea bream chitinase is a family 18 chitinase (Table 3). This estimation is strongly supported by our cloning data for the cDNA encoding chitinase from the stomach of the red sea bream (unpublished data). It includes the conserved regions I and II of family 18 chitinases, and has active sites such as DWE (-Asp-Trp-Glu-) in conserved region II. Therefore it is strongly supported on the basis of amino acid sequence\textsuperscript{15} that red sea bream chitinase belongs to family 18.

Regarding another formation in the reactions of N-acetyltchitooligosaccharide such as tetrasaccharide, pentasaccharide, and hexasaccharide, all reaction products were $\beta$-anomers (Table 1). These results strongly support the conclusion that family 18 chitinases produce the $\beta$-anomer by the retaining mechanism. Regarding allosaminid inhibition, red sea bream chitinase was inhibited by allosaminid in a competitive fashion, like B. mori chitinases.\textsuperscript{26} Since allosaminid has a configuration similar to the intermediate of the substrate during chitinase action, $\beta$-anomer formation is also closely related to allosaminid-inhibition.

**The physiological role of red sea bream chitinase**

Red sea bream chitinase has high activity at both pH 2.5 and 5.0 toward a short substrate such as N-acetyltchitopentasaccharide, while high activity toward a long substrate such as glycolchitin was found at pH 2.5 and 9.0. High activity was observed at a low pH of 2.5.

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**Table 3.** Comparison of Amino Acid Sequences of Red Sea Bream Chitinase with Other Family 18 Chitinases and Family 19 Chitinases

<table>
<thead>
<tr>
<th></th>
<th>Pagrus major</th>
<th>Todarodes pacificus (42kDa)</th>
<th>Todarodes pacificus (38kDa)</th>
<th>Penaeus japonicus</th>
<th>Homo sapiens</th>
<th>Manduca sexta</th>
<th>Bombyx mori</th>
<th>Nicotiana tabacum</th>
<th>Arabidopsis thaliana</th>
<th>Dioscorea oppositifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>D</td>
<td>A</td>
<td>E</td>
<td>E</td>
<td>Q</td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>V</td>
<td>V</td>
<td>S</td>
<td>R</td>
<td>C</td>
<td>Q</td>
<td>N</td>
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<td>10</td>
<td>S</td>
<td>V</td>
<td>Y</td>
<td>Y</td>
<td>Q</td>
<td>Y</td>
<td>S</td>
<td>T</td>
<td>Y</td>
<td>C</td>
</tr>
<tr>
<td>15</td>
<td>G</td>
<td>T</td>
<td>F</td>
<td>T</td>
<td>R</td>
<td>Y</td>
<td>V</td>
<td>Y</td>
<td>Y</td>
<td>R</td>
</tr>
</tbody>
</table>

A homology search was conducted using the BLAST program of the National Center of Biotechnology Information (NCBI). Alignment of the amino acid sequences of red sea bream chitinase is done with other family 18 and 19 chitinases such as Homo sapiens (AAG60019.1),\textsuperscript{20} Bombyx mori (BAB20017.1),\textsuperscript{20} Manduca Sexta (AAC30492.1),\textsuperscript{20} Todarodes pacificus,\textsuperscript{20} Penaeus japonicus,\textsuperscript{20} Nicotiana tabacum (AAB23374), Arabidopsis thaliana (BAA82818), and Dioscorea oppositifolia (BAC56863). Identical residues are enclosed in boxes. X is an unidentified amino acid.
toward both short and long substrates. As far as we know, this is the first report to this effect. Fishes secrete proteases such as pepsin in the acidic conditions of the stomach in the process of food digestion. Hence it is reasonable to suppose that red sea bream chitinase has high activity at pH 2.5 to digest chitosinous foods in the stomach. On the other hand, the other optimum pH, such as 5.0 and 9.0, were observed toward the short substrate and the long substrate respectively. The double optimum pH has been also observed specially toward the long substrate such as glycolchitin in the actions of yam chitinase isozymes\(^{13}\) and fish chitinase.\(^{7}\) Yam chitinase E showed high activity at pH 4.0 and 7.5, and yam chitinase F at pH 3.0 and 9.0.\(^{14}\) Japanese common squid 38 kDa chitinase showed high activity at pH 1.5 and 8.5.\(^{30}\) The optimum pH of about 8.0 might well be reasonable to suppose that red sea bream chitinase has related to self-defense like plant chitinases.\(^{14}\) Hence red sea bream chitinase might have another role in self-defense, against oceanic bacilli in addition to its role in the digestion of chitosinous foods.

**Acknowledgment**

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**References**


