Preliminary Communication

HPLC Analysis of Anomeric Formation and Cleavage Pattern by Chitinolytic Enzyme

Daizo Koga,¹* Takanori Yoshioka,¹ and Yasuyuki Arakane²

¹Laboratory of Biochemistry, Faculty of Agriculture, Yamaguchi University, Yoshida, Yamaguchi 753-8515, Japan
²Research Laboratory, Bankaku So-honpo, Tokai, Aichi 476-0003, Japan

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The reactions of N-acetylchitooligosaccharides with chitinolytic enzyme were analyzed by HPLC using a Tosoh TSK-Gel amide-80 column with 70% acetonitrile as an eluent. We separated α and β anomeric forms of N-acetylchitooligosaccharides, and obtain the following advantages of this HPLC method.

1. We can easily identify the reaction mechanism of chitinolytic enzymes by this method, distinguishing the invert mechanism showing α anommer formation from the retaining mechanism showing β anommer formation.

2. We can also estimate the cleavage patterns of N-acetylchitooligosaccharides by chitinolytic enzymes by using natural substrates.

Key words: chitinase; lysozyme; chitinolytic enzyme; N-acetylchitooligosaccharide

Chitinase (EC 3.2.1.14) is widely distributed in living organisms, and has an important role in each organism. Furthermore, many isozymes of chitinase were found in each organism, and classified into families 18 and 19 of glycosyl hydrolases, and into class I to V, especially in higher plants, according to the amino acid sequence. Their three-dimensional structure and the enzymatic reaction were recently discussed.¹,²,³ Especially, the anomeric form produced in the hydrolytic reaction of the substrate is interesting, since an oxazoline ring would be formed as an intermediate in the enzymatic reaction. If such an oxazoline ring would be formed, water enters from the other side of the oxazoline ring to form the β anomer and complete the hydrolysis.³ To analyze its anomeric form, ¹H-NMR analysis was recently used and the detection succeeded.⁴,⁵ However, for ¹³C-NMR analysis, it is required to exchange water protons in the enzyme solution with deuterium by lyophilization, which sometimes inactivates the enzyme. Furthermore, ¹H-NMR analysis gave us the information only on the anomeric form of the mixture of N-acetylchitooligosaccharides produced in the reaction but not any information on the cleavage pattern.

On the other hand, the analysis of cleavage patterns is also important for characterization and classification of chitinolytic enzymes with respect to the enzymatic action. Previously we used artificially synthesized p-nitrophenyl N-acetylchitooligosaccharides to identify the cleavage patterns.⁶ Therefore, it is important and interesting to compare the cleavage patterns between the natural substrates and such artificial substrates.

In this study, therefore, we did an HPLC analysis of the reaction of N-acetylchitooligosaccharides with hen egg-white lysozyme and yam chitinase E (family 19 of glycosyl hydrolases, class IV) using a Tosoh TSK-Gel amide-80 column.

Yam chitinase E was purified by the method of Tsukamoto et al.,⁷ with a small modification, and this enzyme corresponds to chitinase E3 in that paper. Hen egg-white lysozyme was purchased from Wako Pure Chemical, Osaka, Japan. N-Acetylchitooligosaccharides were generous gifts from Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan. All of the reagents were of analytical grade.

The enzymatic reactions were done as follows: One hundred micro-liters of 0.11 mM N-acetylchitooligosaccharide dissolved in 4.4 mM sodium acetate buffer, pH 4.0 for the reaction for yam chitinase E or pH 5.5 for hen egg-white lysozyme, was reacted with 10 μl yam chitinase E or the lysozyme at 25°C. After the appropriate time, the reaction mixture was immediately cooled in an ice bath, and a 10-μl portion was analyzed by HPLC (Shimadzu LC-10) on a Tosoh TSK-Gel amide-80 column (0.46 ID × 25 cm) at 28°C. The elution of N-acetylchitooligosaccharides was done with 70% acetonitrile at a flow rate of 0.7 ml/min, and monitored at 210 nm. The calculation of molar concentrations of the N-acetylchitooligosaccharides eluted was done by using the relationship between the concentration of each N-acetylchitooligosaccharide and its total peak area of both anomers. Furthermore, the calculation of the anomeric ratio was done by comparing the peak areas of both anomers in each N-acetylchitooligosaccharide.

Identification of α and β anomeric forms. N-Acetylglucosamine and N-acetylchitooligosaccharides had two peaks each by this HPLC method. To identify the anomeric forms of these two peaks, the enzymes that produced either α or β anomer were used. One is hen egg-white lysozyme, which produces the β anomer,⁸ and the other is yam chitinase E, which produces the α

* To whom correspondence should be addressed. Tel: +81-839-33-5862; Fax: +81-839-33-5820; E-mail: koga@agr.yamaguchi-u.ac.jp

Abbreviations: I, 2-acetamido-2-deoxy-D-glucopyranoside (GlcNAc); II to VI, β (1→4) linked di- to hexa saccharides of GlcNAc (N-acetylchitooligosaccharide).
We analyzed the reaction products of N-acetylchitoheexasaccharide with the lysozyme and yam chininate E by this HPLC method. The results are shown in Fig. 1A. By comparing with the peaks of N-acetylchitoooligosaccharides produced by the lysozyme and yam chininate E, we tried to identify the anonomic forms. The anonomic forms of N-acetylchitoooligosaccharides from dimer to pentamer were easily identified as shown in Fig. 1B. Although the anonomic forms of N-acetylglucosamine (the monosaccharide) and N-acetylchitoheexasaccharide could not be identified in these reactions, we may deductively estimate their anonomic forms like other N-acetylchitoooligosaccharides. Therefore, we conclude that the earlier peaks show α anomers and the later peaks β anomers as shown in Fig. 1B. The ratios of anomic forms in naturally occurring N-acetylchitoooligosaccharides were calculated from their peak areas as shown in Fig. 1B. Their ratios of α anomer to β anomer were similarly about 1:0.6. This result indicates that Tosoh TSK-Gel amide-80 column is useful to identify the anomeric form produced by chitinolytic enzymes such as chininate (EC 3.2.1.14), lysozyme (EC 3.2.1.17) and N-acetylhexosaminidase (EC 3.2.1.52).

The reaction time for identification of anomeric form. These anomic forms were gradually equilibrated with time and reached an equilibrated value such as about 1:0.6. Therefore, we followed the course of the reaction of N-acetylchitoheexasaccharide by yam chininate E. The α anomers of the disaccharide, trisaccharide, tetrasaccharide and pentasaccharide were
preferentially observed until 30 min. After 30 min, however, their ratios gradually reached the equilibrated value of about 1:0.6 (data not shown). These results suggest that the enzymatic reaction should be finished by 30 min by immediate cooling. When the reaction is too slow for recognition of anomeric forms, a higher concentration of the enzyme is recommended to be used for the enzymatic reaction.

Analysis of reaction mechanism by yam chitinase E. To investigate the reaction mechanism by yam chitinase E, N-acetylchitooligosaccharides were reacted with yam chitinase E and analyzed by this HPLC method. The results are shown in Fig. 2 and Table 1. The reaction products from N-acetylchitotrisaccharide by yam chitinase E were the monosaccharide and disaccharide. Comparing the anomer ratios of \( \alpha \) to \( \beta \) between the monosaccharide and disaccharide, the \( \alpha \) ratio in the disaccharide is higher than that in the monosaccharide. This result indicates that N-acetylchitotrisaccharide is cleaved at the first linkage from the reducing end-side of the substrate by yam chitinase E to produce the \( \alpha \) anomer of the disaccharide. Furthermore, the anomer ratios in the substrate and product interestingly changed: \( \beta \) Anomer of the substrate trisaccharide decreased, while the \( \beta \) anomer of the product monosaccharide increased. This means that yam chitinase E preferentially hydrolyzed the \( \beta \) anomer of N-acetylchitotrisaccharide, and produced the \( \alpha \) anomer of the disaccharide and the \( \beta \) anomer of the monosaccharide that originates from the preferential \( \beta \) anomer of the substrate. We analyzed the HPLC results of other N-acetylchitooligosaccharides in the same way. When N-acetylchitotetrasaccharide was hydrolyzed, the disaccharide with a high ratio of \( \alpha \) anomer was produced, suggesting that N-acetylchitotetrasaccharide is cleaved to two molecules of the disaccharide, half of which is the \( \alpha \) anomer. In the case of N-acetylchitopentasaccharide, the high ratios of \( \alpha \) anomer were observed in both products such as the disaccharide and trisaccharide. Therefore, we estimated that the pentasaccharide would

be cleaved in two patterns such as O0100 (case 1, A\% ) and O00100 (case 2, B\% ) as shown in Table 1, and tried to calculate the concentrations of both anomers of the products such as the disaccharide and trisaccharides under two assumptions. First, we assume that the anomer ratios of the reducing end-side oligosaccharides produced from the substrate are the same as those of naturally occurring N-acetylchitooligosaccharides such as 1:0.66 for the disaccharide and 1:0.65 for the trisaccharide as shown in Fig. 1B. Second, we assume that the anomer ratios of the newly produced oligosaccharides with \( \alpha \) anomer would change to 1:0.1 by mutarotation, that is the ratio value of the disaccharide produced from the substrate trisaccharide in a 30-min reaction (Fig. 2A). Using these two assumptions, we made two equations on the ratio of \( \alpha \) to \( \beta \) with respect to the disaccharide and trisaccharide. For the disaccharide that comes A\% from case 1 with newly formed anomer and B\% from case 2 with equilibrated anomer, as its ratio of \( \alpha \) to \( \beta \) was 1:0.33, the following equation comes.

\[
[A/(1+0.1)+B/(1+0.66)]:
\[
0.1A/(1+0.1)+0.66B/(1+0.66)]=1:0.33
(1)
\]

For the trisaccharide that comes A\% from case 1 with equilibrated anomer and B\% from case 2 with newly formed anomer, as its ratio of \( \alpha \) to \( \beta \) was 1:0.31, the following equation comes.

\[
[A/(1+0.65)+B/(1+0.1)]:
\[
0.65A/(1+0.65)+0.1B/(1+0.1)]=1:0.31
(2)
\]

From equations 1 and 2, B/A was calculated to be 1.052 and 1.079, respectively. These values are very similar, suggesting that these assumptions are reasonable. As A + B = 100\%, we could calculate A and B to be 48.4\% and 51.6\%, respectively, using the average B/A value of 1.065. As the result, we could estimate that N-acetylchitopentasaccharide is cleaved to the disaccharide and trisaccharide at the second linkage (48.4\%) and third linkage (51.6\%) from the non-reducing end-side of the

Table 1. Comparison of Cleavage Patterns by Yam Chitinase E between N-Acetylchitooligosaccharides and Their Derivatives

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Tetramer</th>
<th>Pentamer</th>
<th>Hexamer</th>
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<tr>
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<td><img src="image7" alt="image" /></td>
<td><img src="image8" alt="image" /></td>
<td><img src="image9" alt="image" /></td>
<td><img src="image10" alt="image" /></td>
</tr>
</tbody>
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The percentages of cleaving rates are indicated below the arrows. The left side of these saccharides is the non-reducing end.

\( \circ \), N-Acetylglucosamine; \( \mathcal{P} \), p-nitrophenyl group.
substrate. In the case of N-acetyltichitohexasaccharide, we could easily estimate the three cleaving patterns from the products, that is, to the monosaccharide plus pentasaccharide, the disaccharide plus tetrasaccharide, and two molecules of the trisaccharide. First, we calculated the their cleaving percentage by using the concentrations of substrate and products without considering the discrimination between $\alpha$ and $\beta$ anomers to be 59.6, 26.7 and 13.7%, respectively. Since high ratios of the $\alpha$ anomer were also observed in the pentasaccharide, tetrasaccharide, trisaccharide, and disaccharide, we could easily estimate one cleavage pattern to the monosaccharide plus pentasaccharide and one cleavage pattern to two trisaccharides as shown in Table 1. However, we must consider two patterns on the cleavage of the hexasaccharide to the disaccharide plus tetrasaccharide, since high ratios of $\alpha$ anomer were observed in both products such as the disaccharide and tetrasaccharide. Therefore, we then tried to calculate the percentage of these two patterns such as OOIOOOO (A%) and OOOIOOOO (B%) by using the same method used for the pentasaccharide. By resolving the equations, we obtained similar values of B/A such as 0.856 and 0.838 with respect to the disaccharide and tetrasaccharide, respectively, suggesting again that these assumptions are reasonable. Using the average B/A value of 0.847, we could calculate A and B to be 54.1% and 45.9%, respectively. We could estimate the cleavage patterns for the hexasaccharide as follows: N-Acetyltichitohexasaccharide was cleaved at the second linkage (26.7\times54.1/100 = 14.5%), the third linkage (13.7%), the forth linkage (26.7\times45.9/100 = 12.2%), and the fifth linkage (59.6%) from the non-reducing end-side of the substrate. Due to a 30-min reaction, however, the product pentasaccharide would be further cleaved to the disaccharide and trisaccharide. Although we cannot completely eliminate this possibility, the percentage of the cleavage pattern may not be largely affected because these second products such as disaccharide and trisaccharide may be small in amount. On the other hand, the $\beta$ anomer of the substrate N-acetyltichitohexasaccharide decreased, while the $\beta$ anomer of the monosaccharide produced increased. This also means that yam chitinase E prefers to hydrolyze $\beta$ anomer of the substrate when it cleaves the substrate at the first linkage from the reducing end-side. This may suggest that only $\beta$ anemic form of the first N-acetyltiglucosamine in the reducing end-direction from the cleavage site is able to bind to yam chitinase E for its hydrolysis. It also suggests that yam chitinase E may not form an oxazoline ring as the intermediate in the enzymatic reaction and act in the inverting mechanism, since the newly produced N-acetyltichitooligosaccharides are all $\alpha$ anomers.

Comparison of cleavage patterns between natural N-acetyltichitooligosaccharides and their p-nitrophenyl derivatives. Previously we analyzed the cleavage patterns of N-acetyltichitooligosaccharides by yam chitinase E by using their $\beta$-linked p-nitrophenyl derivatives.48 To compare the cleavage patterns between the natural substrate and their artificial substrates, the results are shown in Table 1. The same result was obtained only in the case of the tetramer such as N-acetyltetraotetrasaccharide and p-nitrophenyl N-acetyltetrotetrasaccharide. In the case of the pentamer such as N-acetyltihoptotetrasaccharide and p-nitrophenyl $\beta$-N-acetyltetrotetrasaccharide, the cleavage sites are the same, but the ratio of cleavage are different. In other cases such as the trimer and hexamer, from which the monosaccharide is produced from the reducing end-side of the substrate, the cleavages site and their ratios are largely different. The linkage between N-acetyltiglucosamine moiety and p-nitrophenyl group probably cannot be cleaved by this enzyme, because the configuration of the p-nitrophenyl group is largely different from that of N-acetyltiglucosamine. When we use such artificial substrates to estimate the cleavage patterns of N-acetyltichitooligosaccharides by chitinolytic enzymes, we have to take care not to be misled in our conclusions. Therefore, we recommend this HPLC method to identify the anomer form and cleavage pattern at the same time.

Recently such a HPLC analysis was tried using other columns.29 However, the separation of the anomeric peaks was not good enough for further analysis of the enzymatic reaction such as cleavage patterns. We are now investigating the anomer formation and cleavage pattern to compare different family and class chitinases and other chitinolytic enzymes by this HPLC method.

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References