Note

Comparative Biochemistry of Chitinases—Anomeric Form of the Reaction Products

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Anomeric form of the products from chitinase-catalyzed hydrolysis was analyzed by \(^{1}H\)-NMR spectroscopy. When the chitinase from \(\textit{Streptomyces griseus}\) was added to the solution of substrate, \(N,N,N,N\)-pentaacetylchitotetraosylactose, \(\beta\)-anomer was first produced by the enzymatic hydrolysis, and then transformed to \(\alpha\)-anomer by mutarotation. In contrast, the chitinase from \(\textit{Dioscorea opposita}\) (yam) produced only \(\alpha\)-anomer, which was transformed to \(\beta\)-anomer by mutarotation.

Chitinase (EC 3.2.1.14) hydrolyzes \(\beta\)-1,4-glycosidic linkages of chitin with an endo-splitting manner. The chitinases are widely found in living organisms, and are involved in various biological processes, for example, self-defence against pathogens, degradation of old cuticle, and chinum assimilation.\(^{11}\) Hence, there may be some difference in the specificities of chitinases obtained from various sources. The specificities of chitinases have been studied by structural analysis of products from polysaccharide or oligosaccharide digestion.\(^{2}\) However, not much variation in the specificity has been found for many chitinases investigated thus far. On the other hand, information on the anomeric specificity of products from chitinase-catalyzed hydrolysis have been obtained only for \(\textit{Bacillus circulans}\) chitinase,\(^{35}\) and are quite limited.

Since the anomeric specificity should be dependent upon the fine structure of catalytic site in chitinase molecule, it is expected that the anomeric specificities of chitinases obtained from various sources are not identical. In this study, we analyzed the anomeric specificities of chitinases from a microbe, \(\textit{Streptomyces griseus}\), and from a plant, \(\textit{Dioscorea opposita}\) (yam), by \(^{1}H\)-NMR spectroscopy.

The substrate, \(N,N,N,N\)-pentaacetylchitotetraosylactose \([R-(\text{GlcNAc})_4]_1\), was lyophilized three times from 99.8% \(D_2\)O, and then dissolved in 0.5 ml of 0.01 M sodium deuteroacetate buffer (pH 4.5). The substrate solution was put into a 5-mm NMR tube. \(\textit{Streptomyces griseus}\) chitinase purchased from Sigma Chemical Co. was dissolved in the same buffer, and a portion of the enzyme solution was added to the substrate solution in an NMR tube. The NMR tube was immediately set into the NMR probe, which was thermostatted at 22°C. After an appropriate reaction time, \(^1\)H-NMR spectrum was obtained with a JEOL EX-270 spectrometer. The solvent peak was suppressed with a pulsed presaturation procedure. Accumulation of a spectrum required 3 min. The substrate concentration was 8.7 mM and the enzyme concentration was adjusted so that the enzymatic hydrolysis was completed in 60–100 min. The enzymatic reaction was also done with chitinase E3 purified from a yam, \(\textit{Dioscorea opposita}\) \(\text{Thuinb.}\), instead of \(\textit{S. griseus}\) chitinase.

A time-dependent profile of the \(^1\)H-NMR spectrum of \(\textit{S. griseus}\) chitinase reaction mixture is shown in Fig. 1. According to the assignments reported thus far,\(^7\) the signals at 5.14 ppm (H1\(\alpha\)) and 4.64 ppm (H1\(\beta\)) are derived from the anomeric proton of reducing end GlcNAc residue in \(\alpha\)-form and that in \(\beta\)-form, respectively. The signals at 4.5–4.6 ppm (H1) are from the anomeric protons of the other GlcNAc residues. With progress of the reaction time, the peak area of the signals of H1 decreased, and those of H1\(\alpha\) and H1\(\beta\) increased. However, H1\(\beta\) increased rapidly in the early stage of the reaction, and decreased gradually after 120 min of reaction time. The increment of H1\(\alpha\) was quite gradual, and continued even at 600 min of reaction time. As easily expected, \(\textit{S. griseus}\) chitinase should produce oligosaccharides in \(\beta\)-anomer,

![Chemical shift (ppm)](image-url)

**Fig. 1.** Time-Dependent Profile of Anomeric Proton Signals of the Substrate and the Products in Chitinase Reaction Mixture. Chitinase was from \(\textit{Streptomyces griseus}\). Substrate \([R-(\text{GlcNAc})_4]_1\) concentration was 8.7 mM. The enzymatic reaction was done in 0.01 M sodium deuteroacetate buffer, pH 4.5, and at 22°C. H1\(\alpha\) and H1\(\beta\) indicate the signals derived from the anomeric proton of the reducing end GlcNAc residue in \(\alpha\)-form and that in \(\beta\)-form, respectively. H1 indicates the signals derived from the anomeric protons of the other GlcNAc residues.

**Abbreviations:** GlcNAc, 2-acetamido-2-deoxy-\(\alpha\)-glucopyranose; (GlcNAc)\(_n\), \(\beta\)-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of \(n\); R-(GlcNAc)\(_n\), (GlcNAc)\(_n\), wherein the reducing end residue is reduced to sugar alcohol with NaBH\(_4\); NMR, nuclear magnetic resonance.
which should be transformed to \( \alpha \)-anomer by mutarotation. After completion of the enzymatic reaction (1440 min), the relative peak area of the signals of reducing end GlcNAc residue (H1\( \alpha \) + H1\( \beta \)) to the total peak area of anomeric proton signals (H1\( \alpha \) + H1\( \beta \) + H1) was 24\%. R-(GlcNAc)\(_5\) has four glycosidic bonds and four anomeric protons, whose signals are designated by H1, and does not have a reducing end proton. If the substrate is hydrolyzed only at one specific glycosidic bond among the four, one of the four anomeric protons (H1) is converted to a reducing end proton (H1\( \alpha \) or H1\( \beta \)) and the relative peak area of the reducing end signals (H1\( \alpha \) + H1\( \beta \)) to the total peak area of anomeric proton signals (H1\( \alpha \) + H1\( \beta \) + H1) should theoretically be 25\% after completion of the hydrolytic reaction. Thus, the relative peak area experimentally obtained from 1440 min (24\%) indicates that R-(GlcNAc)\(_5\) is hydrolyzed only at one specific glycosidic bond.

In contrast to \textit{S. griseus} chitinase, \textit{D. opposita} chitinase gave a rapid increase in H1\( \alpha \) and a gradual increase in H1\( \beta \) as shown in Fig. 2. At 60 min of reaction time, H1\( \alpha \) signal reached a maximum of peak area and thereafter decreased gradually. \textit{D. opposita} chitinase had the opposite specificity of anomer production to that of \textit{S. griseus} chitinase. At 1440 min, the relative peak areas of the reducing end signals (H1\( \alpha \) + H1\( \beta \)) to the total peak area of anomeric proton signals (H1\( \alpha \) + H1\( \beta \) + H1) was 26\%. The value indicates that the chitinase hydrolyzes R-(GlcNAc)\(_5\) only at one specific glycosidic bond.

Since R-(GlcNAc)\(_5\) is hydrolyzed only at one specific glycosidic bond, the molar concentration of the reducing end produced by the enzymatic hydrolysis is theoretically equal to the initial concentration of substrate (8.7 mM) after completion of the enzymatic reaction. Thus, the molar concentrations of H1\( \alpha \) and H1\( \beta \) at each reaction time could be calculated from the relative peak areas of the individual signals, and were plotted against reaction time (Fig. 3). The experimental time-courses of anomer formations were analyzed by a curve fitting procedure.

The enzymatic reaction and the mutarotation reaction can simply be represented by the reaction scheme shown in Fig. 4. The differential equation for \( S, P_\alpha \), and \( P_\beta \) can be written as

\[
\begin{align*}
\frac{dS}{dt} &= -k_1S + k_2P_\alpha + k_3P_\beta \\
\frac{dP_\alpha}{dt} &= -k_1S - k_2P_\alpha + k_4P_\beta \\
\frac{dP_\beta}{dt} &= -k_3P_\alpha - k_4P_\beta + k_5P_\beta 
\end{align*}
\]
\[
\frac{d[S]}{dt} = -(k_s + k_p)[S]
\]

\[
\frac{d[P_\alpha]}{dt} = k_s[S] + k_1[P_\alpha] - k_1[P_\gamma]
\]

(1)

\[
\frac{d[P_\gamma]}{dt} = k_p[S] + k_1[P_\alpha] - k_2[P_\gamma]
\]

These equations were numerically solved by a computer to obtain the theoretical time-courses. The Runge-Kutta-Gill method was used for a numerical solution of the equation. By use of a Gauss-Newton nonlinear least-squares curve fitting procedure, Eq. (1) was fitted to the experimental time-courses to obtain the optimal values of the rate constants shown in the reaction scheme.

From our knowledge of the anomeric specificity of glycosyl hydrolases investigated thus far, (GlcNAc)_m should be produced through either of the \(k_s\) and \(k_p\) processes. Thus, either of the \(k_s\) and \(k_p\) values was assumed to be zero, and the curve fitting was done by changing the values of the other three rate constants.

The theoretical time-courses best fitted to the experimental ones are also shown in Fig. 3. Assuming \(k_s = 0\) (for \(S. griseus\) chitinase) or \(k_p = 0\) (for \(D. opposita\) chitinase), the calculated time-courses were successfully fitted to the experimental ones, indicating that the two chitinases have strict specificity of anom production but a pronounced contrast in their anomeric specificities. The rate constant values for each chitinase reaction were obtained by the curve fitting procedure, and are listed in the figure. The rate constants for mutarotation thus obtained are consistent with the reported values of \(k_1\) and \(k_2\), which are obtained by HPLC analysis.

Anomeric specificity of the products for each chitinase could be qualitatively evaluated from the time-dependent profile of \(^1\)H-NMR spectra. However, the kinetic analysis of the experimental time-courses of \(\alpha\)- and \(\beta\)-anomer productions is needed for quantitative evaluation, because the mutarotation of \((\text{GlcNAc})_m\) cannot be neglected under the reaction conditions used in this study. By these procedures, we have first found a pronounced difference in anomeric specificity between the chitinases from a plant and a microbe. Although most enzymes classified as lysozymes have a specificity of anom retention in their hydrolytic reaction, only the lysozyme from papaya latex has a specificity of anom inversion.\(^9\) The chitinase from yam also exhibits an inversion of anomeric form as observed for lysozyme from papaya latex. The chitinolytic enzymes from plants may universally have a specificity of anom inversion. There may be some physiological implication in the specificity of \(\alpha\)-anomer production of plant chitinolytic enzymes. For further investigation of physiological function of chitinases, it is important to identify the anomeric specificity of chitinases from various biological sources.

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References