Analysis of products digested by glycosyl hydrolases helps understanding of the hydrolysis mechanism and the substrate recognition in the enzymes. We developed a new universal technique, which consists of ruthenium (II) complex labeling and mass spectrometry analysis, to identify the reducing sugars released from oligosaccharides by enzymatic digestion. This method was applied to enzymatic digestion by chitinase and cellulase of the hyperthermophilic archaea Pyrococcus furiosus and Pyrococcus horikoshii respectively.

Key words: ruthenium complex; mass spectrometry; amination of saccharide; chitinase; cellulase

The most useful abundant biomass materials on Earth are polysaccharides such as starch, chitin, and cellulose. Biological conversion of polysaccharides has been studied extensively during recent decades for industrial purposes. A number of polysaccharide-degradable glycosyl hydrolases have been reported. Understanding of their catalytic mechanisms is important for the efficient utilization of biomass.

Conventionally, isotope-labeled substrates are used to analyze the reactions of glycosyl hydrolases. In previous studies, preparation of isotope-labeled substrate has made possible detailed analysis of the reaction mechanism of glycosyl hydrolases, but this traditional method is not recommended, due to technical difficulties and safety concerns. Consequently, universal analytical methods for the analysis of product sugars have been awaited to study glycosyl hydrolases in detail.

In order to develop industrially applicable glycosyl hydrolases, we have studied hyperthermophilic chitinase and cellulase from the hyperthermophilic archaea Pyrococcus furiosus and Pyrococcus horikoshii respectively. Chitinase and cellulase cleave /C12 1,4 glycosidic linkages of polymeric N-acetylglucosamine (chitin) and glucose (cellulose). This archaeal chitinase and archaeal cellulase are not only stable at high temperatures, but are effective to both colloidal and crystalline polysaccharides. Since both enzymes are industrially useful, their mechanisms of catalysis and substrate recognition are informative, but their detailed hydrolytic patterns remain unclear and need to be elucidated through analysis of the product released from the substrate.

For the analysis of the enzyme-digested products, we developed a new technique based on a method we have established for the N-terminal sequencing of proteins. According to this sequencing method, the amino groups of the protein are selectively labeled with an active ester of bis(terpyridine)ruthenium (II) [(Ru)-CO-labeling, Fig. 1]. The labeled molecules are easily recognized by mass spectrometry (MS), since Ru has a characteristic isotope distribution due to stable isotopes, 96Ru, 98Ru–102Ru, and 104Ru.

The outline of our method is shown in Fig. 2. In the first step, we introduced an amino group at the reducing end of the substrates (Fig. 2, 1–4) because (Ru)-CO-labeling requires an amino group at the position of interest. This amination step was performed by Lubineau’s method, with minor modifications. The oligosaccharide (0.01 mmol) and ammonium bicarbonate (0.35 mmol) were dissolved in 50 ml of 30% ammonia solution and the solution was incubated at 42°C for 16 h. White precipitants were collected by filtration and dissolved in 100 ml of water. The resulting glycosylamines (Fig. 2, 1–4) were analyzed by mass spectrometry.
digested with hyperthermostable chitinase and with cellulase from *P. furiosus* and *P. horikoshii* respectively.6,9) Glycosylamine (1.0 mol) was dissolved in 100 μl of 20 mM potassium phosphate buffer (pH 6.0), and then 10 μl of approximately 10 μM chitinase or cellulase was added. The enzyme reaction mixtures were incubated overnight at 40°C. Although the enzymes used are effective at about 90°C, the enzyme reaction was performed at relatively low temperature because the reducing group formed by the enzymatic digestion can

![Diagram of enzymatic digestion](image)

**Fig. 2.** (Ru)-CO-Labeling Procedure for Digested Product with Chitinase or Cellulase. Initial oligosaccharide substrates and glycosylamine derivatives are shown by 1–4 and 1’–4’ respectively.

![ESI-Qq-TOF-MS Spectra](image)

**Fig. 3.** ESI-Qq-TOF-MS Spectra of (Ru)-CO-Labeled Glycosylamines after Enzymatic Digestion. The spectra of products of chitinase (1’ and 2’, Fig. 2) are shown in the top and bottom panels in (A) respectively, and those of cellulase (3’ and 4’, Fig. 2) in the top and bottom panels in (B) respectively. The (Ru)-CO-labeled peaks appeared in divalent ion. M1 and M2 indicate (Ru)-CO-labeled chitobiosylamine and cellobiosylamine respectively. Insets are spectra at around m/z 546 (A) and m/z 505 (B) expanded to show their isotope distribution patterns.
After enzymatic digestion, the mixture of the products was subjected to (Ru)-CO-labeling. The digested product was dissolved in 10 μl of 100 mM sodium bicarbonate, followed by the addition of 10 μl of a solution containing 1.26 mM (Ru)-COONSuc and 100 mM sodium bicarbonate. This labeling reaction was carried out at 40 °C overnight. The final reaction mixture was analyzed by ESI-Qq-TOF-MS (Fig. 3) with a Q-Star spectrometer (Applied Biosystems, Foster City, CA) equipped with a nanospray ion source. The digested products of the chitotriosylamine (Fig. 2, 1’) and chitopentaosylamine (Fig. 2, 2’) substrates showed the peaks at m/z 546.6 and 546.7 respectively (Fig. 3A), in good agreement with the (Ru)-CO-labeled disaccharide (M1, chitobiosylamine). The digested products of cellotriosylamine (Fig. 2, 3’) and cellopentaosylamine (Fig. 2, 4’) were also analyzed by the MS (Fig. 3B). The main peaks, at m/z 505.8 and 505.9, were assigned to the (Ru)-CO-labeled disaccharide (M2, chitosylamine). No other (Ru)-CO-labeled species than the labeled disaccharide (Fig. 2, m = 1) was detected with the MS. These results indicate that both archaeal chitinase and archaeeal cellulase liberate disaccharides from the reducing ends.

Fluorogenic and chromogenic substrates, such as a synthetic sugar containing p-nitrophenol, are often used in the analysis of enzymatic degradation by glycosyl hydrolases. However, these substrates can affect the binding mechanism, since the heterologous group cause steric hindrance and/or additional affinity.3,4 Our new method overcomes this problem through minimal structural modification of the substrate: only the substitution of the hydroxyl group to the amino group. Moreover, synthesis of glycosylamines by ammonium hydrogen carbonate and (Ru)-CO-labeling of the amino group are simple reactions with high reproducibility. These features mean that this analytical method makes possible easy, rapid, and selective determination of reducing sugars and supplies reliable information on the substrate binding mechanism of glycosyl hydrolases.

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References


