Methanolysis of 7-Acetoxy-4-Methylcoumarin Catalyzed by Cyclosophoraoses Isolated from *Rhizobium meliloti*

Heylin Park,[†] Lin-woo Kang,[†] and Seunho Jung[†,‡,*]

[†]*Department of Advanced Technology Fusion,* [‡]*Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea.* [*]*E-mail: shjung@konkuk.ac.kr*
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Cyclosophoraoses (cyclic-(1→2)-β-D-glucan, collectively "Cys") are unique molecules that are synthesized by members of the family *Rhizobiaceae* as an *intra-* and *extra-*oligosaccharide.[1] In *Agrobacterium* and *Rhizobium* species, this molecule contains glucose residues linked solely by β-1,2-glycosidic bonds, and the predominant ring size distribution is between 17 and 27 glucose residues (Figure 1). Additionally, Cys may become modified with anionic substituents.[1] The biosynthesis of Cys is osmotically regulated, with high levels synthesized during growth at low osmolarity. This osmotic regulation was first observed in *Agrobacterium tumefaciens* cultures, and it was proposed that the Cys may function during hypo-osmotic adaptation.[2] Cys is also involved in the initial stage of the root-nodule formation of the *Rhizobium* species in nitrogen fixation.[3,4] Cys is suspected to be involved in complexation with various plant flavonoids throughout the process of root-nodule formation.[5] Several reports have shown that Cys has a good potential as a host molecule for various inclusion complexation technologies; for example, as a solubility enhancer of poorly soluble guest molecules,[6-8] a chiral nuclear magnetic resonance (NMR) solvating agent[9] and a chiral additive in capillary electrophoresis (CE).[10] Recently, a novel function of Cys as a catalyst for methanolysis reaction has been reported.[11] Cys enhanced the rates of the methanolysis reactions of both oxazolone compounds and phospholipids. Even if the exact three-dimensional structure of Cys is not clearly identified, NMR[12] and conformational studies[12,13] suggest that Cys has flexible backbone structures and narrower cavity sizes than expected. The characteristic scaffold induced by β-(1→2)-glycosidic linkages of Cys would provide an appropriate space for the binding of substrates, thus affording the possibility of a catalytic reaction.[11]

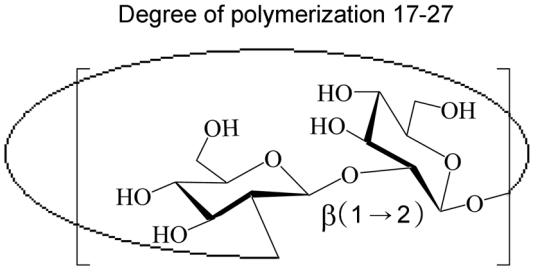

Figure 1. Chemical structure of Cys.

Herein, we report on the methanolysis of 7-acetoxy-4-methylcoumarin, one of the coumarin derivatives, catalyzed by Cys employed as a biological catalytic carbohydrate. Coumarin and its derivatives, flavonoids isolated from various plants, have been generally reported as antioxidants.[14] The reaction was monitored by ^{1}H NMR spectroscopy and high-performance liquid chromatography (HPLC). The determination of the reaction intermediate formed on Cys during methanolysis of 7-acetoxy-4-methylcoumarin was also investigated by mass spectrometric analysis.

First, we conducted isolation, purification, and structural analyses of Cys as described previously.[5,8,15] Through NMR spectroscopy and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, we confirmed that Cys is composed of unbranched cyclic β-(1→2)-D-glucans with various sizes from 17 to 27 in degree of polymerization (DP). Based on the MALDI-TOF mass

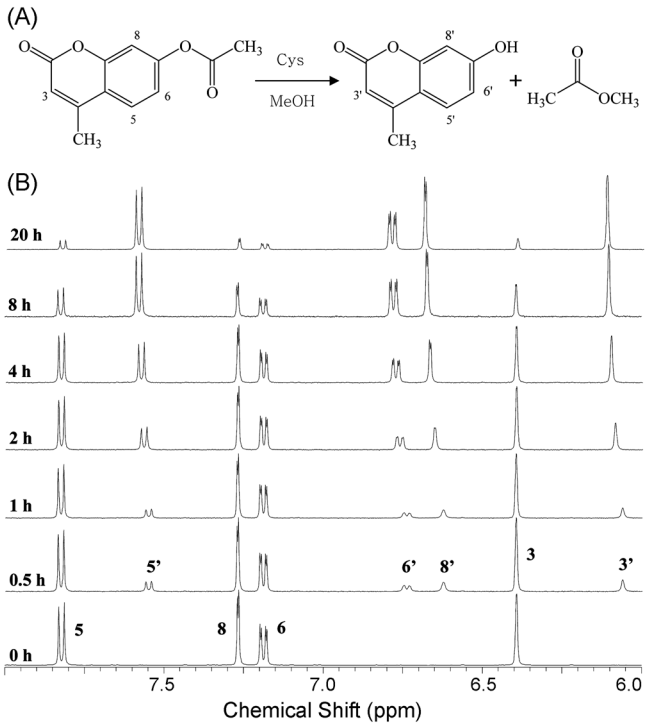

Figure 2. Scheme of methanolysis (A) and ^{1}H NMR spectra (B) of 7-acetoxy-4-methylcoumarin and cleaved coumarine derivative in presence of 0.1 equiv of Cys at different periods of time.

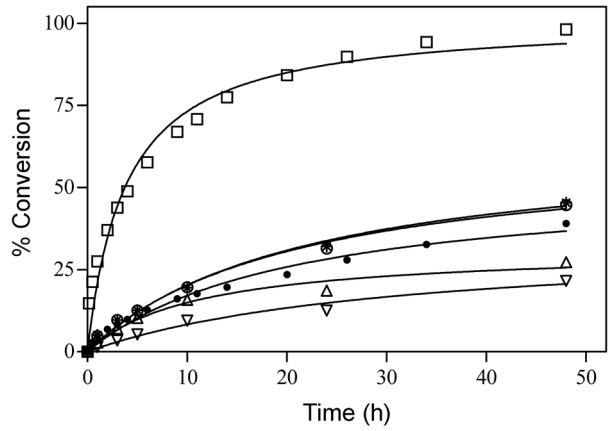

Figure 3. Time-course of methanolysis of 7-acetoxy-4-methylcoumarin in the absent and present of 0.1 equivalent Cys at 30 °C. The data were fitted to a single exponential to obtain k_{cat_Cys} and k_{uncat}. Symbols; Cys (□), 4 mM glucose (○), 88 mM glucose (*), 4 mM β-CD (△), and 12 mM β-CD (▽), without catalyst (●).

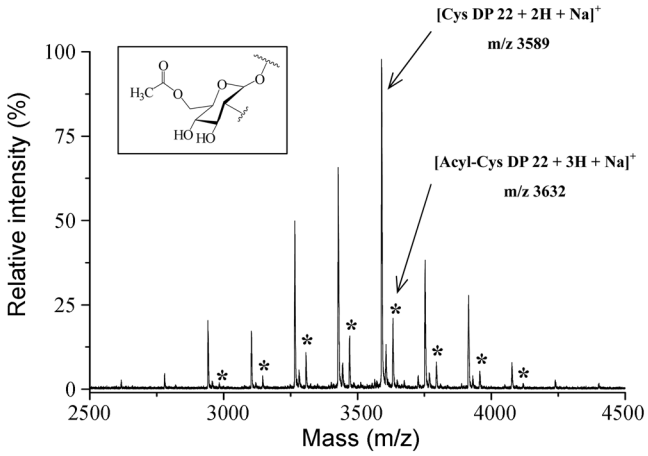

Figure 4. MALDI-TOF mass spectra of reaction mixtures containing Cys and potential acyl intermediates formed on Cys (Acyl-Cys). The each signal of at m/z 2778, 2941, 3103, 3265, 3427, 3589, 3752, 3914, 4076, and 4238 corresponds to the DP of 17 to 26 Cys with cationized with one sodium, respectively. The asterisks indicate the sequential peaks, which were proposed to be Acyl-Cys containing acetyl (m/z 2820, 2982, 3145, 3307, 3470, 3632, 3794, 3956, and 4117). The chemical structure of a possible Acyl-Cys is shown in a square box.

spectrometry, the number-average molecular weight, M_n of Cys was determined as 3568.6.[8]

Cys, one of the microbial carbohydrates, catalyzed the ester cleavage of 7-acetoxy-4-methylcoumarin in methanol solvent as shown in Figure 2(A). Figure 2(B) shows the partial ^{1}H NMR spectra of 7-acetoxy-4-methylcoumarin and cleaved coumarin derivative (product) in presence of 0.1 equiv of Cys at different periods of time. The proton peak of the exchanged methyl group of the product appeared at 30 min and increased with reaction time. Cys-induced methanolysis of 7-acetoxy-4-methylcoumarin was also monitored by HPLC and then time-course curves were obtained where the reaction was enhanced about 11.4-fold (k_{cat}/k_{uncat}) in the presence of 0.1 equiv of Cys in methanol at 30 °C (Figure 3). Artificial enzymes generally have been reported to achieve rate enhancements between 10^1 and 10^5. In this respect, this rate acceleration is modest compared to natural enzyme equivalent.[16-18] In addition, methanolysis of 7-acetoxy-4-methylcoumarin was enhanced about 6-fold by succinoglycan, acid polysaccharide from *Rhizobium meliloti*.[19] We also investigated the methanolysis reaction by glucose (4 and 88 mM) and β-cyclodextrin (β-CD, 4 and 12 mM) to identify the effect of other typical carbohydrates. But the catalytic effects of glucose or β-CD were hardly detected when the glucose or β-CD of same mole concentration or same amount (weight) with Cys were added (Figure 3). Besides, % conversion of 7-acetoxy-4-methylcoumarin to product was decreased with concentration of β-CD. Although β-cyclodextrin (β-CD) is known to be an interesting enzyme model, it was reported that β-CD did not accelerate, but rather retarded some reactions such as hydrolysis of aniline compounds depending on the substrate structure.[20] These results would be due to the fact that β-CD inhibited the methanolysis of 7-acetoxy-4-methylcoumarin by undesirable interaction with the substrate.

The ester cleavage of the 7-acetoxy-4-methylcoumarin by a nucleophile can occur through a nucleophilic pathway with the formation of intermediates that are produced from the release of the acetyl group of the substrate. MALDI-TOF mass spectrometric analysis of the reaction mixture was performed to assess whether intermediate of Cys was formed during reaction. In the mass spectrum of the reaction mixture of methanolysis of 7-acetoxy-4-methylcoumarin by Cys, peaks at m/z 2820, 2982, 3145, 3307, 3470, 3632, 3794, 3956, and 4117 were newly detected (Figure 4). These molecular ions correspond to the results from m/z shift of 42, which could be attributed to the acyl moiety (acetyl) of 7-acetoxy-4-methylcoumarin, as compared to the native Cys with DP 17 to 25. Through the mass analysis, these sequential peaks were proposed to be acyl intermediates formed on Cys (Figure 4).

In this study, we have described that Cys isolated from a soil microorganism, *Rhizobium meliloti*, functioned as a catalytic carbohydrate in the methanolysis of 7-acetoxy-4-methylcoumarin, a derivative of flavonoid. Cys enhanced the ester cleavage of 7-acetoxy-4-methylcoumarin about 11.4-fold (k_{cat}/k_{uncat}) in methanol at 30 °C. In addition, possible acyl intermediates formed on Cys during reaction were revealed by MALDI-TOF mass spectrometric analysis, suggesting that Cys-catalyzed methanolysis likely proceed through a nucleophilic pathway with an intermediate formation step. Although the exact reaction mechanism remains unclear, we assume that the catalytic methanolysis could be attributed to not only an appropriate space based on the characteristic scaffold induced by β-(1→2)-glycosidic linkages within Cys for the binding of substrates but also an attack of hydroxyl group of Cys as a nucleophile. This result and previous report of methanolysis catalyzed by Cys suggest that a microbial catalytic carbohydrate, Cys, has catalytic ability for transesterification of ester compounds

through nucleophilic pathway in organic solvents and its ability is dependent on the structure of substrate. The study made it possible to advance further studies on catalytic functions of other microbial carbohydrates which are comparable to typical enzyme models.[23]

Experimental Sections

Materials. All the chemicals containing 7-acetoxy-4-methylcoumarin were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). D2O (99.9 atom % D), $CDCl_3$ (99.9 atom % D), and DMSO-d_6 (99.9 atom % D) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Preparation of cyclosophoraoses. *Rhizobium meliloti* were cultured in a 5-L jar fermenter containing glutamic acid-mannitol-salts (GMS) medium to a late logarithmic phase at 30 °C.[21] Cells were harvested by centrifugation (8,000 rpm, at 4 °C) and then extracted with 75% (v/v) ethanol at 70 °C for 30 min. After centrifugation, the supernatant was concentrated on a vacuum rotary evaporator. The concentrated sample was chromatographed on a Sephadex G-50 column (3 × 130 cm) at a rate of 1 mL/min and eluant fractions (7 mL) were assayed for carbohydrate by the phenol-sulfuric acid method. The fractions containing Cys were pooled, concentrated, and desalted using a Sephadex G-10 column (2 × 20 cm). The desalted sample was then applied to a column (2 × 20 cm) of DEAE-cellulose to separate neutral and anionic Cys. After the neutral Cys were desalted using a Sephadex G-10 column (2 × 20 cm), they were also confirmed on thin layer chromatography (TLC, BuOH:EtOH:water = 5:5:4, v/v),[22] NMR spectroscopy,[7,8] and MALDI-TOF mass spectrometry.[6]

General experimental procedures. NMR spectra were recorded in D_2O, CD_3Cl or DMSO-d_6 with a Bruker AMX spectrometer (operated at 500 MHz for 1H, 125 MHz for 13C) at 25 °C. All NMR measurements were performed with 0.7 mL samples in 5 mm NMR tubes. Tetramethylsilane (TMS, Me_4Si) was used as an external reference, and chemical shifts were calibrated with an accuracy of 0.05 ppm. A Jupiter C18 column (5 μM, 250 × 4.60 mm) was used for HPLC (Shimadzu, Japan) experiments. The analysis was carried out at 30 °C and a flow rate of 1 mL/min with the mobile phase (water:acetonitrile = 60:40, v/v). Elution was monitored at 270 nm. MALDI-TOF mass spectra were obtained with a MALDI-TOF mass spectrometer (Voyager-DETM STR Bio-Spectrometry, Applied Biosystems, Framingham, MA, USA) in the positive ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix. Approximately 0.5 μL of the sample/matrix mixture was applied to the MALDI probe, and the solvent was removed by evaporation.

General procedure for the methanolysis by Cys. The reaction was carried out in MeOH (2 mL) containing 40 mM of 7-acetoxy-4-methylcoumarin at 30 °C in the absence or presence of 0.1 equiv of Cys. To identify the effect of other typical carbohydrates, 4 or 88 mM glucose and 4 or 12 mM β-CD were also tested. The glucose or β-CD of same mole concentration or same amount (weight) with Cys was added. Aliquots were periodically withdrawn and analyzed by NMR spectroscopy or HPLC. The values of k_{cat_Cys} and k_{uncat} were calculated by integration of the respective HPLC peaks were assigned to substrate and product. The data were fitted to a single exponential to obtain k_{cat_Cys} and k_{uncat}. To measure the possible acyl intermediates, 7-acetoxy-4-methylcoumarin (70 mM) was dissolved in 1mL of MeOH, and Cys (5 mg) was then added. At 1 h, the reaction mixture was completely evaporated at room temperature, and subjected to extraction after adding 1mL of water and chloroform, respectively. The intermediates dissolved in the water layer were then analyzed by MALDI-TOF mass spectrometry.

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