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Methanolysis of ethyl esters of *N*-acetyl amino acids catalyzed by cyclosophoraoses isolated from *Rhizobium meliloti*

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Abstract—Methanolysis of four ethyl esters, *N*-acetyl-L-phenylalanine ethyl ester, *N*-acetyl-L-tyrosine ethyl ester, *N*-acetyl-L-tryptophan ethyl ester, and ethyl phenylacetate was catalyzed by a mixture of microbial cyclooligosaccharides termed cyclosophoraoses isolated from *Rhizobium meliloti*. Cyclosophoraoses [cyclic-(1→2)-β-D-glucans, collectively 'Cys'] are a mixture of large-ring molecules consisting of various numbers of glucose residues (17–27) linked by β-(1→2)-glycosidic bonds. Cys as a catalytic carbohydrate enhanced the methanolysis about 233-fold for *N*-acetyl-L-tyrosine ethyl ester in comparison with a control. The effect of dry organic solvents on the methanolysis of *N*-acetyl-L-tyrosine ethyl ester was investigated by high-performance liquid chromatography (HPLC), and it was found that the rate enhancement correlated closely with the hydrophobicity of the solvent.

Keywords: *N*-Acetyl amino acids; Ethyl esters; Methanolysis; *Rhizobium meliloti*; Cyclosophoraoses; Catalytic oligosaccharides

1. Introduction

Cyclosophoraoses (collectively 'Cys') are a family of unbranched cyclic-(1→2)-β-D-glucans that are found almost exclusively in fast-growing soil bacteria of the *Agrobacterium* and *Rhizobium* species as *intra*- and *extra*-oligosaccharides.[1,2] Cys is produced as a mixture of large-ring molecules, each of which has various degrees of polymerization (DPs).[3,4] Cys is synthesized in the cytosol and is transported to the periplasmic space where it plays an important role in regulating the osmolarity in response to external osmotic shocks.[5] In addition, Cys is involved in the initial stage of the root-nodule formation of the *Rhizobium* species in nitrogen fixation.[6,7] Cys is suspected to be involved in the complexation with various plant flavonoids throughout the process of root-nodule formation.[8] Several reports have shown that Cys has good potential as a host molecule for various inclusion complexation technologies, primarily owing to its characteristic scaffold dictated by the β-(1→2)-glycosidic linkage (Fig. 1A).

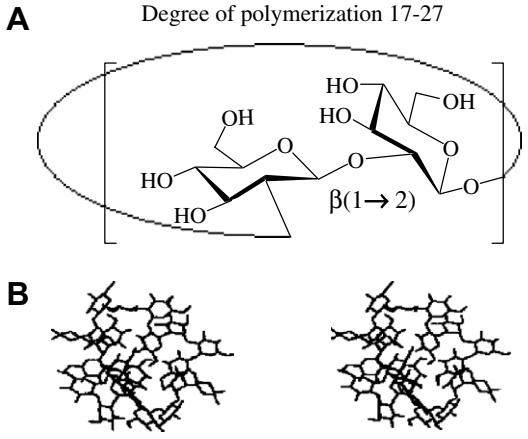

Figure 1. Chemical structure of Cys (degree of polymerization 17–27) (A) and stereoview of the proposed molecular model of Cys (B).

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Examples include application as a solubility enhancer of poorly soluble guest molecules,[9–11] a chiral NMR solvating agent[12] a chiral additive in capillary electrophoresis (CE),[13] and a catalyst for methanolysis.[14] Even if the exact three-dimensional structure of Cys is not clearly identified, nuclear magnetic resonance (NMR)[15] and conformational studies[15,16] suggest that Cys has flexible backbone structures and narrower cavity sizes than expected (Fig. 1B).

In general, protein enzymes, ribozymes,[17] DNAzymes,[18] and catalytic antibodies[19] as typical biocatalysts have been studied by many researchers in various fields; however, catalytic carbohydrates derived from living organisms are the exception. Cyclomaltooligosaccharides [cyclodextrins, cyclic-(1→4)-α-D-glucans] as nonnatural carbohydrates have been known to show catalytic functions.[20,21] Recently, it has been reported that a few microbial carbohydrates, Cys,[14] α-cyclosophorohexadecaose [a cyclic-(1→2)-β-D-glucan with one α-(1→6)-linkage],[22] succinoglycan,[23] and *Zoogloeal* zooglans[24] function as novel biological catalysts for the methanolysis of ester compounds. Among them, Cys enhanced the rates of the methanolysis reactions of both oxazolone compounds and phospholipids. 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.[14]

Herein, we investigated catalytic ability of Cys for the methanolysis of three ethyl esters of *N*-acetyl amino acids and ethyl phenylacetate. The esters of *N*-acetyl amino acids have been used as low-molecular-weight substrates for transesterification in organic solvents or hydrolysis in water by nucleophilic catalysts such as serine proteases.[25–27] The effect of nonaqueous solvents on the methanolysis was also investigated.

2. Results and discussion

2.1. Identification of neutral cyclosophoraoses

The isolation, purification, and structural analyses of Cys were carried out as described previously.[9–11] Purified Cys was separated with a R_f value of 0.13 on TLC. We confirmed that Cys is composed of unbranched cyclic β-(1→2)-D-glucans with various sizes from 17 to 27 in DP by NMR spectroscopy and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectometry. Based on MALDI-TOF mass spectrometry, the number-average molecular weight M_n of Cys was determined as 3568.6.[10]

2.2. Methanolysis of ethyl esters catalyzed by Cys

The methanolysis (Fig. 2) of four ethyl esters, *N*-acetyl-L-phenylalanine ethyl ester (APEE), *N*-acetyl-L-tyrosine ethyl ester (AYEE), *N*-acetyl-L-tryptophan ethyl ester (AWEE), and ethyl phenylacetate (EPA), catalyzed by Cys was monitored by ^{1}H NMR spectroscopy. Figure 3 shows the ^{1}H NMR spectra of APEE (substrate) and *N*-acetyl-L-phenylalanine methyl ester (APME, product) in the 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. After 11 h, methanolysis of APEE by Cys was almost completed (⩾96%), but in the absence of Cys the reaction proceeded slowly (⩽6%). The Cys-induced methanolysis of AYEE, AWEE, and EPA was also monitored by in similar manner of APEE (data not shown). The results in Figure 4 and Table 1 show that the reactions were enhanced approximately 53-, 233-, 38-, and 19-fold for APEE, AYEE, AWEE, and EPA, respectively, in the presence of 0.1 equiv of Cys in

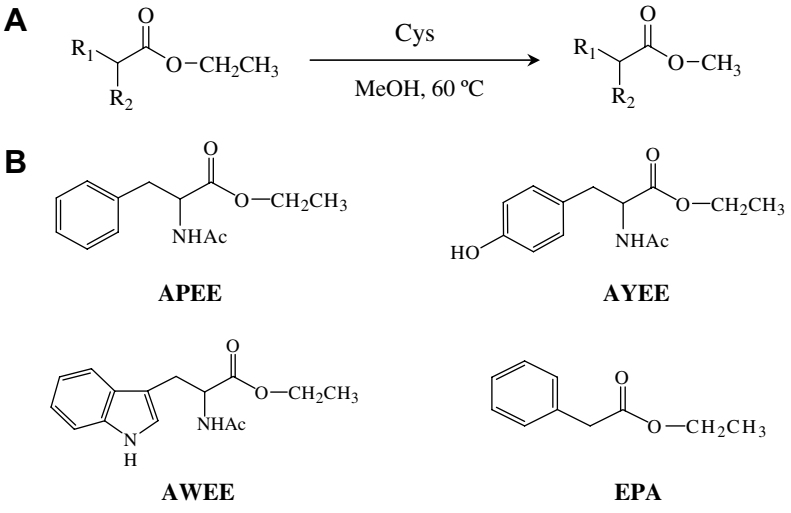

Figure 2. Scheme of methanolysis (A) and chemical structures (B) of ethyl esters.

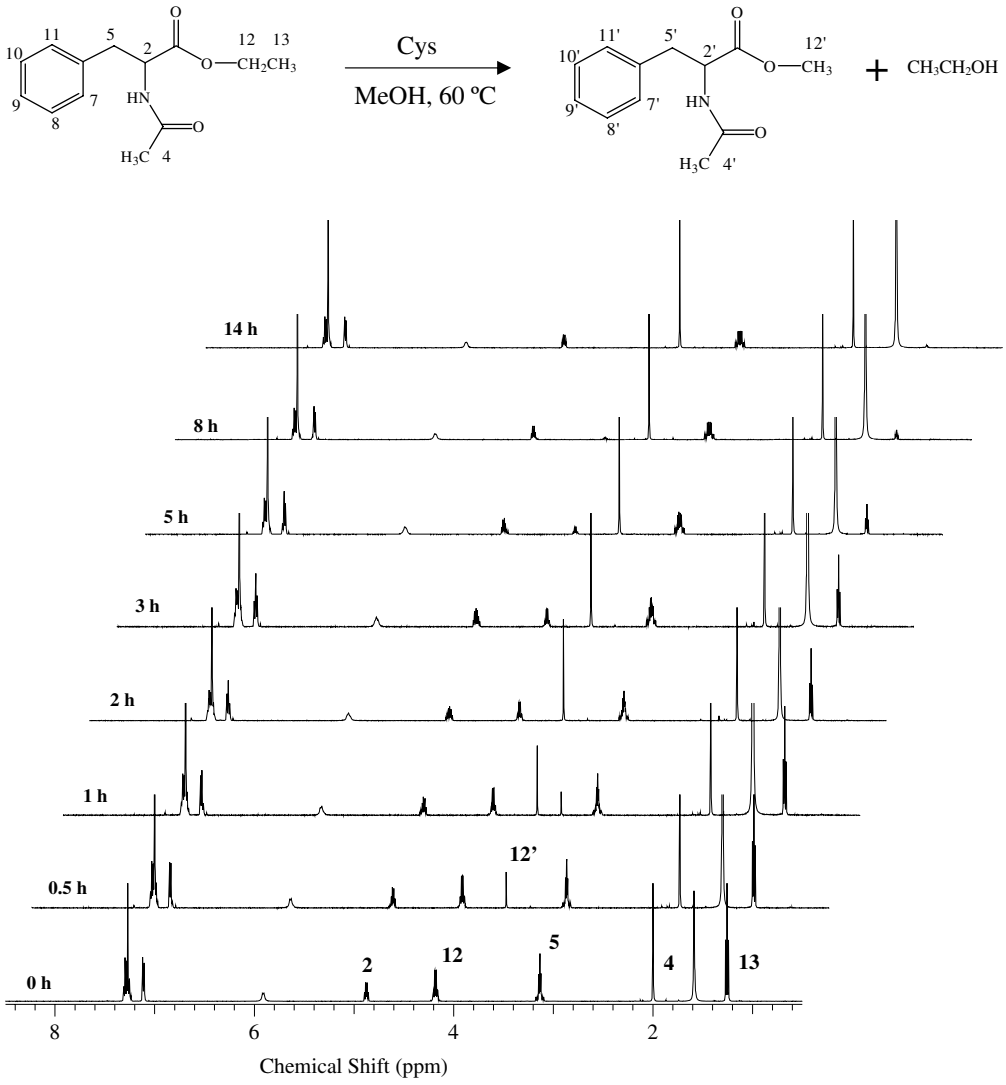

Figure 3. ^{1}H NMR spectra of APEE (substrate), and *N*-acetyl-L-phenylalanine methyl ester (APME, product) in the presence of 0.1 equiv of Cys at different periods of time.

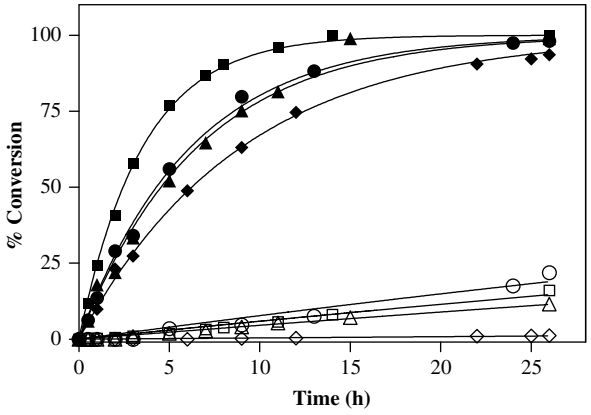

Figure 4. Time-course of methanolysis of ethyl esters in the absence or presence of 0.1 equiv of Cys at 60 °C. The data were fitted to a single exponential to obtain k_{cat_Cys} and k_{uncat} for each substrate. Symbols: APEE (□), AYEE (◇), AWEE (△), and EPA (○) in the absence of Cys, and APEE (■), AYEE (◆), AWEE (▲), and EPA(●) in the presence of Cys.

MeOH. Depending on the chemical functional group linked to ethyl esters, the extent of the rate enhancement for the methanolysis of esters by Cys was different. Cys showed the most effective catalytic activity for AYEE among the four ethyl esters. Although the uncatalyzed methanolysis of esters of *N*-acetyl amino acids (APEE, AYEE, and AWEE) was slower than that of EPA (Table 1), the extent of the rate enhancement for methanolysis of esters of *N*-acetyl amino acids by Cys was higher. The esters containing the N-acetylated amino group were found to be more suitable substrates than EPA for methanolysis by Cys. Glucose, linear-(1→2)-β-D-glucan, and amylose were also tested as catalysts to identify the effect of noncyclic carbohydrates for the methanolysis of AYEE, which had the highest value of k_{cat_Cys}/k_{uncat}. The reactions were monitored by HPLC. Linear-(1→2)-β-D-glucans were prepared by acid hydrolysis of Cys, and then their structural analysis

Table 1. Methanolysis of ethyl esters catalyzed by Cys

	k_{uncat} (min^{-1})	k_{cat_Cys} (min^{-1})	k_{cat_Cys}/k_{uncat}
APEE	0.319	17.034	53.40
AWEE	0.240	9.230	37.96
AYEE	0.028	6.610	233.36
EPA	0.516	9.702	18.81

was investigated by NMR spectroscopy (Fig. 5A) and MALDI-TOF mass spectrometry (data not shown), indicating that the oligosaccharides are β-(1→2)-linked linear glucans with various DP values from 4 to 22. Figure 5B shows the comparison of catalysis by Cys and noncyclic carbohydrates. Glucose and amylose showed no catalytic effect on the methanolysis. Cys and linear-(1→2)-β-D-glucan enhanced the reaction about 180- and 16-fold, respectively. These results can be caused by the structural differences of carbohydrates used in the reaction. Glucose and amylose may lack proper space for the binding of substrate, and linear-(1→2)-β-D-glucan could not completely form the suitable structure to accept the substrate. From the results, we suggest that both of the characteristic scaffolds induced by β-(1→2)-glycosidic linkages and the cyclic structures would provide an appropriate space for the binding of substrates, thus affording the possibility of a catalytic reaction. Additionally, to confirm the catalytic properties of Cys species with different DPs, each cyclosophoraose (from 17 to 27) was fractionated by preparative HPLC. Then the catalytic activity of each cyclosophoraose fraction with DP 19, DP 22, a mixture

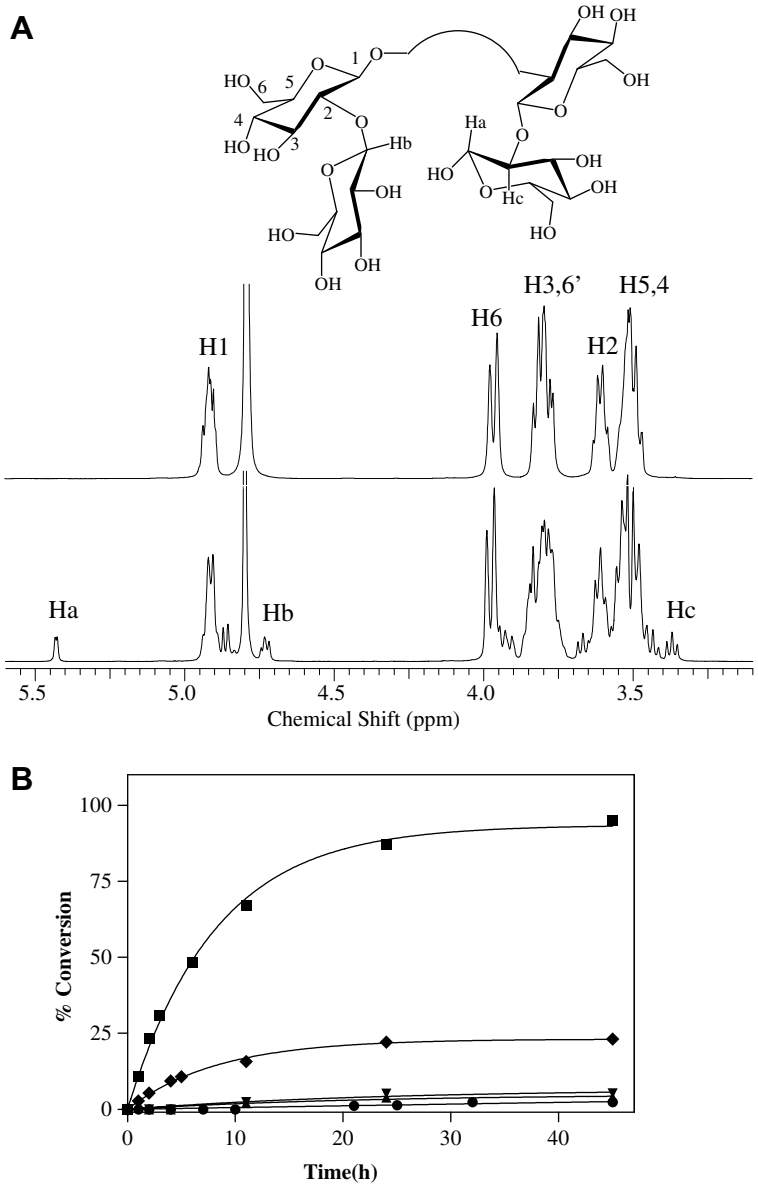

Figure 5. ^{1}H NMR spectra of Cys and linear (1→2)-β-D-glucan (A) and time-course of methanolysis of AYEE (B) in the absence (●) or presence of carbohydrates used as catalysts: Cys (■), linear-(1→2)-β-D-glucan (◆), amylose (▼), and glucose (▲).

of DP 19 and 22, and DP 17–27 was investigated for the methanolysis of AYEE by HPLC. We determined that Cys species with different degrees of polymerization possess almost the same catalytic properties (data not shown). The results can be explained by the fact that Cys has a flexible structure owing to the β-(1→2)-glycosidic linkages and a great water solubility.

2.3. Effect of nonaqueous media on the methanolysis of ethyl esters catalyzed by Cys

The effects of eight organic solvents on the methanolysis of AYEE which had the highest value of k_{cat_Cys}/k_{uncat} were investigated by HPLC. As a control experiment, MeOH was used as both a substrate and an organic solvent ($k_{uncat} = 0.034\ min^{-1}$, $k_{cat\_Cys} = 5.55\ min^{-1}$). HPLC analysis revealed that the reaction progress in each organic solvent was different. Cys was catalytically active ($k_{cat\_Cys} = 6.30\ min^{-1}$) and enhanced the methanolysis of AYEE about 180-fold (k_{cat_Cys}/k_{uncat}) in dry hexadecane. Similar kinetic analyses were carried out for the Cys-catalyzed methanolysis of AYEE in other organic solvents. The effect of each organic solvent was analyzed by comparing the values of k_{rel}, which is the ratio of k_{cat_Cys} in each organic solvent to k_{cat_Cys} in MeOH. The values of k_{rel} broadly correlated with the logarithm of the octanol–water ($\log P$) partition coefficient for a given solvent as shown in Figure 6. This plot shows that Cys-catalyzed methanolysis of AYEE is dependent on the nature of the solvent, and that there is a correlation with the hydrophobicity of the organic solvent. The best solvents were water-immiscible hydrophobic solvents, while the worst were hydrophilic solvents.

2.4. Potential acyl intermediates formed on Cys during the methanolysis

The methanolysis of the ethyl esters by a nucleophile can occur through a nucleophilic pathway with the formation of intermediates that are produced from the release of the ethyl group of the substrate. To assess whether Cys was incorporated into the intermediate, a MALDI-TOF mass spectrometric analysis of the reaction mixture was performed. In the mass spectrum of the reaction mixture for the methanolysis of APEE by Cys, peaks at m/z 2969, 3131, 3293, 3455, 3617, 3779, 3941, 4103, and 4266 were newly detected (Fig. 7A). These molecular ions correspond to the results from a m/z shift of 189, which could be attributed to the acyl moiety (*N*-acetyl-L-phenylalaninyl) of APEE, as compared to the native Cys with DP 17–25. Through the mass analysis, these sequential peaks were proposed to be acyl intermediates formed on Cys (Fig. 7A). New peaks for the potential acyl intermediates of AWEE (Fig. 7B) and AYEE (data not shown) were also detected, showing m/z shift of 228 and 205, respectively.

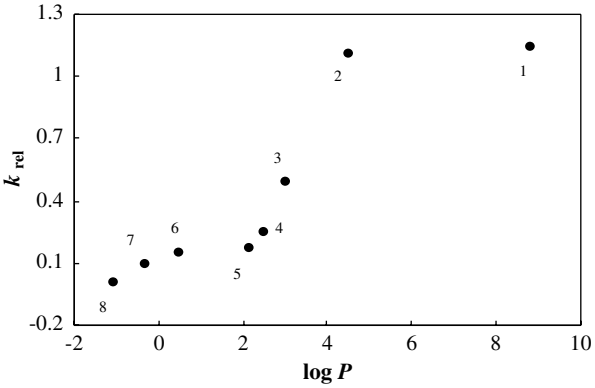

Figure 6. The effects of organic solvents on the methanolysis of AYEE (1, hexadecane; 2, octane; 3, carbon tetrachloride; 4, toluene; 5, benzene; 6, tetrahydrofuran; 7, acetonitrile; 8, dioxane).

In conclusion, we have described that Cys, as a cyclic catalytic carbohydrate, isolated from a soil microorganism, *Rhizobium meliloti*, catalyzed the methanolysis of four ethyl esters at 60 °C. Among the tested substrates, Cys enhanced the methanolysis of AYEE with the highest value of k_{cat_Cys}/k_{uncat}. A comparison of the esters of *N*-acetyl amino acids and EPA indicated that Cys showed a catalytic effect with preference for the substrates containing an *N*-acetamido group. We also investigated the effect of nonaqueous organic solvents on the methanolysis of AYEE. The best solvent was the most hydrophobic solvent, hexadecane. In addition, possible acyl intermediates formed on Cys during reaction were revealed by MALDI-TOF mass spectrometric analysis, suggesting that Cys-catalyzed methanolysis likely proceeds through a nucleophilic pathway with an intermediate formation step.

3. Experimental

3.1. Materials

N-Acetyl-L-phenylalanine ethyl ester (APEE) and *N*-acetyl-L-tyrosine ethyl ester (AYEE) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Fluka Chemical Co. (St. Gallen, Switzerland), respectively. *N*-Acetyl-L-tryptophan ethyl ester (AWEE), ethyl phenylacetate (EPA), D_2O (99.9 atom % D), and $CDCl_3$ (99.9 atom % D) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All the other chemicals containing anhydrous glucose, amylose, and anhydrous organic solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. General experimental procedures

NMR spectra were recorded in D_2O or CD_3Cl with a Bruker AMX spectrometer (operated at 500 MHz for

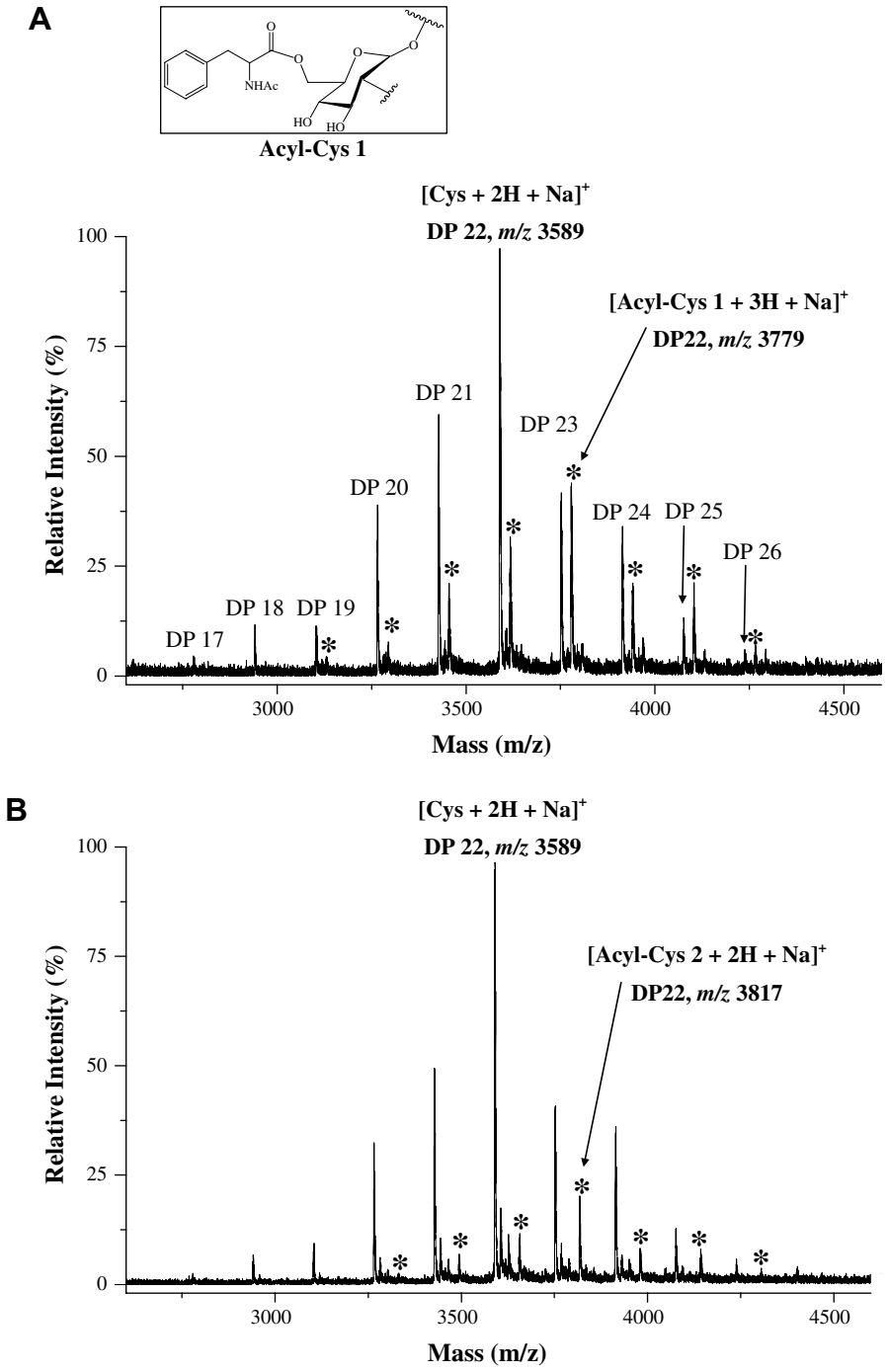

Figure 7. MALDI-TOF mass spectra of reaction mixtures containing Cys and potential acyl intermediates (Acyl-Cys 1 for APEE and Acyl-Cys 2 for AWEE) of the methanolysis of APEE (A) and AWEE (B). Each signal at *m*/*z* 2778, 2941, 3103, 3265, 3427, 3589, 3752, 3914, 4076, and 4238 corresponds to the DP of 17–26 Cys cationized with one sodium ion, respectively. The asterisks indicate the sequential peaks, which were proposed to be Acyl-Cys 1 (A) containing an *N*-acetyl phenylalanine group (*m*/*z* 2969, 3131, 3293, 3455, 3617, 3779, 3941, 4103, and 4266) and Acyl-Cys 2 (B) containing an *N*-acetyl tryptophan group (*m*/*z* 3170, 3332, 3494, 3656, 3817, 3980, 4143, and 4303). The chemical structure of a possible Acyl-Cys 1 is shown in the square box.

^{1}H and 125 MHz for ^{13}C) 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 C_{18} 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 (70:30 water–acetonitrile). Elution was monitored at 278 nm for AYEE. 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.

3.3. Preparation of cyclosophoraoses (Cys)

R. meliloti were cultured in a 5-L jar fermenter containing glutamic acid and mannitol salts (GMS) medium to a late logarithmic phase at 30 °C.[28,29] Cells were harvested by centrifugation (8000 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 carbohydrates 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 was desalted using a Sephadex G-10 column (2 × 20 cm), they were also confirmed on thin-layer chromatography (TLC, 5:5:4 BuOH–EtOH–water), NMR spectroscopy, and MALDI-TOF mass spectrometry. For further separation, each cyclosophoraose with different DP was fractionated by preparative HPLC (LC-6AD, Shimadzu, Japan). The column used was a Bondclone C_8 column (10 μM, 250 × 21.2 mm), and the eluant was 96:4 water–MeOH with flow rate of 4 mL/min.

3.4. Preparation of linear (1→2)-β-D-glucan from cyclosophoraoses (Cys)

Linear-(1→2)-β-D-glucan was prepared by acid hydrolysis (0.08 M trifluoroacetic acid, 100 °C, 1 h) and subsequent fractionation by using a Sephadex G-10 column (2 × 20 cm). The structural analysis of linear (1→2)-β-D-glucan was investigated by NMR spectroscopy and MALDI-TOF mass spectrometry.

3.5. General procedure for the methanolysis of ethyl esters

The reaction was carried out in MeOH (2 mL) containing 40 mM of each ethyl ester at 60 °C in the absence or presence of 0.1 equiv of carbohydrates such as Cys, glucose, linear (1→2)-β-D-glucan, and amylose. To test the effect of organic solvents (hexadecane, octane, carbon tetrachloride, toluene, benzene, THF, acetonitrile, and dioxane), 4 mM of Cys was placed in 2 mL of each organic solvent containing 40 mM of AYEE and 4 M of MeOH. 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 ^{1}H NMR signals, or HPLC peaks were assigned to the ethyl ester and methyl ester. To measure the possible acyl intermediates, each ethyl ester of *N*-acetyl amino acid (200 mM) was dissolved in 1 mL of MeOH, and Cys (14 mg) was then added. At 3 h the reaction mixture was completely evaporated at room temperature and subjected to extraction after adding 1 mL of water and chloroform, respectively. The intermediates dissolved in the water layer were then analyzed by MALDI-TOF mass spectrometry.

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