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Cyclosophoraose as a catalytic carbohydrate for methanolysis

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Abstract—A novel catalytic methanolysis can be induced by a natural cyclooligosaccharide, a cyclosophoraose (cyclic-(1 → 2)-β-D-glucan, Cys), which is a member of a family of unbranched cyclooligosaccharides produced as *intra*- or *extra*oligosaccharides by soil microorganisms of the genus, *Rhizobium*. Cys catalyzed the methanolysis for 5(4*H*)-oxazolones and various phospholipids. Cys enhanced the methanolysis reaction about 9200-fold for a benzylidene oxazolone or 250-fold for dipalmitoylphosphatidylcholine comparing with control. In this study, we describe that natural cyclosophoraoses isolated from the *Rhizobium* species function as catalytic carbohydrates for the methanolysis.

Keywords: Cyclosophoraoses; *Rhizobium meliloti*; Methanolysis; Catalysis; Benzylidene oxazolone; Dipalmitoylphosphatidylcholine

1. Introduction

A catalyst is a substance that enhances chemical reactions. Catalysts reduce the free energy of activation, and thus stabilize the transition state of the reaction. Particularly, biological catalysts show remarkable catalytic functions. Protein catalysts, enzymes, enhance the reaction rate at least a million fold. RNA catalysts, ribozymes or catalytic antibodies, increase reaction rates up to the level of typical enzymes depending on the kinds of reaction.[1,2] Due to a recent discovery of catalytic DNA,[3] nucleotides come to be representative molecules for biocatalysts as well as protein catalysts. However, no report on a natural catalytic carbohydrate isolated from living organisms has been made.

Cys is from a family of unbranched cyclooligosaccharides that are produced as *intra*- or *extra*oligosaccharides with various degrees of polymerization (DP) by soil microorganisms of the family, *Rhizobiaceae*.[4] The first report of Cys came in 1942 with its discovery in the extracellular media of *Agrobacterium tumefaciens* cultures.[5] Cys generally functions in the periplasmic place as an osmoprotectant against osmotic stress.[6] Cys has also been investigated as a host for inclusion complexation against various guest chemicals because of its characteristic scaffold.[7–9] A suggested molecular model (Fig. 1) of Cys indicated the presence of a much narrower hole than that expected.[10,11] This unusual narrow cavity, in which it would appear almost impossible for guest molecules to fit, originates from its characteristic β-(1 → 2)-glycosidic linkage. However, Cys is able to make complexes with various fairly hydrophobic (or nonpolar) guest molecules and even in many cases make much stronger complexes,[7,9] than a typical inclusion complexation agent, β-cyclodextrin (β-CD).

Herein, we report a novel catalytic methanolysis induced by natural Cys isolated from soil microorganisms, which are *Rhizobium* species such as *R. meliloti*, *R. leguminosarum*, and *R. phaseoli*.

2. Results and discussion

2.1. Isolation, purification, and identification of Cys from *R. meliloti*

The isolation, purification, and structural analyses of cyclosophoraoses were carried out as described previously.[7–9] Ring sizes of neutral cyclosophoraoses were

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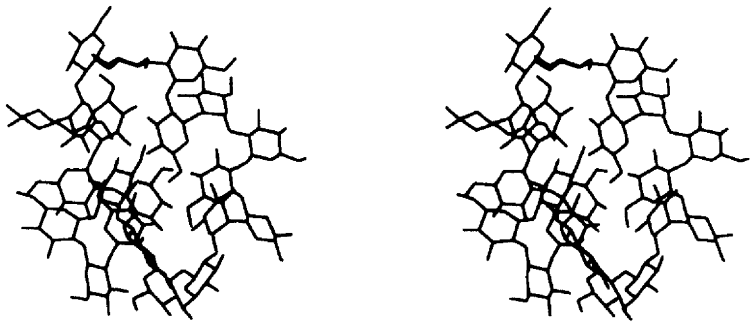

Figure 1. Stereoview of a proposed model[11] of Cys of DP 21.

confirmed ranging from DP 17 to 27 through matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS).[7] Based on the MALDI-MS, the number-average molecular weight M_n of neutral cyclosophoraoses was determined as 3568.6.[8]

2.2. Methanolysis of 4-benzylidene-2-phenyloxazolone (1) catalyzed by Cys

Cys catalyzed the methanolysis of a benzylidene oxazolone ($k_{cat} = 4.16 \times 10^{-1}\,min^{-1}$) as shown in Figures 2A and 3A. Figure 3A shows time curves of **1** where the reaction was enhanced about 9200-fold (k_{cat}/k_{uncat}) in the presence of 0.020 equiv of Cys in MeOH. When we performed the similar complexation experiments with 5(4*H*)-oxazolones, 4-benzyl-2-phenyloxazolone (BPO), or 4-ethoxymethylene-2-phenyloxazolone (EMPO), both of which have two bulky nonpolar moieties adjacent to the oxazolone ring, the methanolysis reaction was also induced by Cys in a similar manner of **1** (Fig. 3A). The reaction was enhanced about 950-fold for BPO and 3100-fold for EMPO in the presence of 0.020 equiv of Cys in MeOH. However, no cleavage reaction was induced with the 3-phenyl-5-isooxazolone, which has only one bulky nonpolar moiety (data not shown). These results would indicate that Cys catalyzed the methanolysis regioselectively. Figure 3B shows the dependence of the cleavage reaction as added equivalents of Cys. After 30 min, the catalytic rate was saturated when the ratio of the equivalent of Cys to the

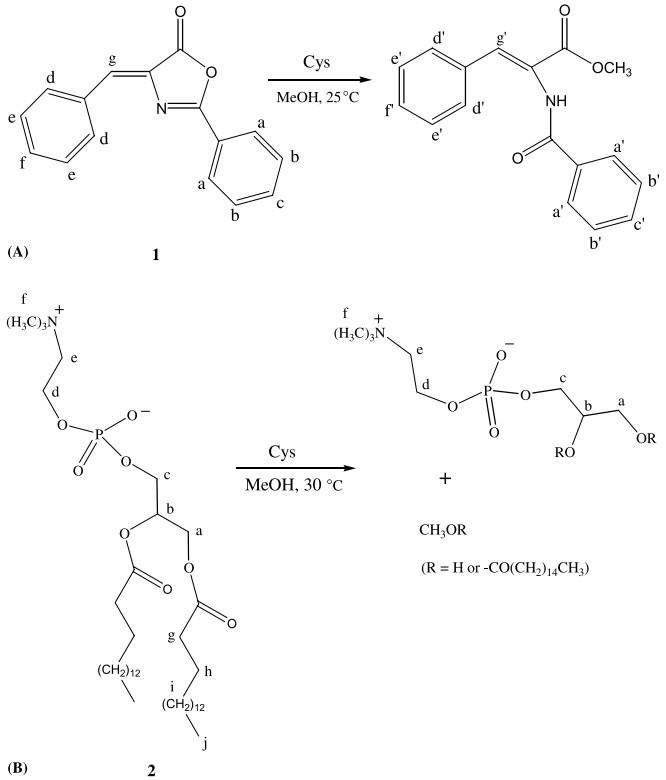

Figure 2. Scheme for the methanolysis of **1** (A) and **2** (B) by Cys.

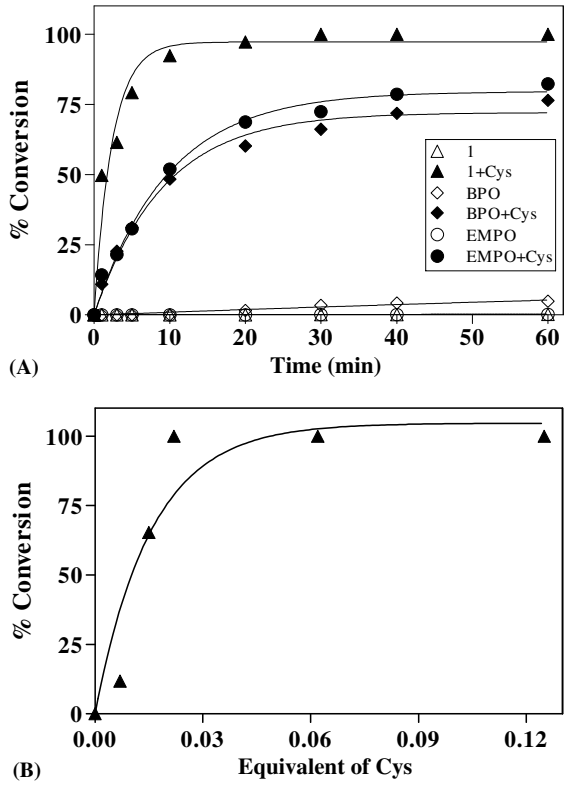

Figure 3. (A) Time-course of methanolysis of **1**, BPO and EMPO in the absence or presence of 0.020 equiv Cys. The reaction was carried out in MeOH (3 mL) at 25 °C with or without Cys. The concentration of the substrates used was 40 mM. The data were fitted to a single exponential to obtain a k_{cat_Cys} of $4.16\times10^{-1}\,min^{-1}$, a k_{uncat} of $0.45\times10^{-4}\,min^{-1}$ for **1**, and k_{cat_Cys} of $0.68\times10^{-1}\,min^{-1}$, a k_{uncat} of $0.71\times10^{-4}\,min^{-1}$ for BPO, and k_{cat_Cys} of $1.47\times10^{-1}\,min^{-1}$, a k_{uncat} of $0.46\times10^{-4}\,min^{-1}$ for EMPO, respectively. (B) The methanolysis of **1** with increasing equivalents of Cys. The reaction was carried out with **1** in MeOH for 30 min at 25 °C in the presence of 0, 0.007, 0.015, 0.023, 0.062, and 0.126 equiv of Cys, respectively.

substrate **1** was >0.02. This result suggests a possibility that more than one oxazolone molecule could bind to one molecule of Cys, thus not forming a 1:1 complex between Cys and the oxazolones to catalyze the methanolysis reaction. Figure 4 shows the partial ^1H nuclear magnetic resonance (NMR) spectra that monitor the progress of the catalytic reaction at different periods of time. The proton peaks of the product, the amine proton (–NH) and the *ortho* proton (a′) of the benzyl group, clearly appeared at 1 min in the presence of Cys. Complexation of Cys with **1** seemed to make the ester group more accessible to methanolysis reagent, and then the reaction could occur via an acyl intermediate. Figure 5 shows the molecular ions (m/z) of the acylated Cys-**1** intermediates detected in the MALDI-TOF spectrum. The measured masses are observed as m/z 3035, 3197, 3359, 3521, 3684, 3846, 4008, 4170, 4332, and 4494, respectively. These sequential ions represent the [acylated Cys-$\mathbf{1}^+ + Na^+ + 9H^+$]. The intermediates were also confirmed in a UV absorption spectrum during the methanolysis. The UV absorption spectra of the fractions containing the acyl intermediate (acylated Cys-**1**) showed λ_{max} at 202, 220, and 280 nm (data not shown).

2.3. Methanolysis of dipalmitoylphosphatidylcholine (DPPC, 2) catalyzed by Cys

Based on the methanolysis of **1**, we applied the same reactions for various phospholipids such as **2**, dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylethanolamine (DPPE), and dipalmitoylphosphatidic acid (DPPA). Interestingly, most phospholipids except DPPG experienced successful catalytic methanolysis in the presence of Cys and then produced lyso-phospholipids and a fatty acid methyl ester. Figure 6 shows the time curves of fractions reacted by Cys for each phospholipid. Depending on its functional head group, catalytic efficiency was changed. Particularly, Cys enhanced the methanolysis of **2** about 250-fold at 30 °C ($k_{cat} = 3.78\times10^{-2}\,h^{-1}$ and $k_{uncat} = 1.5\times10^{-4}\,h^{-1}$) (Figs. 2B and 6). This would be due to the difference in binding between the charged headgroups and hydroxyl groups of Cys. Figure 7 shows the process of methanolysis of **2** by Cys based on NMR spectral analyses in which a proton peak for the methoxy group was clearly produced. This peak increased with time. ^{31}P NMR analysis also showed that the signal corresponding to lyso-palmitoylphosphatidylcholine methanolyzed catalytically by Cys was newly observed as time went by (data not shown). One of the fatty acyl groups (at the position *sn*-1 or *sn*-2) was methanolyzed to a fatty acid methyl ester, which was confirmed by MALDI-TOF spectrometry (data not shown). NMR spectral analyses based on chemical shifts indicated that the complexation of Cys with **2** was made around two major regions such as the phosphocholine group and the protons of the α-carbon of the ester group of **2** (Fig. 8). It could be interpreted that the complexation occurred through the hydrogen bonding, electrostatic or van der Waals interactions between the head group of DPPC and the hydroxyl group of Cys, which made the fatty acyl group of **2** more accessible to the neighboring MeOH environment.

Figure 9 shows the hypothetical reaction mechanism of catalysis for **2**, where the acylated Cys-**2** intermediate was actually detected in the MALDI-TOF spectrum as shown in Figure 10. The measured masses are observed as m/z 3200, 3361, 3523, 3686, 3847, 4009, and 4172, respectively. These sequential ions corresponded to the [acylated Cys-$\mathbf{2}^+ + 2Na^+ + nH^+$] ($n = -1$ or 0) (Fig. 10).

The molecular shape of charged head groups of phospholipids are very important for initiation of the reaction process. That is why the DPPG did not experience the methanolysis in comparison with other phospholipids.

Thus this reaction was catalytically enhanced via complexation by Cys. From this point of view, this

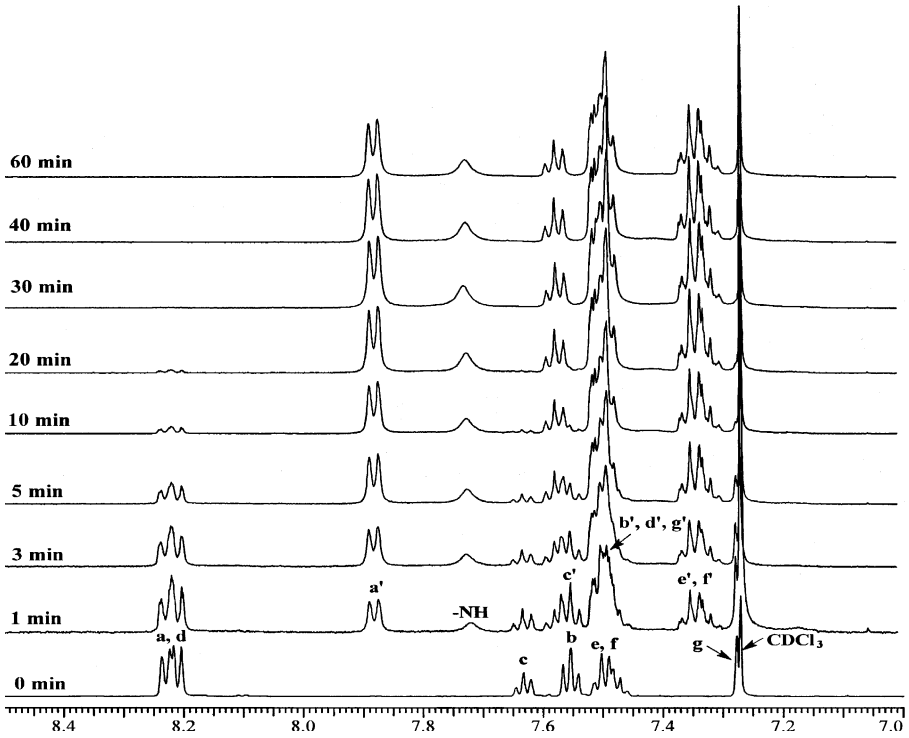

Figure 4. Partial ^{1}H NMR spectra of **1** in the absence or presence of Cys as catalyst at 0, 1, 3, 5, 10, 20, 30, 40, and 60 min. The reaction was carried out in MeOH at 25 °C in the absence or presence of 0.020 equiv Cys.

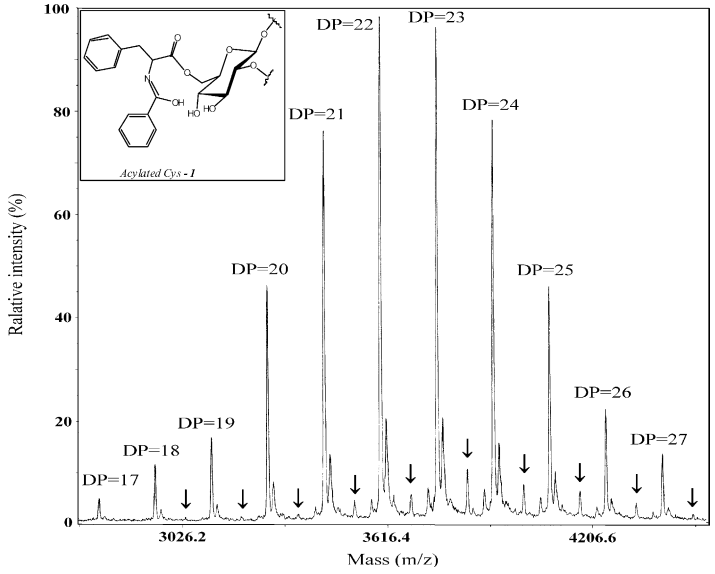

Figure 5. MALDI-TOF MS containing acylated Cys-**1**. The arrows are the intermediates corresponding to the acylated Cys-**1**. The arrows indicate the intermediates formed on Cys, of which the measured masses are observed as m/z 3035, 3197, 3359, 3521, 3684, 3846, 4008, 4170, 4332, and 4494, respectively. These sequential ions represent the [acylated Cys-$\mathbf{1}^{+} + Na^{+} + 9H^{+}$]. The inset indicates a possible acylated Cys-**1**.

reaction could be called, "the catalytic complexation by Cys." This type of catalytic reaction would be accelerated once the complexation is achieved by a complex carbohydrate molecule that has the appropriate hydrogen-bonding network and exhibits the necessary regioselectivity and enantioselectivity. Cys possesses many intramolecular hydrogen-bonding networks based on the published molecular models.[10,11] Furthermore, Cys seems to be regioselective and enantioselective toward the target chemicals in that chiral separations by Cys have been successfully performed for some enantiomers.[12,13]

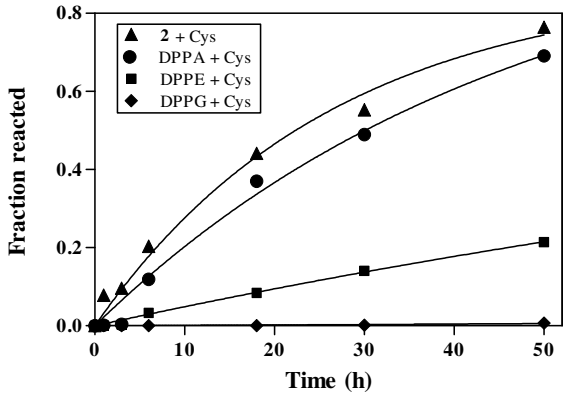

Figure 6. Time-course for the methanolysis of **2**, DPPA, DPPE, and DPPG with MeOH in the presence of Cys. The reaction was carried out in MeOH (1 mL) at 30 °C in the absence or presence of 0.500 equiv Cys. The data were fitted to a single exponential to obtain a k_{cat_2} of $3.78\times10^{-2}\,h^{-1}$, a k_{cat_DPPA} $2.13\times10^{-2}\,h^{-1}$, k_{cat_DPPE} $6.59\times10^{-3}\,h^{-1}$, and a k_{cat_DPPG} $1.59\times10^{-4}\,h^{-1}$.

In this study, we showed that Cys isolated from a *Rhizobium* species catalyzed the methanolysis of the 5(4*H*)-oxazolones and phospholipids via acyl intermediates. Cys enhanced the methanolysis reaction about 9200-fold for one of the 5(4*H*)-oxazolones, **1**, or 250-fold for **2** in comparison with a control. Once complexation occurred, a catalytic effect is easily induced with **1** or **2** positioned favorably toward further reaction. The characteristic scaffold[10,11] induced by β-(1→2)-glycosidic linkages of Cys would provide the appropriate space for the binding of **1** or **2** so that a catalytic reaction, which occurs via a Cys-acyl intermediate, could be allowed. We hope that, beginning with this work, many novel biological catalytic functions induced by complex carbohydrates will be discovered in the near future.

3. Experimental

3.1. Bacterial cultures and isolation of Cys

R. meliloti 2011 was cultured in a 5-L jar fermenter containing GMS medium[14,17] at 30 °C for 72 h. Isolation and purification of Cys were achieved as in previous reports.[7–9]

3.2. Chemicals

4-Benzylidene-2-phenyloxazolone (**1**), 4-ethoxymethylene-2-phenyloxazolone, and 3-phenyl-5-isooxazolone were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The 4-benzyl-2-phenyloxazolone was prepared according to the previous report.[15] DPPC (**2**), DPPG, DPPE, DPPA, β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine, and β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

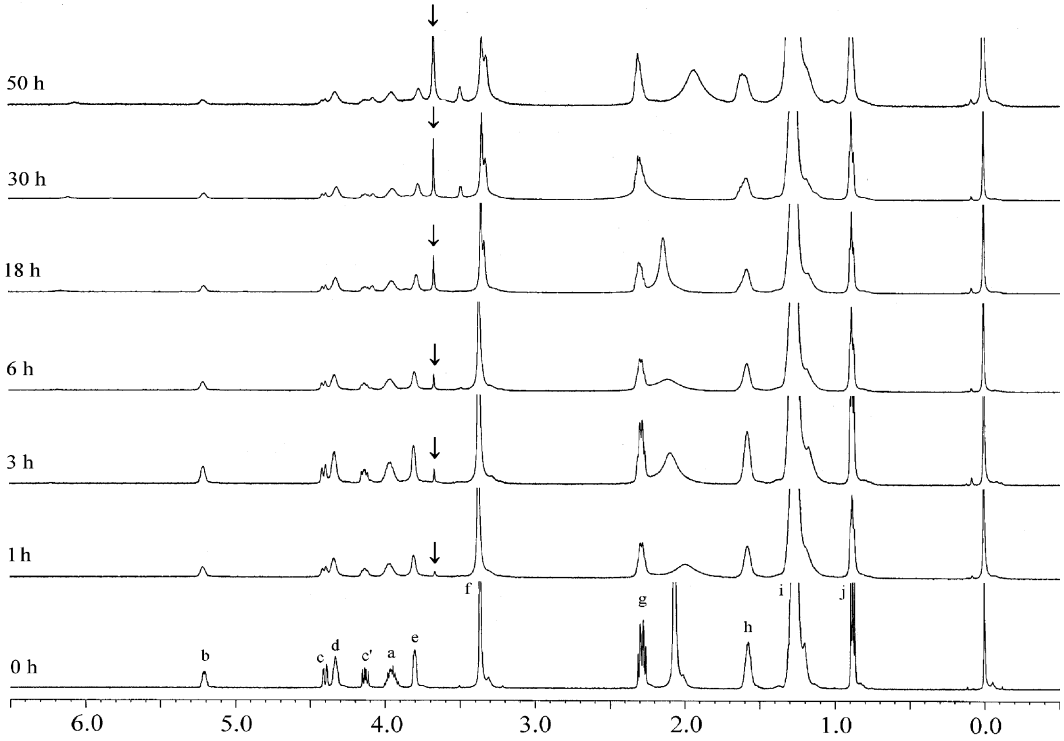

Figure 7. ^{1}H NMR spectra for methanolysis of **2** catalyzed by Cys with increasing reaction time. The arrows indicate the resonances of methoxy protons of methyl palmitate made from methanolysis of **2** catalyzed by Cys.

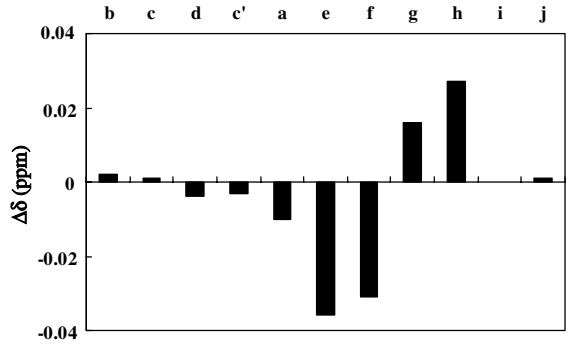

Figure 8. Chemical shifts (ppm, $\Delta\delta = \delta_{complex} - \delta_{free}$) of **2** complexed with Cys after 50 h.

3.3. Thin-layer chromatography (TLC)

To monitor the methanolysis reaction, the 5(4*H*)-oxazolones and phospholipids were assayed by TLC. Silica Gel 60 F_{254} glass-backed TLC plates were spotted with the analytes and developed with two solvent systems (3:1 hexane–EtOAc for 5(4*H*)-oxazolones and 65:25:5 $CHCl_3$–MeOH–H_2O for phospholipids). The 5(4*H*)-oxazolones were identified by irradiation with ultraviolet light (254 nm). Ammonium molybdate–copper solution in concentrated sulfuric acid was used to identify the phospholipids.[16]

3.4. MALDI-TOF mass spectrometric analysis

Mass spectra of Cys were obtained with a MALDI-TOF mass spectrometer (Voyager-DE^{TM} STR BioSpectrometry, Applied Biosystems, Framingham, MA, USA) in the positive-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix. For the identification of the cleavage position of the phospholipids, β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine and β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine their mass spectra were also determined on the MALDI-TOF mass spectrometer.

3.5. Identification of acylated Cys

To measure the acyl intermediates, **1** (30 mg) and **2** (4 mg) were dissolved in 3 and 1 mL of MeOH, respectively, and Cys (10 mg) was then added. The reaction mixture was stirred for 10 min, completely evaporated at room temperature, and subjected to the extraction after adding each 1 mL of both ultrapure water and $CHCl_3$. The intermediate dissolved in the water layer was then filtered through a small glass-wool plug. The resulting solution was chromatographed using a 3×30-cm column packed with Sephadex G-25. The column was developed by eluting with ultrapure H_2O. Successive 7 mL fractions were collected, tested for the presence of

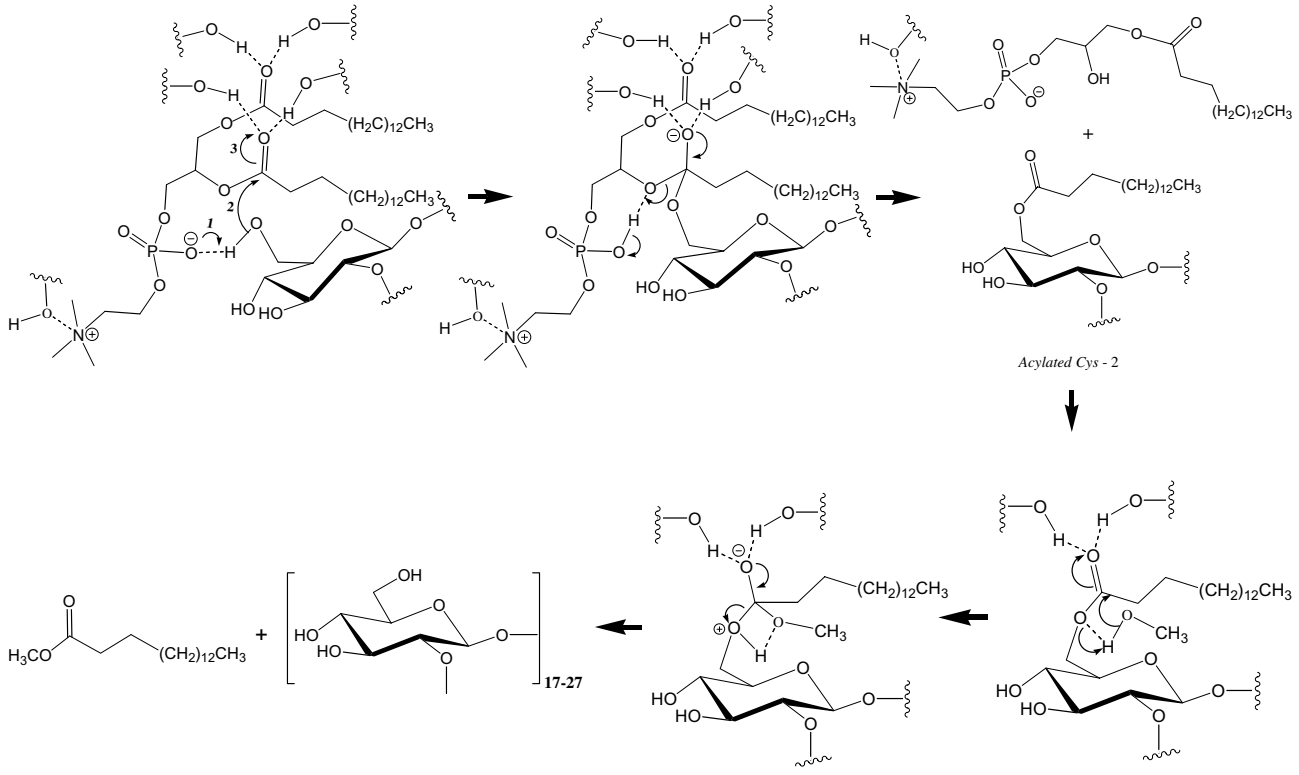

Figure 9. Hypothetical mechanism for the methanolysis reaction of **2** catalyzed by Cys. Acylated Cys-**2** intermediate was detected in the MALDI-TOF MS as shown in Figure 10. Dotted lines indicate hydrogen bonds between hydroxyl groups of Cys and **2** during the methanolysis.

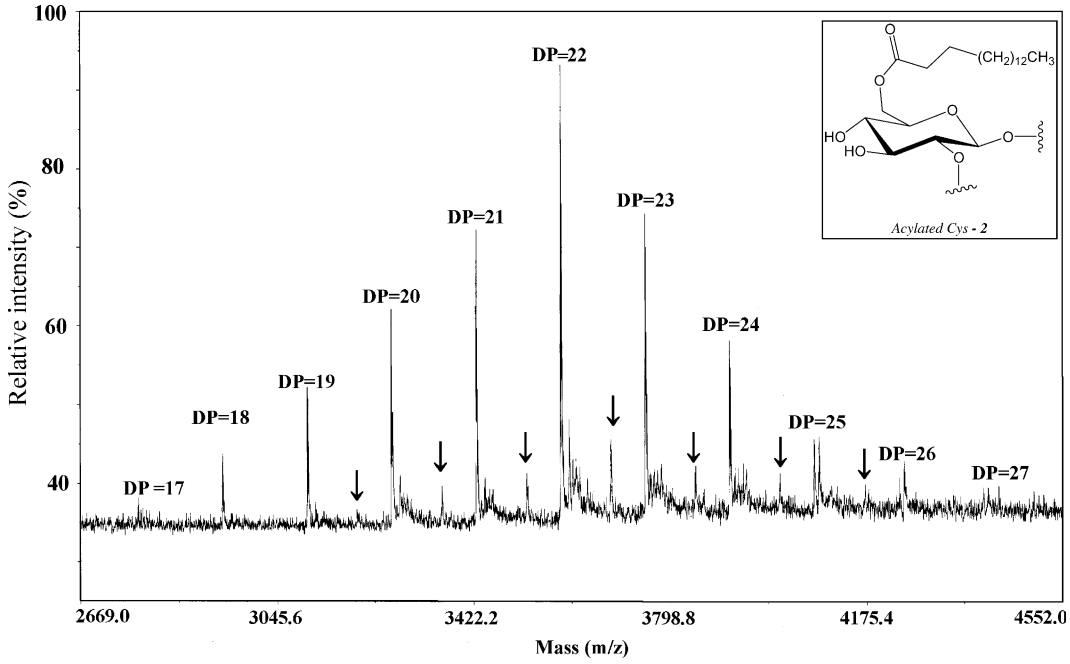

Figure 10. MALDI-TOF MS containing acylated Cys-**2**. The arrows are the intermediates corresponding to the acylated Cys-**2**. The arrows indicate the intermediates formed on Cys, of which the measured masses are observed as m/z 3200, 3361, 3523, 3686, 3847, 4009, and 4172, respectively. These sequential ions corresponded to the [acylated Cys-$\mathbf{2}^{+} + 2Na^{+} + nH^{+}$] ($n = -1$ or 0). Inset indicates a possible acylated Cys-**2**.

Cys by developing on TLC (E. Merck, solvent: 5:5:4 BuOH–EtOH–H_2O) and then spraying with 5% phenol–H_2SO_4. Cys was found to be present in first three fractions taken after elution of the void volume. The three fractions were also subjected to scanning by UV spectroscopy (Hitachi, Japan). The MALDI-MS sample was prepared by mixing the lyophilized sample (1 mg) and 2,5-dihydroxybenzoic acid (DHB) in 0.1% trifluoroacetic acid (TFA) as matrix. The sample was dried and then analyzed.

3.6. NMR spectroscopic analysis

NMR spectroscopic analyses were carried out on a Bruker Avance 500 or 600 spectrometry. Conversion of **1** was determined by measuring the decrease of the integral area of the resonance at 8.21 ppm [*ortho* protons of reactant (a and d of **1**)] and the increase of the integral area of the resonance at 7.87 ppm [*ortho* protons (a′) of product] by 600-MHz ^{1}H NMR spectroscopy. The reacted fractions were then quantified. In the case of the methanolysis of the phospholipids including **2**, the conversion was analyzed by 500 MHz ^{1}H NMR spectroscopy by measuring the decrease of the methine proton resonance (b in **2**) and the increase of methoxy proton resonances (3.66 ppm) of methyl palmitates produced from Cys-catalyzed methanolysis. For these two reactions, aliquots were taken at a given time, rapidly evaporated under reduced pressure, and then placed under vacuum to remove the residual water before NMR measurement. All measurements were done in $CDCl_3$. ^{1}H chemical shifts are expressed in δ (ppm) downfield from the tetramethylsilane (Me_4Si, internal) resonance (0.000 ppm). The time-course of the two reactions was fitted to a single exponential to obtain k_{cat} or k_{uncat}.

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