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Non-enzymatic Self-acetylation of α-Cyclosophorotridecaoses Isolated from *Ralstonia solanacearum*: Mass Spectrometric Study

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The periplasm is the cellular space between the inner and outer membrane in gram-negative bacteria and thus serves as a protective buffer between the external environment and the inside of the bacterium. Periplasmic glucans aid in maintaining a constant internal cellular environment, even in the presence of dynamic external pH, osmolarity, and ionic surroundings.[1] Thus, several studies on the structural analysis and function of periplasmic glucans have been carried out to date.[2-4] In general, they are classified into four families depending on the polyglucose backbone.[1] Family I glucans are linear, β-1,2 glucans containing 5–12 glucose residues, whereas those of family II are β-1,2-linked cyclic glucans containing 17–25 glucose residues. Family III comprises β-1,6 and β-1,3 cyclic glucans containing 10–13 glucose units. The periplasmic glucans in family IV are β-1,2 cyclic glucans containing one α-1,6 linkage, and are produced by *Rhodobacter sphaeroides*, *Xanthomonas campestris*, and *Ralstonia solanacearum* with backbone structures composed of 18, 16, and 13 glucose units of homogenous size, respectively.[2,5-8]

R. solanacearum is a plant pathogen causing bacterial wilt, and produces α-cyclosophorotridecaoses (α-C13, Figure 1(a)) as a family IV series. Because of its homogeneous and small size, it is commonly chosen as an experimental cyclic glucan molecule.[7,8] The single α-1-6 linkage might allow the steric constraints imposed by molecules smaller than a degree of polymerization (DP) of 17 to be overcome.[7,8] Elucidating the structural identity of α-C13 will help to identify its biological role in pathogenesis and its capability of inducing complex with host molecular signals.

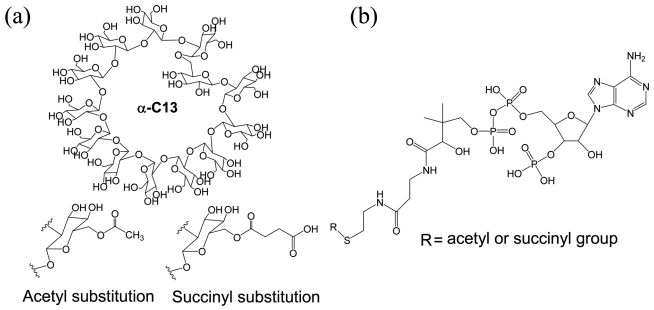

Figure 1. Chemical structures of α-C13 (a) and acetyl/succinyl coenzyme A (b).

In most Proteobacteria, periplasmic glucans are modified by non-glucose residues such as phosphoglycerol, phosphoethanolamine, acetyl, or succinyl groups.[4,5,9,10] Depending on the particular bacterial strain or growth conditions, glucans can be found either in unsubstituted or substituted form. *Pseudomonas syringae* produce succinylated linear β-1,2-glucans (family I) in low osmolarity media, whereas they produce acetylated ones in high osmolarity media.[11,12] Growth of *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* in phosphate-limited media leads to succinylation and no substituent, respectively, in place of phosphoglycerol substitution on the cyclic β-1,2-glucans.[13]

Although the specific effect of the substituent is not clear, it has been reported that succinyl substituents in family II periplasmic glucans of *Brucella abortus* are necessary for hypo-osmotic adaptation.[10] Recently, we identified a novel acetylated α-C13;[14] however, the mechanism of α-C13 acetylation has not yet been investigated. The presence of acyl-transferase has long been accepted as the main driver of this process.[15] In fact, some acyl-transferases that transfer substituents such as phosphoglycerol or succinate to backbone glucans have been reported,[16,17] and acetyl coenzyme A has been suggested as the leading donor for acetyl substitution to periplasmic glucans.[1] Acetyl coenzyme A is the thioester between coenzyme A and acetic acid, which serves as an important metabolic intermediate and is thus used in many biochemical reactions (Figure 1(b)). Herein, we suggest a potential hypothesis to explain the mechanism of acetylation of α-C13 *via* acetyl coenzyme A.

One possibility for this mechanism might be related to the catalytic activity of microbial carbohydrates. To date, some microbial carbohydrates have been reported to show potential as catalysts in specific organic reactions.[18-21] The family II periplasmic glucan member cyclosophoraose (Cys) of *Rhizobium* species functions as a catalytic carbohydrate for the methanolysis of 5(4*H*)-oxazolones and 7-acetoxy-4-methylcoumarin.[18,19] In addition, family IV glucans from *Xanthomonas oryzae* showed a catalytic effect on benzoate

methanolysis and Strecker reactions.[20,21] These results were mostly based on the conformation and hydroxyl group of the glucans, and thus the possible intermediates of these reactions were identified as acyl glucans. Considering this evidence, we here investigated whether α-C13 can be acetylated by using only the suspected acyl donor molecule acetyl coenzyme A.

The isolation and purification of α-C13 were performed in the present study. The structure of α-C13 is shown in Figure 1(a), and the novel acetylated α-C13 was also confirmed with nuclear magnetic resonance (NMR) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as described previously.[14] These results showed that one or two acetyl groups are linked to the C6 position of the glucose residue in α-C13. We hypothesized that chemical collision between acetyl coenzyme A and α-C13 is a potential mechanism for the formation of acetyl α-C13.

To confirm this hypothesis, the prepared α-C13 was reacted with acetyl coenzyme A in Tris buffer (pH 8.3). α-C13 could be substituted with an acetyl moiety released from acetyl coenzyme A in Tris buffer. The result was detected by MALDI TOF MS, and the detailed analysis was performed by ESI MS. The pseudo-molecular ion peaks of original α-C13 and the reacted α-C13 were compared by MALDI-TOF MS as shown in Figure 2(a) and 2(b).

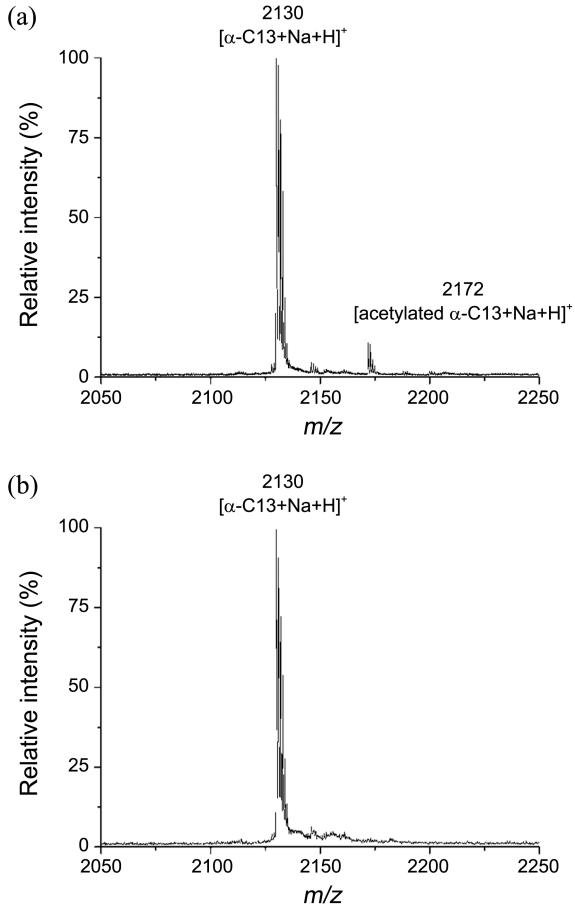

Figure 2. MALDI-TOF mass spectra of the reacted α-C13 (a), and original α-C13 (b).

Furthermore, Figure 3(a) shows the electrospray ionization (ESI) MS result of the reaction mixture, and protonated molecular weights of *m/z* 2107 and 2149 were observed in positive mode. Because the mass difference with the acetyl group was 42, the form with a molecular weight of *m/z* 2149 was suspected to be the acetylated α-C13. To confirm the structure of one acetylated α-C13, the ion of *m/z* 2149 was subjected to collision-induced dissociation (CID) with a collision energy (CE) of 70 eV. As a result, the molecular ion corresponding to *m/z* 2149 was fragmented to 2107 (Figure 3(b)). This result clearly indicates the release of an acetyl moiety and that the peak corresponding to *m/z* 2149 is the acetylated form of α-C13. The MS/MS data showed that the acetyl moiety linked to α-C13 is dissociated at 70 eV, although it was not released even when a CE of 50 eV was applied. Furthermore, the acetyl moiety of linear octassaccharides was not released even after a CE of 70 eV was applied.[22] These facts provide evidence that the acetyl linkages attached to cyclic glucans are less stable than those attached to linear glucans when subjected to ESI collision-activated dissociation (CAD).

The α-C13 was reacted with another candidate enzyme, succinyl coenzyme A, in Tris buffer (pH 8.3). We found that α-C13 can be substituted with not only an acetyl group but also a succinyl group. Figure 4(a) shows the ESI MS data of the reaction mixture, in which the peaks at *m/z* 2207 and 2107 correspond to the succinylated and unsubstituted forms of α-C13, respectively. The mass difference of *m/z* 100 between these two forms is attributed to a single succinyl group. CAD of the succinylated α-C13 led to the loss of a succinyl substituent and glucose from the α-C13 backbone

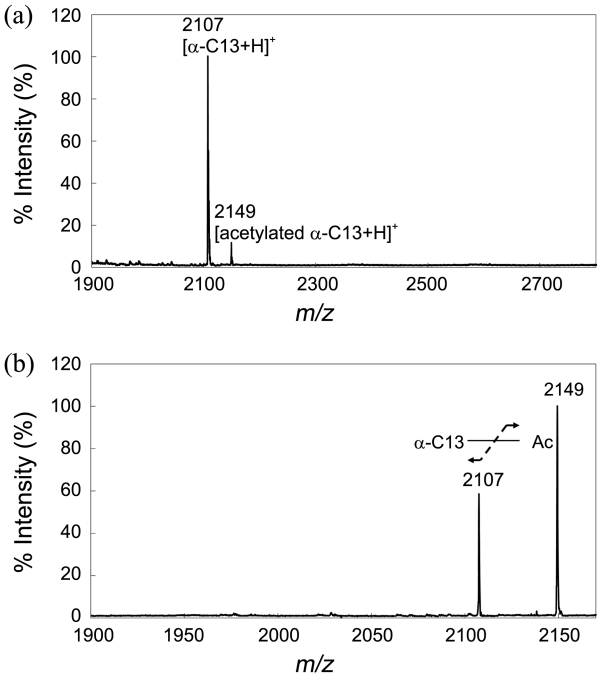

Figure 3. ESI mass spectrum (a) and ESI-CAD mass spectrum (b) of acetyl α-C13 at 70 eV. The scheme in the inset indicates the fragmentation pattern of acetyl α-C13.

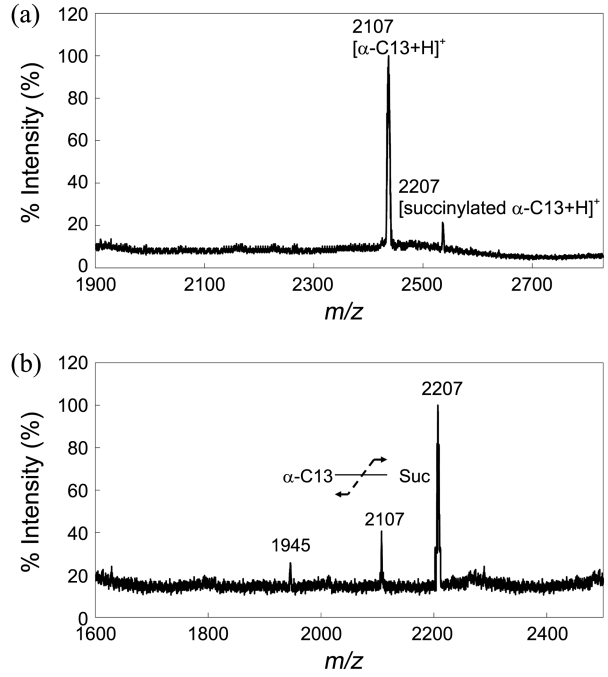

Figure 4. ESI mass spectrum (a) and ESI-CAD mass spectrum (b) of succinyl α-C13 at 55 eV. The scheme in the inset indicates the fragmentation pattern of succinyl α-C13.

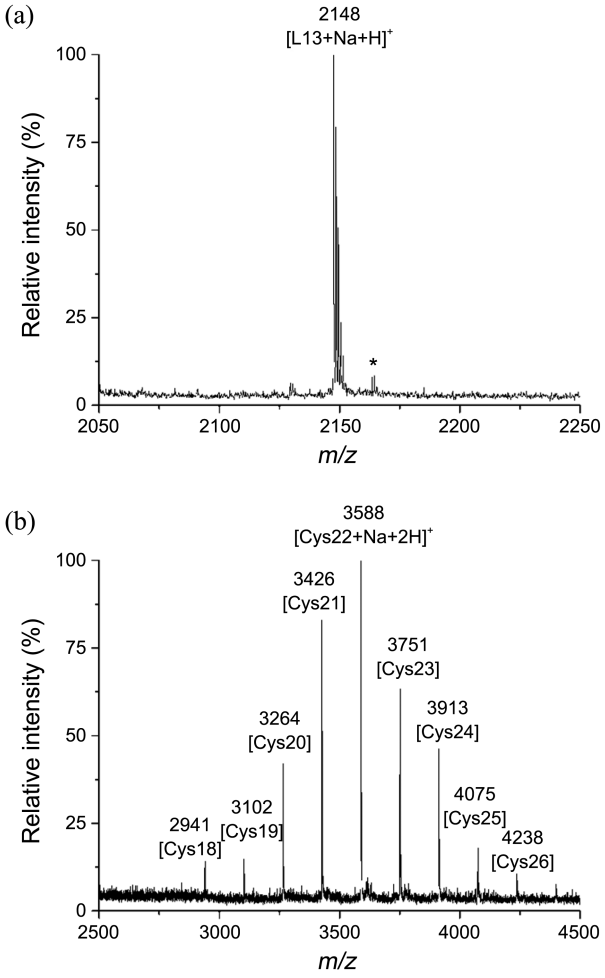

Figure 5. MALDI-TOF mass spectra of L13 (a) and Cys (b) observed after the reaction with acetyl coenzyme A. Asterisk (*) in Figure 5(a) indicates the potassium adduct of L13. The detailed description of other sodium adducts except Cys22 is omitted in Figure 5(b), for clarity. L13: linear DP13 glucans, Cys: cyclosophoraoses.

structure at a CE of 55 eV, and then the fragmented ions were observed at *m/z* 2107 and 1945 (Figure 4(b)). The additional loss of glucose can be attributed to the action of the original glucans, as shown in a previous study.[22]

In this respect, ESI MS/MS is a very useful structural analysis technique for scarce samples, and the structural analyses with MS have been often performed.[23] In addition, structural analysis based on the decomposition patterns of acetyl α-C13 has not been reported previously, and the succinylated form of α-C13 is a novel product. In this study, we investigated the low-energy CAD patterns of acetylated and succinylated α-C13 using ESI tandem MS.

To compare the α-C13 forms with other carbohydrates, linear DP13 glucans (L13) were prepared by acid hydrolysis of α-C13. Cys, consisting of 18–26 glucose units with β-1,2 linkages (family II), were also purified from *S. meliloti*. The L13 and Cys were reacted with acetyl coenzyme A for acetylation. As aforementioned, the presence of acetyl α-C13 was confirmed with a mass peak of 2172 in MALDI-TOF MS (Figure 2). In addition, the mass range below *m/z* 1000 indicated that the acetyl group was released from acetyl coenzyme A by a mass subtraction of 42. However, L13 and Cys were not acetylated at all (Figure 5). These results might have been caused by structural differences of the carbohydrates used in the reactions. L13 may lack the space or an appropriate cavity for efficient binding of coenzyme A, whereas Cys (only β-1,2 linkages) have no adequate positioning for the interaction between nucleophilic –OH groups of Cys and carbonyl carbon of acetyl coenzyme A. The characteristic cyclic scaffold induced by the single α-1,6 linkage of α-C13 may provide the appropriate space needed for the binding of acetyl or succinyl coenzyme A, leading to acyl α-C13. Although the reaction was performed in a buffer system, the results demonstrated that self-acetylation of a carbohydrate is possible without requiring additional help from a transferase enzyme. The bacterial periplasm is not an empty space, but is rather filled with periplasmic fluid that has a gel-like consistency.[24] Therefore, we expect an increased collision frequency between biomolecules compared to that observed in buffer.

In conclusion, this study confirmed the presence of acetyl α-C13 with ESI MS/MS and that acetylated α-C13 could be produced in a minimal reaction buffer containing acetyl coenzyme A. Based on these results, we suggest a novel method for the acetylation of α-C13. The nature of the periplasm differs substantially from that of an isotropic aqueous solution, and there is a possibility of various chemical collisions between biomolecules including periplasmic glucan. Thus, as one approach for acetylation, we introduce the concept of 'self acetylation of periplasmic glucans' based on

the intrinsic properties of catalytic carbohydrates.[18-21] Further studies to determine the exact mechanism driving the acetylation reaction and to develop an *in vivo* mimic system are in progress. In addition, functional analyses of the substitution of periplasmic glucans are required.

Experimental

Bacterial Strains and Culture Conditions. *R. solanacearum* KACC 10698 was grown in a TGY medium at 24 °C with agitation. *S. meliloti* was cultured in a GMS medium to late logarithmic phase at 30 °C.

Preparation of α-C13. α-C13 produced by *R. solanacearum* KACC 10698 was purified as described in our previous report.[14] The acetylated and unsubstituted forms of α-C13 were treated with 0.1 M KOH at 37 °C for 1 h. The alkaline-treated materials were neutralized and desalted on a Bio-Gel P-4 column. The final unsubstituted α-C13 was confirmed with NMR spectroscopy and MALDI-TOF MS.

Preparatin of L13 from α-C13. L13 were prepared by acid hydrolysis (0.08 M trifluoroacetic acid, 100 °C, 1 h) and semi-preparative high-performance liquid chromatography (HPLC). After the hydrolysis, the hydrolysates were evaporated to dryness to remove trifluoroacetic acid, and desalted by using a Sephadex G-10 column.[25] The desalted material was subjected to semi-preparative HPLC (Agilent Technologies 1200 series) on a C18 column (5 μm, 250 × 9.4 mm; Eclipse XDB-C18) at 15 °C, and detected with an RI detector using 99:1 (v/v) water:methanol as the solvent system at a flow rate of 1 mL/min. The product was analyzed with MALDI-TOF MS.

Purification of Cys from *S. meliloti*. The isolation and purification of Cys were carried out as previously described.[26]

Reaction Condition. Reactions were performed in a minimal reaction buffer containing 300 mM NaCl, 5 mM $MnCl_2$, and 20 mM Tris-chloride buffer (pH 8.3) at 30 °C for 1 h.[27] The concentrations of both substrates, coenzyme A (acetyl and succinyl), and α-C13 were 500 μM. After 1 h, the reaction mixture was lyophilized and the mass was detected with MALDI-TOF MS.

MALDI-TOF MS Analysis. The reaction mixture was first analyzed with MALDI-TOF MS using 2,5-dihydroxybenzoic acid as the matrix with a MALDI-TOF mass spectrometer (Voyager-DE™ STR BioSpectrometry; PerSeptive Biosystems; Framingham, MA, USA) in positive ion mode.

ESI MS/MS Analysis. For detailed structural analysis, the ESI MS/MS technique was used. The reaction mixtures described above were desalted with Sephadex G-10 and lyophilized. The materials were dissolved in a 1:1 solution of water and MeOH and directly infused into the ESI source at a rate of 1 mL/min. The low-energy CAD experiments were carried out on an API 4000™ triple quadrupole LC/MS/MS system (Applied Biosystems; Foster City, CA, USA) equipped with a turbo ESI source. Ionization was performed in positive ion mode and nitrogen was used as the drying and nebulizing gas. The spray voltage was set at 4500 eV in product ion scan mode (MS2) and the scan range was *m/z* 1600 to 2500. The applied CEs were 50, 55, and 70 eV.

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