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Benzoate methanolysis catalyzed by α-cyclosophorohexadecaose isolated from *Xanthomonas oryzae*

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Abstract

Benzoate methanolysis was accelerated by α-cyclosophorohexadecaose (α-C16), which is a neutral cyclic hexadecaglucoside containing fifteen β-(1 → 2)-linkages and one α-(1 → 6)-linkage. This α-C16, a periplasmic cyclooligosaccharide isolated from rice pathogenic bacteria, *Xanthomonas oryzae*, enhanced the methanolysis of three different benzoates (vinyl benzoate, phenyl benzoate and 4-chlorophenyl benzoate) about 69-fold, 24-fold and 14-fold comparing with control, respectively. A possible intermediate formed during the reaction was detected on MALDI-TOF spectra. Through this study, we suggest that a microbial cyclic glucan, α-C16, can function as a catalytic carbohydrate for the methanolysis of various ester compounds.

Keywords: Benzoate methanolysis; α-Cyclosophorohexadecaoses; *Xanthomonas oryzae*; Catalytic carbohydrate; Catalysis

1. Introduction

α-Cyclosophorohexadecaose (α-C16), the cyclic osmoregulated periplasmic glucans (OPGs) produced by *Xanthomonas* genus (Amemura & Cabrera-Crespo, 1986), is an unbranched cyclooligosaccharide with fifteen β-(1 → 2)-linkages and one α-(1 → 6)-linkage and it functions as an osmotic regulator. The presence of a single α-(1 → 6)-linkage was known to make ring closure feasible and induce structural constraints in the cyclic OPG. Such constraints may significantly simplify the conformational analysis of this cyclic OPG and may also endow them with unique physical properties (Choi & Jung, 2005; Lippens, Wieruszeski, Talaga, Bohin, & Desvausx, 1996; Talaga et al., 1996; York, 1995). Recently, a novel structure of anionic α-C16 isolated from *X. campestris* pv. *campestris* (Jung, Park, Cho, & Jung, 2005) also has been reported.

Methanolysis is a kind of transesterification using an ester and methanol as substrates. Transesterification is an important organic reaction where an ester is transformed into another ester through interchange of the alkoxy moiety. The transesterification is an equilibrium reaction and the transformation occurs essentially by mixing the reactants. However, the presence of catalysts reduces the free energy of activation, and stabilizes the transition state of the reaction, then thus accelerates considerably the adjustment of the equilibrium. So far, catalysts which have been used for transesterification are acids (Freedman, Butterfield, & Pryde, 1986; Freedman, Pryde, & Mounts, 1984), alkaline metal hydroxides, alkoxides (Gryglewicz, 2000), carbonates (Mouloungui, Elmestour, Delmas, & Gaset, 1992), non-ionic bases (Flynn & Nenortas, 1963) and enzymes mainly serine protease (Glazer, 1966; Gryglewicz, Jadownicka, &

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Czerniak, 2000; Roy, Sharma, & Gupta, 2004; Zaks & Klibanov, 1985; Zaks & Klibanov, 1988) and acyl intermediates could be formed by the catalysts, during the catalytic reactions. (Ema, Maeno, Takaya, Sakai, & Utaka, 1996; Gryglewicz, 2000; Nakatani, Hiratake, Yoshikawa, Nishioka, & Oda, 1992) In this respect, artificial enzymes have been reported to achieve rate enhancements (k_{cat}/k_{uncat}) between 10^1 and 10^5 (Ikeda, Horimoto, Nakata, & Ueno, 2000; Ortega-Caballero, Bjerre, Laustsen, & Bols, 2005; Rousseau, Nielsen, & Bols, 2004), while this rate acceleration is modest compared to natural enzyme equivalent.

Recently, we have reported that some microbial carbohydrates have potentials as catalysts in specific organic reactions (Lee & Jung, 2004; Lee, Kang, Kwon, & Jung, 2006). Both cyclosophoraose (Lee & Jung, 2004) isolated from *Rhizobium* and zooglan (Lee et al., 2006), the acidic exopolysaccharide produced by *Zoogloea ramigera*, function as catalytic carbohydrates for methanolysis of 4-benzylidene-2-phenyloxazolone, one of 5 (4H)-oxazolones.

In this study, we report that, as another catalytic carbohydrate, α-C16 isolated from rice pathogenic microorganism, *X. oryzae* pv. *oryzae* catalyzed benzoate methanolysis.

2. Experimental

2.1. Bacterial cultures and preparation of neutral α-C16

Xanthomonas oryzae pv. *oryzae* KACC 10331 were from the Korean Agricultural Culture Collection (KACC), and they were grown in a TGY medium (Sunish Kumar & Sakthivel, 2001) at 28 °C with 150 rpm agitation. The microorganisms were grown for 2 days (Vonjnov, Slater, Newman, Daniels, & Dow, 2001) and collected by centrifugation at 4 °C for 10 min at 8000 rpm. The cell pellets were extracted with 5% trichloroacetic acid, and after centrifugation, the supernatant was neutralized with NH_4OH (Miller, Kennedy, & Reinhold, 1986) and chromatographed on a Sephadex G-25 (2.5 × 34 cm) column at a rate of 1 mL/min. The fractions containing the putative cyclic glucan were pooled, concentrated and then applied to a column (2 × 35 cm) of DEAE-Sephadex to separate the anionic form of α-C16. Neutral one is collected and desalted on a Bio-Gel P-4 column (Bio-Rad). The column (2.4 × 54 cm) was eluted at room temperature with distilled water at a flow rate of 18 mL/h and then the desalted material was finally lyophilized.

2.2. Chemicals

Phenyl benzoate (**1**), 4-chlorophenyl benzoate (**2**), vinyl benzoate (**3**) and glucose were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). α-CD and amylose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and *N*,*N*-dimethylformamide were purchased from Sigma–Aldrich (St. Louis, MO, USA). The solvents were obtained in anhydrous form.

2.3. Thin-layer chromatography (TLC)

TLC analysis was carried out to monitor benzoate methanolysis. Silica Gel G-60 (E. Merck, 400–240 mesh) plates were spotted with the analytes and developed under the solvent systems (1:1 hexane–methylene chloride). The reactants and products were detected by irradiation with ultraviolet light (254 nm).

2.4. MALDI-TOF mass spectrometry analysis

The mass spectra of the oligosaccharides were obtained with a MALDI-TOF mass spectrometer (Voyager-DE™ STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

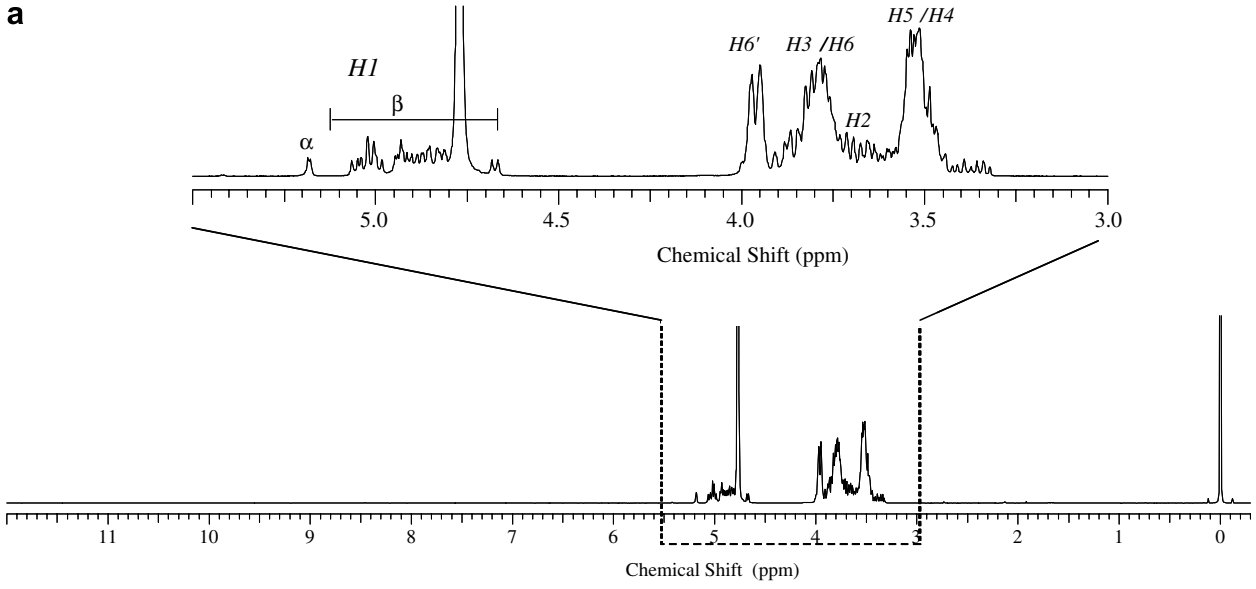
2.5. Identification of acylated α-C16

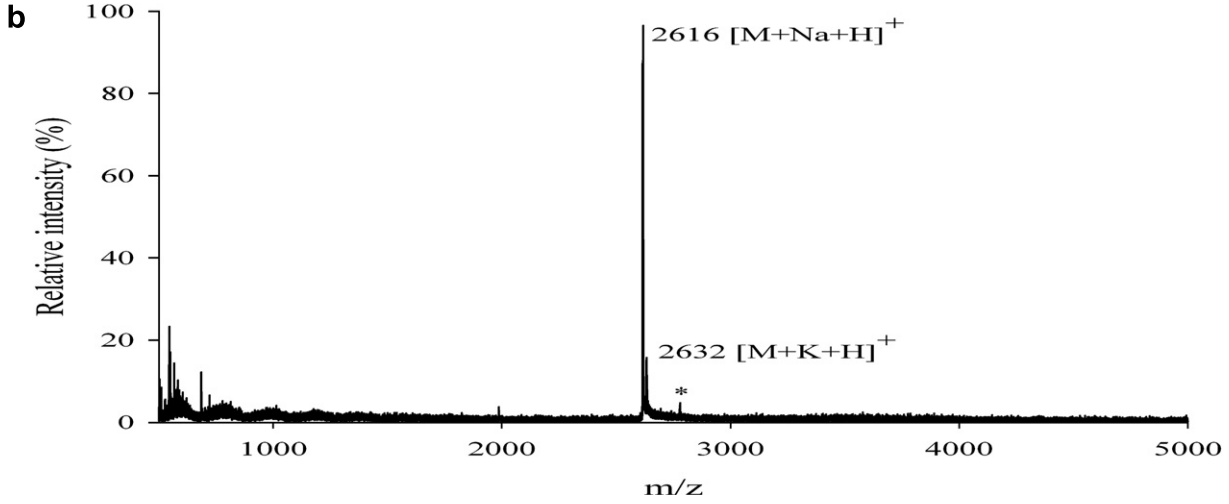
To measure the acyl intermediate, **1** (100 mM) was dissolved in 0.5 mL of MeOH and then α-C16 (5.2 mg) was added. The reaction mixture was magnetically stirred at 40 °C. After 3 h, the liquid was discarded from the settling by means of a centrifuge and the remaining liquid was completely evaporated to dryness with N_2 gas. The residual pellet containing putative acyl α-C16 was dissolved in ultrapure water. The intermediate dissolved in water layer was detected by MALDI-TOF MS analysis in the positive mode using 2,5-dihydroxybenzoic acid (DHB) as matrix.

For another acyl intermediate, phenyl salicylate (100 mM) was dissolved in 0.5 mL *N*,*N*-dimethylformamide and then α-C16 (5.2 mg) was added. The reaction mixture was stirred at 40 °C. After 2 h, the solution was detected by MALDI-TOF MS analysis in the positive mode using 2,5-dihydroxybenzoic acid (DHB) as matrix.

2.6. NMR spectroscopic analysis

For NMR spectroscopic analysis, we used a Bruker Avance 500 spectrometry. The conversion of **3** was determined by measuring the decrease of the integral area of the resonance at 7.99 ppm [*ortho* protons (a) of reactant (vinyl benzoate)] and the increase of the integral area of the resonance at 7.92 ppm [*ortho* protons (a′) of product (methyl benzoate)] by 500 MHz ^{1}H NMR spectroscopy. The conversions of **1** and **2** were determined in the same way. The reacted fractions were then quantified. For these three reactions, aliquots were taken at a suitable time intervals and the reaction was stopped by freezing immediately after centrifugation to remove catalyst. All NMR spectroscopic analyses were done in $CDCl_3$. The time course of three reactions was fitted to one phase exponential association to obtain k_{cat} or k_{uncat}.





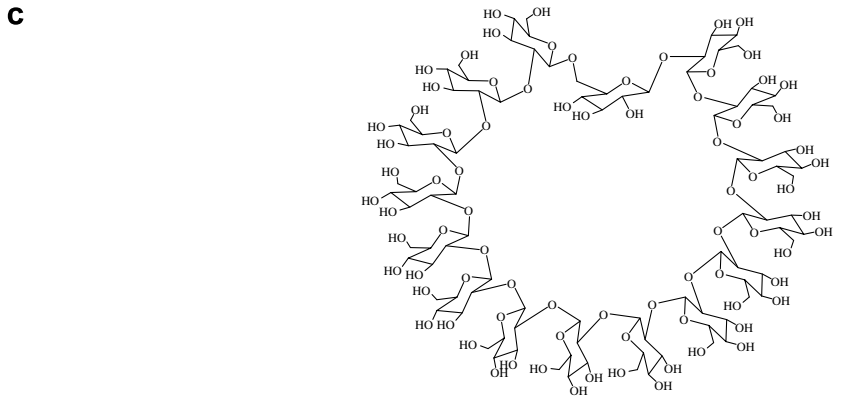
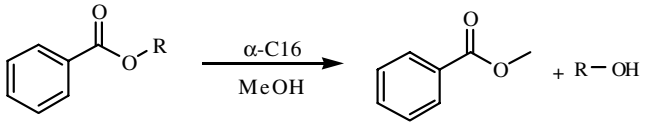


Fig. 1. Structural identification of α-C16 isolated from *X. oryzae* pv. *oryzae* KACC 10331. (a) ^{1}H NMR spectrum of α-C16. The peak at 5.18 ppm is indicative of the anomeric proton (H-1) of the α-glucose residue and the peaks from 4.67 to 5.06 ppm are indicative of the H-1 protons of the glucose residues engaged in β-(1 → 2)-linkages. Other proton signals of glucoses are found to appear between 3.3 and 4.0 ppm. (b) Positive-ion MALDI-TOF MS of α-C16. A neutral α-C16 is present with *m*/*z* 2616 and 2632 calculated masses representing for an $[M + Na + H]^{+}$ and $[M + K + H]^{+}$, respectively. (Asterisk (*) means cyclic glucans of DP 17 (*m*/*z* 2778) isolated from *X. oryzae* pv. *oryzae* KACC 10331.) (c) Chemical structure of α-C16 isolated from *X. oryzae* pv. *oryzae* KACC 10331.



1 : R= phenyl

2 : R= 4-chlorophenyl

3 : R= vinyl

Fig. 2. Scheme for methanolysis of phenyl benzoate (**1**), 4-chlorophenyl benzoate (**2**), vinyl benzoate (**3**) by α-C16.

3. Results and discussion

3.1. Confirmation of neutral α-C16

The structure (Fig. 1) of α-C16 was identified with NMR spectroscopy and MALDI-TOF MS analyses as reported previously (Talaga et al., 1996; York, 1995).

Table 1
Rate constants for α-C16-catalyzed methanolysis of **1**, **2** and **3**

Substrate	$k_{cat} \times 10^{-1}$ (h^{-1})	$k_{uncat} \times 10^{-2}$ (h^{-1})	k_{cat}/k_{uncat}
1	1.10	0.45	24
2	1.75	1.22	14
3	6.04	0.88	69

3.2. Methanolysis of phenyl benzoate (*1*), 4-chlorophenyl benzoate (*2*), vinyl benzoate (*3*) catalyzed by α-C16

The reaction was carried out in MeOH (3 mL) at 40 °C in the absence or presence of 0.2 equiv. of α-C16. Each substrate concentration was fixed at 20 mM. α-C16 catalyzed the methanolysis of three benzoates (Fig. 2) with different rate constants (Table 1). Each different R group of substrates (Fig. 2) made a distinct effect on k_{cat} and k_{uncat}. In the presence of α-C16, after 4 h, vinyl benzoate (**3**) methanolysis was completed more than ninety

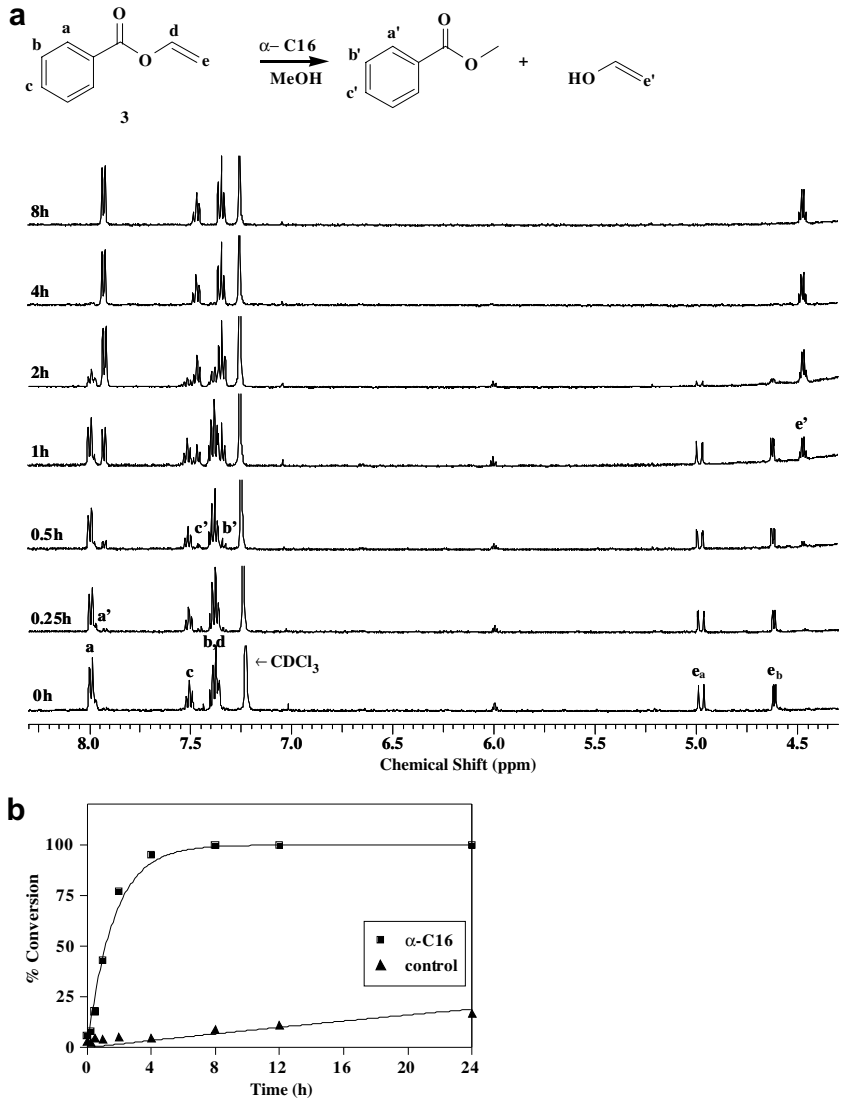


Fig. 3. (a) Partial ^{1}H NMR spectra of **3** in presence of α-C16 as a catalyst at 0, 0.25, 0.5, 1, 2, 4, and 8 h. The reaction was carried out in MeOH at 40 °C in the absence or presence of 0.2 equiv. of α-C16. (b) Time course of methanolysis of **3** in the absence or presence of 0.2 equiv. of α-C16. The concentration of used substrate was fixed at 20 mM. The data were fitted to one phase exponential association.

five percent (⩾95%), but in the absence of α-C16, the conversion to product was less than 5% (Fig. 3(b)). Thus, the reaction rate was enhanced by 69-fold (k_{cat}/k_{uncat}). Fig. 3(a) shows the partial ^{1}H nuclear magnetic resonance (NMR) spectra that monitor the progress of the catalytic reaction at different periods of time.

After 24 h, methanolysis of 4-chlorophenyl benzoate (**2**) with α-C16 was completed more than 95% and 14 times (k_{cat}/k_{uncat}) faster than the reaction without α-C16 (Table 1). After 34 h, phenyl benzoate (**1**) methanolysis was ⩾95% done in the presence of α-C16 and 24 times (k_{cat}/k_{uncat}) faster than the reaction in the absence of α-C16 (Table 1). Comparing vinyl group (**3** R) with phenyl (**1** R) and 4-chlorophenyl group (**2** R), catalytic methanolysis of benzoate containing vinyl group (**3** R) was much faster than the others. We propose that a larger R group interrupts α-C16 approach to the substrate for the intermediate formation and a smaller R group gives a favorable

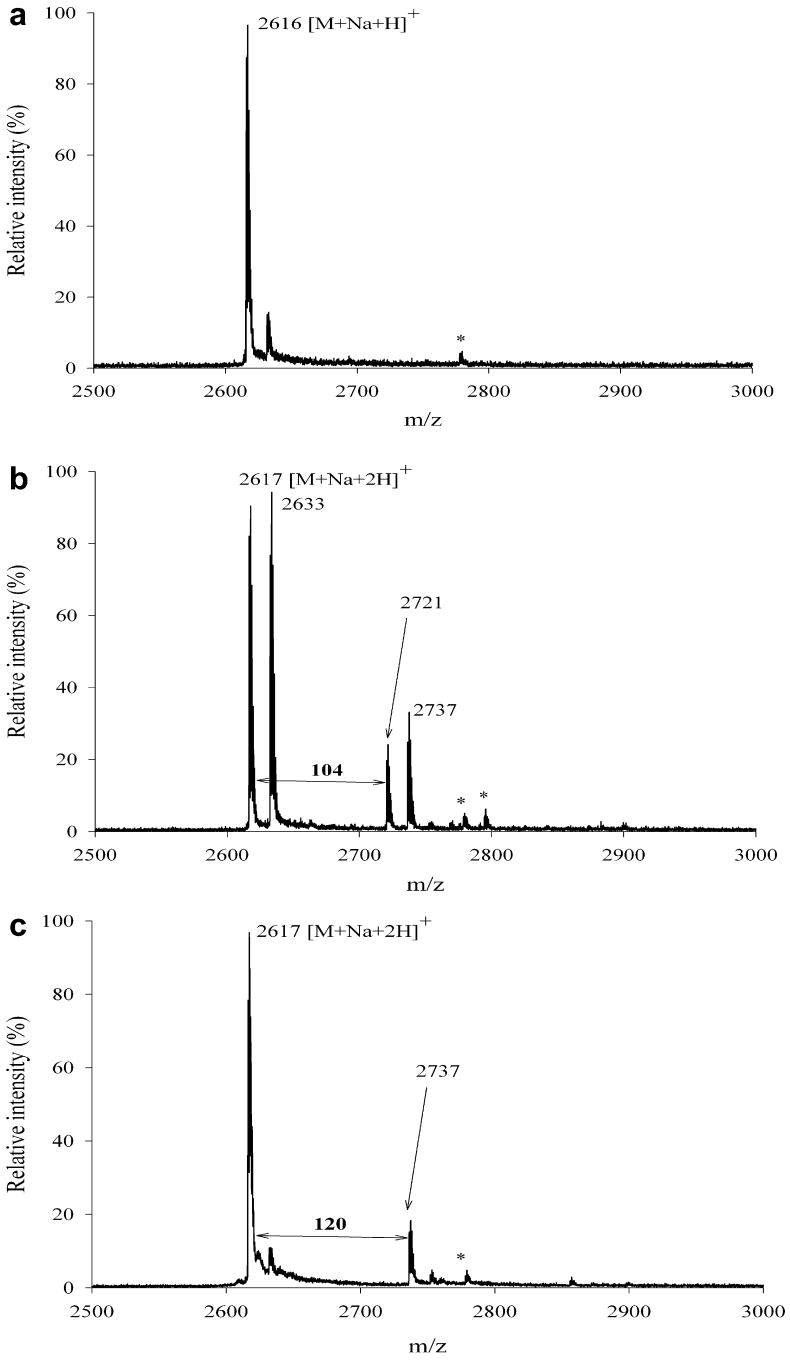


Fig. 4. Mass spectra of (a) original α-C16, (b) α-C16 used in methanolysis of **1** and (c) α-C16 used in methanolysis of phenyl salicylate. MALDI-TOF MS in the positive ion mode with 2,5-DHB as matrix. (b) Mass spectra containing *m/z* 2721 [acylated α-C16 + Na + 2H]$^+$ and *m/z* 2737 [acylated α-C16 + K + 2H]$^+$. (Asterisk (*) means cyclic glucans of DP 17 (*m/z* 2779 and 2795 corresponding to sodium and potassium adducts, respectively) isolated from *X. oryzae* pv. *oryzae* KACC 10331.) (c) Mass spectra containing *m/z* 2737 [acylated α-C16 + Na + 2H]$^+$ and 2753 [acylated α-C16 + K + 2H]$^+$.

space for catalytic action of α-C16. Additionally comparing phenyl group (**1** R) with 4-chlorophenyl group (**2** R), k_{cat}/k_{uncat} of **1** was higher than that of **2** since the k_{uncat} value of **2** was higher than that of **1**. Rather, it may be due to the electronic and resonance effects of the *para*-chloro substituent of **2** R.

As a result, α-C16 enhanced the methanolysis of three different benzoates about 24-fold for **1**, 14-fold for **2** and 69-fold for **3** in comparison with a control. In this study, we suggest α-C16 functions as a catalytic carbohydrate for methanolysis of benzoates.

3.3. A possible intermediate

To investigate how α-C16 participates in the reaction, MALDI-TOF MS analysis was carried out. MS analysis has been often performed to show evidence for the existence of the covalent acyl intermediates (Ashton et al., 1991; Kim et al., 2002; Viladot, Canals, Batllori, & Planas, 2001). In this study, the possible intermediate peaks were detected on the MALDI-TOF spectra. Fig. 4(a) shows original α-C16 mass data, and no peak was observed between α-C16 and cyclic glucan of DP 17. Fig. 4(b) shows mass spectrum of α-C16 used in phenyl benzoate (**1**) methanolysis and measured values at *m*/*z* 2721 and 2737 represent [acylated α-C16 + Na + 2H]$^+$ and [acylated α-C16 + K + 2H]$^+$, respectively. To confirm the presence of intermediate again, phenyl salicylate methanolysis by α-C16 was also carried out as monitoring the reaction with TLC analysis (data not shown) and α-C16 also catalyzed the reaction. Phenyl salicylate has –OH group at the benzoate *ortho* position and therefore, mass difference of the acyl intermediate of phenyl salicylate with the intermediate of **1**, **2** or **3** methanolysis is 16. Fig. 4(c) shows this mass difference as *m*/*z* 2737 [acylated α-C16 + Na + 2H]$^+$ and 2753 [acylated α-C16 + K + 2H]$^+$. Based on Fig. 4(b) and (c), the MALDI-TOF spectra showing the possible intermediate indicate definitely the existence of the covalent compounds formed between α-C16 and the substrates.

3.4. Effect of other carbohydrates

Through TLC analysis, we investigated the methanolysis reaction to identify the effect of other carbohydrates, where amylose, α-CD, glucose and α-C16, respectively, were used. Fig. 5 shows TLC analysis of methanolysis for three different benzoates after 10 h at 40 °C. The top spots mean substrates **1**, **2**, and **3**, respectively, from the left side. The bottom spots in the Lane E of substrate **1** and **2** mean phenol and 4-chlorophenol, respectively and the middle spots in the Lane E of substrates **1**, **2** and **3** mean methyl benzoate. In common with three reaction mixtures with α-C16, methyl benzoate was formed as products. Based on the substrate spot concentrations as shown in Lane E, the reaction progress was different. Methanolysis of **3** by α-C16 occurred completely but methanolysis of substrate **1** and **2** occurred incompletely by α-C16, and other control carbohydrates such as glucose, amylose and α-CD did not show any catalytic effect on the reaction.

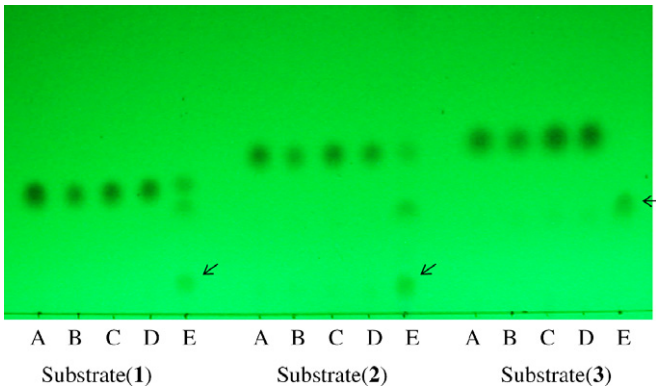


Fig. 5. TLC analysis of reaction mixtures of substrates (**1**, **2** and **3**) and various carbohydrates containing α-C16 after 10 h at 40 °C. The reaction condition is 20 mM substrates (**1**, **2** and **3**) in 0.3 mL methanol with 0.2 equiv. of α-C16 (lane E), without any carbohydrate (lane D), with the same weight of glucose (lane C), α-CD (lane B) and amylose (lane A) as α-C16. Lane E shows the reaction products besides reactants. Arrows indicate the products. (TLC solvent condition, hexane: methylene chloride = 1:1).

Above results can be caused by the structural differences of carbohydrates used in the reactions. Glucose and amylose lack space or appropriate cavity for binding of substrates. We also used α-CD as another control cyclic carbohydrate but it has no effect on the reaction. On the complexation of α-CD with a fixed cavity and substrates, the adequate positioning for the interaction between nucleophilic –OH and carbonyl carbon wouldn't be formed. α-C16 as a microbial cyclic glucan has the backbone of cyclic β-(1 → 2) glucans and single α-(1 → 6) linkage which give more flexible conformation than α-CD. The characteristic scaffold induced by β-(1 → 2)-glycosidic bonds and single α-(1 → 6) linkage of α-C16 could give the appropriate space for the binding of the substrates so that a catalytic reaction which takes place via α-C16-acyl intermediate could be allowed.

4. Conclusion

α-C16, a periplasmic cyclooligosaccharide isolated from rice pathogenic bacteria, *X. oryzae*, enhanced the methanolysis of three different benzoates about 24-fold for **1**, 14-fold for **2** and 69-fold for **3** in comparison with a control. In this study, we suggest α-C16 functions as a catalytic carbohydrate for methanolysis of benzoates. Further study on other applications of α-C16 is in progress.

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