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Carbohydrate Research 343 (2008) 1378–1382
Carbohydrate RESEARCH

Note

Selective phospholipid methanolysis catalyzed by a weakly acidic microbial polysaccharide, zooglan

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Received 21 December 2007; received in revised form 31 March 2008; accepted 5 April 2008
Available online 10 April 2008

Abstract—Zooglan, a weakly acidic exopolysaccharide produced by *Zoogloea ramigera* 115, catalyzed the preferential methanolysis of phospatidylcholine compared to other phospholipids when the reaction was carried out in pure methanol at 30 °C. The reaction was monitored by thin-layer chromatography (TLC) as well as ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectrometry. Zooglan enhanced the rate of methanolysis of dipalmitoylphosphatidylcholine (DPPC) up to about 170-fold compared to controls such as DPPC alone, pyruvic acid, succinic acid and acetic acid. Furthermore, the methanolysis was different depending on the head groups of the phospholipids. Through this study, we have shown that zooglan can act as an environmentally benign catalytic polysaccharide for methanolysis in pure methanol solution.

Keywords: Zooglan; *Zoogloea ramigera*; Catalytic polysaccharide; Catalyst; Phosphatidylcholine; Methanolysis

Zoogloea ramigera is an aerobic gram-negative bacterium that is found primarily in organically enriched aqueous environments; these organisms have the ability to form flocks. *Z. ramigera* I-16-M produces no gelatinous matrix,[1] while *Z. ramigera* 115 produces an extracellular zoogloeal matrix in which the cells are embedded.[2] The structure of the biopolymer produced from *Z. ramigera* 115 has been reported to have a pyruvyl group as a substituent and is composed of glucose and galactose as shown in Figure 1A. The polysaccharide has a molecular weight of about 10^5.[3–7] On the other hand, the polysaccharide produced by the *Z. ramigera* 115SLR mutant has been reported to have more varied substituents than does the wild type organism, for example, inclusion of acetyl, succinyl and pyruvyl groups.[8]

With regard to physiochemical properties, zooglan produced from *Z. ramigera* 115 has the ability to absorb amino acids,[9] metal ions,[10–12] and cholesterol.[13] In addition to these properties, a catalytic ability of zooglan for specific organic reactions has been identified. Recently, we reported that zooglan isolated from *Z. ramigera* 115 catalyzes the methanolysis of the lactone ring in 4-benzylidene-2-phenyloxazolone in pure methanol.[3] We also reported that some microbial carbohydrates have catalytic potential in specific methanolysis reactions.[14–16]

Methanolysis has also been performed during biodiesel production, using catalysts such as acids, alkalis, and lipases.[17] Biodiesel fatty acid methyl esters (FAMEs), which are produced by the methanolysis of triglycerides such as animal fats and vegetable oils, have been of increasing interest as promising renewable alternative energy sources to replace conventional fossil fuels. Thus, environmentally benign catalysts and processes for making the FAMEs are of importance in terms of environmental protection. Thus, zooglan may have potential as an environmentally friendly catalyst for producing biodiesel.

As part of our early work in this area, we recently reported that cyclic β-(1→2)-glucans (cyclosophoraose) isolated from *Rhizobium* species catalyze the methanolysis of biological phospholipids.[14] However, no work on

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doi:10.1016/j.carres.2008.04.008

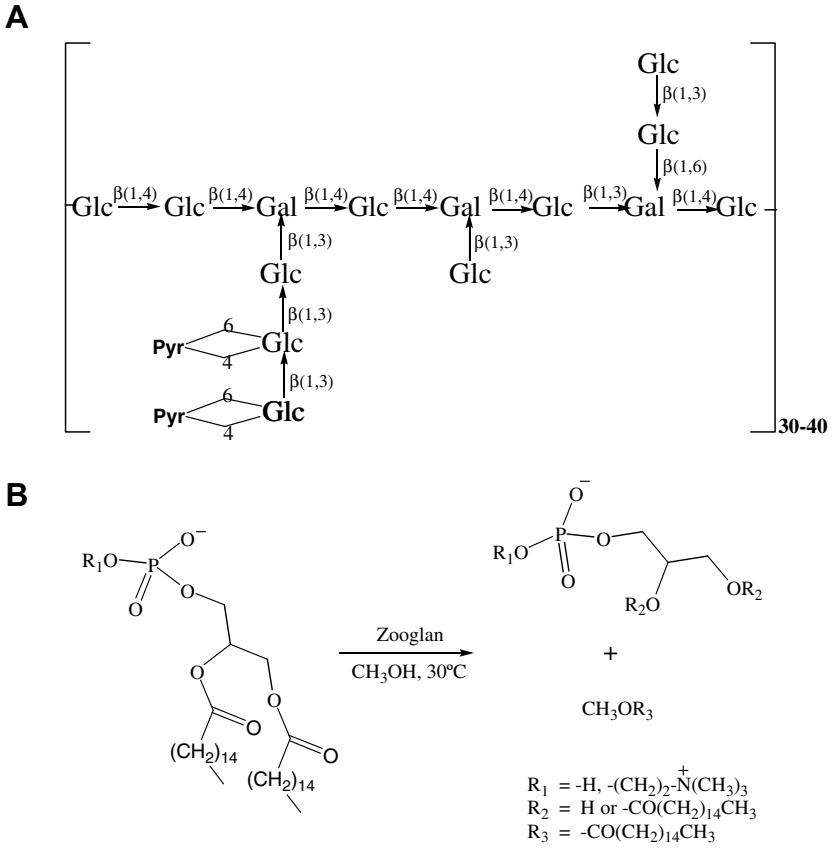

Figure 1. Possible structure[6] of zooglan, which is produced by *Zoogloea ramigera* 115 (A). Glc, Gal and Pyr represent glucose, galactose and pyruvic acid, respectively. Schematic representation of the methanolysis reaction of DPPC catalyzed by zooglan (B).

the catalytic methanolysis of phospholipids induced by microbial linear polysaccharides was described. In this study, we report for the first time that zooglan, which contains a pyruvyl group as a substituent, can function as a catalytic polysaccharide in the production of FAMEs when phospholipids are used as substrates. Furthermore, the methanolysis catalyzed by zooglan showed selectivity for the phospholipids (Fig. 1B). This selective methanolysis process may be useful in the studies on the specific interactions between cellular carbohydrates and lipids depending on head groups, and on quantitative or qualitative analysis of a specific lipid like phospatidylcholines among a mixture of phospholipids.

Prior to the reaction, we characterized both the isolated and the commercial zooglan by FTIR spectroscopy. Based on the previous reports,[3,18] we identified the functional groups of the zooglan. From the spectra, we confirmed no differences between the isolated and the commercial zooglan. The functional groups of zooglan were assigned as following: OH, 3445 cm^{-1}; C–H, 2935–2920 cm^{-1}; C=O of carboxyl groups, 1653 cm^{-1} and 1458 cm^{-1}; tertiary CH–OH, 1165 cm^{-1}; C–O stretching of zooglan bonds, 950–1150 cm^{-1}.[3,18]

Because the zooglan used in this study has a pyruvyl group as a substituent, we measured the pH of the polysaccharide in pure water. A 3% aqueous solution of zooglan has a pH of 6.75. This weakly acidic polysaccharide functioned as a catalyst for the selective methanolysis of phospholipids. Among the phospholipids tested, dipalmitoylphosphatidylcholine (DPPC) was preferentially methanolyzed, while dipalmitoylphosphatidic acid (DPPA) was a weaker substrate for the reaction.

We first identified the reactant and the products on TLC using a colorimetric detection system composed of an ammonium molybdate–copper solution in the absence or the presence of zooglan (Fig. 2). After 48 h, DPPC and DPPA were clearly converted into two spots in the presence of zooglan, while DPPC and DPPA alone were not (Fig. 2A). From the TLC image, we confirmed that dipalmitoylphosphatidylserine (DPPS) and dipalmitoylphosphatidylethanolamine (DPPE) were not methanolyzed by zooglan. The TLC image with DPPA and DPPC was re-run with cospot lanes with the known products for clarity. In Figure 2B, the TLC image of DPPA and DPPC with cospot lanes of the known products in the absence or the presence of zooglan is shown. Lyso-palmitoylphosphatidic acid and the lyso-palmitoylphosphatidylcholine were newly observed in lanes 3 and 6, respectively. The TLC image suggests that the intact DPPC and DPPA are converted

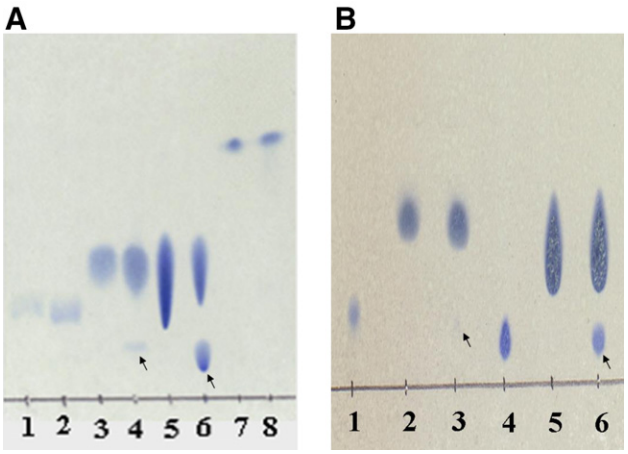

Figure 2. TLC image of the dipalmitoylphospholipids containing DPPC in the absence or the presence of zooglan (3 mg) after 48 h at 30 °C (A). Lane 1, DPPS alone; lane 2, DPPS with zooglan; lane 3, DPPA; lane 4, DPPA with zooglan; lane 5, DPPC; lane 6, DPPC with zooglan; lane 7, DPPE; lane 8, DPPE with zooglan. The TLC image rerun with cospot lanes with the known products for the DDPA and DPPC in the absence or the presence of zooglan (3 mg) (B). Lane 1 is L-α-lyso-oleoylphosphatidic acid alone as the known product, lane 2, DPPA alone, lane 3, DPPA with zooglan, lane 4, L-α-lyso-palmitoylphosphatidylcholine alone as the known product, lane 5, DPPC alone, lane 6, DPPC with zooglan, respectively. Arrows indicate the products newly made from the reaction mixtures in the presence of zooglan. After the reaction mixtures were loaded and developed on TLC, the solution of ammonium molybdate as a detecting agent was sprayed.

into lyso-palmitoylphosphatidylcholine or lyso-palmitoylphosphatidic acid as one of the products by zooglan during the reaction, and then one of the fatty acyl groups (at the position *sn*-1 or *sn*-2) is methanolyzed to the FAME as shown in Figure 1B. To investigate the effect of simple acids on methanolysis, we used succinic acid, acetic acid and pyruvic acid, and then monitored the reaction with TLC. The acid did not have any effect on the methanolysis of DPPC at 30 °C when an excess of the simple acid (100–170 mM) was mixed with DPPC in anhydrous methanol (data not shown).

The catalytic reaction was also confirmed by ^{1}H NMR spectroscopic analysis (Fig. 3A and B). Fig. 3A shows the conversion of DPPC and DPPA catalyzed by zooglan with increasing reaction time, based on the integration analysis of the reactant and the product measured from ^{1}H NMR spectra. In the presence of zooglan, the methanolysis of DPPC was enhanced about 170-fold compared with DPPC alone at 30 °C. With the results obtained from the ^{1}H NMR spectral analysis, the data were fitted to a single exponential to obtain a k_{cat} $(3.15 \pm 0.03) \times 10^{-2}\,h^{-1}$ and a k_{uncat} of $(1.85 \pm 0.05) \times 10^{-4}\,h^{-1}$. Also, the conversion rate of DPPA was determined to have a k_{cat} of $(2.94 \pm 0.07) \times 10^{-3}\,h^{-1}$. DPPA alone in methanol was not converted into products after 48 h and so it was not possible to calculate a rate constant. Figure 3B shows the partial ^{1}H NMR spectrum of DPPC when zooglan was added to a solution of the substrate dissolved in pure methanol. After just 5 min, a signal corresponding to the methoxy protons of the FAME product was clearly observed at 3.66 ppm; the integration of this peak increased with reaction time.

We further confirmed the reaction catalyzed by zooglan through ^{31}P NMR spectral analysis on the methanolysis of DPPC (Fig. 3C). In the presence of zooglan, the reactant (DPPC) and the product (lyso-palmitoylphosphatidylcholine) were observed at 0.456 and 1.042 ppm after just 5 min, respectively. After 48 h, the peaks corresponding to the product increased further.

Because the zooglan itself is a heterogeneous biopolymer, it is difficult to determine the origin of the selectivity of the methanolysis reaction. However, it is clear that the zooglan more favorably catalyzes the methanolysis of DPPC compared to other phospholipids. Although a detailed mechanistic study is complicated by the high molecular weight of zooglan and its insolubility in any solvent, a possible reaction mechanism might be assumed as follows based on the our previous study.[3,14] One possibility is that either the hydroxyl groups or pyruvyl groups of the zooglan, acting as a nucleophile, might attack the ester carbonyl carbons of the DPPC, which is bound to the polysaccharide. This process would result in the formation of an acyl intermediate, which would be subject to attack by the methanol as second nucleophile thus generating the FAME product. A second possibility is that zooglan might activate the methanol via hydrogen bonding, resulting in the attack of the hydroxyl group on the ester group of the DPPC.

In conclusion, we show that the natural polysaccharide zooglan acts as a catalytic carbohydrate for the selective methanolysis of the biological phospholipids. The preferential catalytic methanolysis of zooglan toward phosphatidylcholine was confirmed through TLC, ^{1}H and ^{31}P NMR spectral analysis. Although the exact mechanism remains unclear, zooglan definitely has a potential to act as a catalyst for methanolysis, which is environmentally benign compared to other typical acid or base catalysts. Studies on other functions of zooglan are in progress.

1. Experimental

1.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidic acid (DPPA) and dipalmitoylphosphatidylglycerol (DPPG). L-α-Lysophosphatidic acid, oleoyl and L-α-lysophosphatidylcholine, palmitoyl, anhydrous MeOH (99.8%),

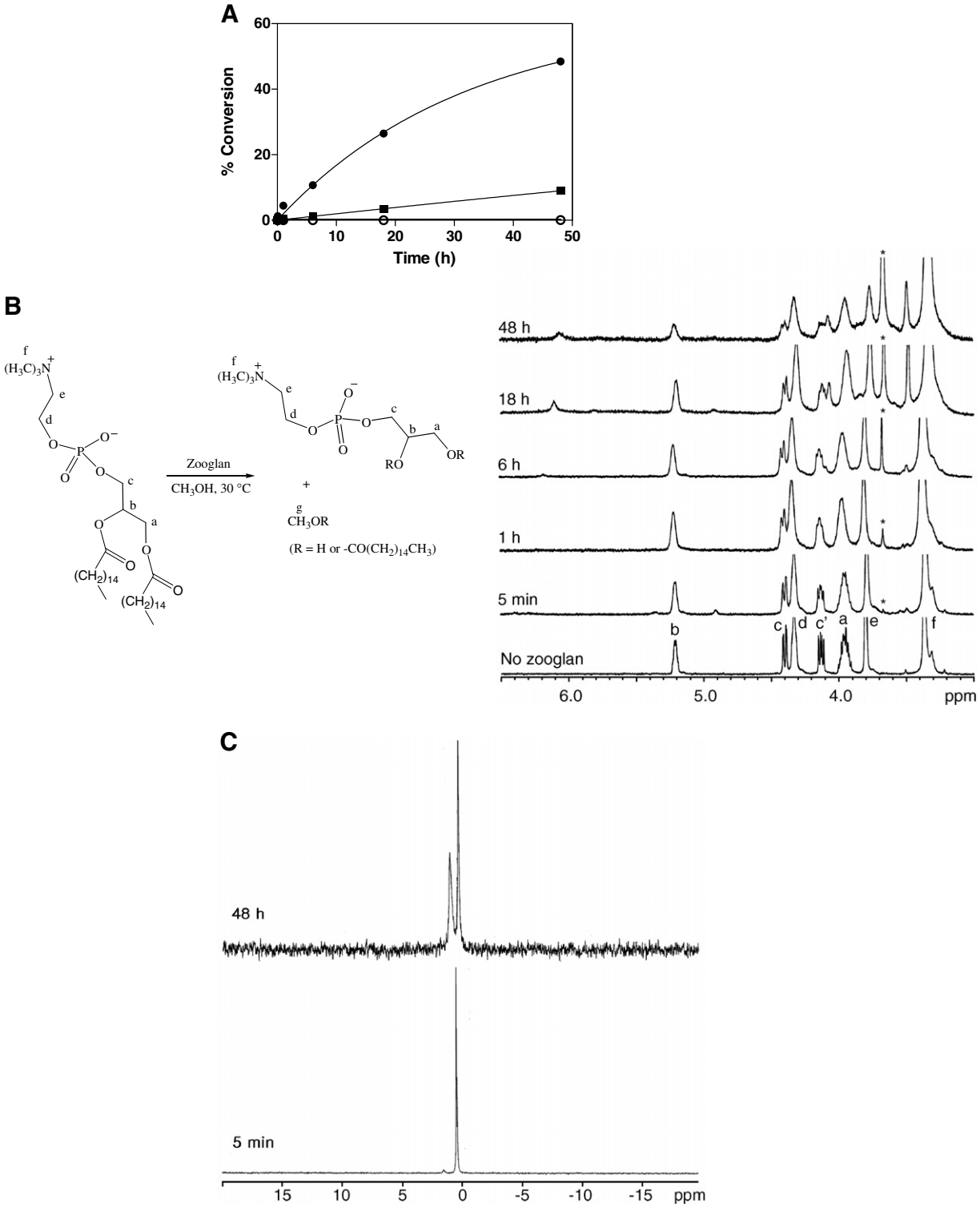

Figure 3. Conversion of DPPC (●) and DPPA (■) into their product with increasing reaction time in the absence (○ for DPPC alone) or the presence of zooglan (A). No product peaks for DPPA alone in anhydrous methanol were observed. Three separate experiments were performed, and then average values at each time were plotted. The data were fitted to a single exponential to obtain a k_{cat} of $(3.15 \pm 0.03) \times 10^{-2}$ h^{-1} and a k_{uncat} of $(1.85 \pm 0.05) \times 10^{-4}$ h^{-1}. Also, the conversion rate of DPPA was determined at a k_{cat} of $(2.94 \pm 0.07) \times 10^{-3}$ h^{-1}. Partial ^{1}H NMR spectra of DPPC with increasing reaction time in the absence or the presence of zooglan (B). The asterisks indicate the resonances of the methoxy protons of the methyl palmitate made from the methanolysis of DPPC catalyzed by zooglan. ^{31}P NMR spectra of the reaction mixtures at 5 min (low view) and 48 h (top view) when zooglan is added at 30 °C (C). All the reactions in this study were carried out in MeOH (1 mL) at 30 °C in the absence or the presence of zooglan (3 mg). Then, the concentration of the phospholipids used was 20 mM.

pyruvic acid, succinic acid and acetic acid were purchased from Sigma–Aldrich Inc. (St. Louis. MO).

1.2. Isolation and characterization of zooglan

For the preparation of zooglan, *Z. ramigera* 115 (ATCC 25935) was grown in 5 L jar fermentor containing 3 L of defined medium for 6 days at 30 °C, isolated and purified, as in our previous report.[3] For this study, commercial zooglan isolated from *Z. ramigera* 115 was also purchased from Sigma–Aldrich Inc.

To characterize both the isolated and the commercial zooglan, Fourier transform infrared (FTIR) spectroscopic analysis was performed. A pellet for FTIR analysis was obtained by grinding 2 mg of the zooglan with a KBr and pressing in a mold. The FT-IR spectrum of zooglan was obtained using a JASCO FT-IR-300E spectrometer (USA) over the wavenumber range of 4000–400 cm^{-1}.

To measure the pH of an aqueous solution containing the zooglan in water, 3 mg of the polysaccharide was suspended in pure water (100 mL) resulting in a 3% aqueous solution. The pH of the solution was determined by using ThermoOrion pH meter, model 410.

1.3. General methods

In this study, the reaction was carried out in anhydrous MeOH (1 mL) at 30 °C in the absence or the presence of 3 mg of zooglan; the concentration of the phospholipids was 20 mM. Pyruvic acid (170 mM), succinic acid (127 mM) and acetic acid (100 mM) were also tested for controls.

To monitor the reaction, the phospholipids were assayed on TLC. Silica Gel 60 F_{254} glass-backed TLC plates were spotted with the reaction mixtures and developed with a solvent system ($CHCl_3$–MeOH–H_2O, 65:25:5). An ammonium molybdate–copper solution in concentrated sulfuric acid was used as a detecting agent.[19]

NMR spectroscopic analyses were carried out on a Bruker AVANCE 500 spectrometer. ^1H or ^{31}P NMR spectroscopy was performed using $CDCl_3$ as an NMR solvent. The conversion of the phospholids was measured by monitoring the decrease of the methine proton resonance (b in Fig. 3B) and the increase of methoxy proton resonances (3.66 ppm) of methyl palmitates produced from the reaction with zooglan. For this test, aliquots were taken at a period of time, rapidly evaporated under reduced pressure. ^1H chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane (Me_4Si, TMS), which was used as an internal standard. The time course of the reaction was fitted to a single exponential to obtain k_{cat} or k_{uncat} using the GRAPHPAD PRISM version 4.0 (GRAPHPAD Software, Inc., San Diego, CA).

Acknowledgements

This research was supported by the National R&D project for Biodiscovery in MOST 2004. Professor S. Jung expresses his thank to Konkuk University for the financial support during his sabbatical year.

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