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Microbial secretion of lactate-enriched oligomers for efficient conversion into lactide: A biological shortcut to polylactide

Camila Utsunomia,[1] Ken'ichiro Matsumoto,[1,2] Sakiko Date,[1] Chiaki Hori,[1,2] and Seiichi Taguchi[1,2,*]

Division of Applied Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan[1] and CREST, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan[2]

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Recently, we have succeeded in establishing the microbial platform for the secretion of lactate (LA)-based oligomers (D-LAOs), which consist of D-LA and D-3-hydroxybutyrate (D-3HB). The secretory production of D-LAOs was substantially enhanced by the supplementation of diethylene glycol (DEG), which resulted in the generation of DEG-capped oligomers at the carboxyl terminal (referred as D-LAOs-DEG). The microbial D-LAOs should be key compounds for the synthesis of lactide, an important intermediate for polylactides (PLAs) production, eliminating the costly chemo-oligomerization step in the PLA production process. Therefore, in order to demonstrate a proof-of-concept, here, we attempted to convert the D-LAOs-DEG into lactide via metal-catalyzed thermal depolymerization. As a result, D-LAOs-DEG containing 68 mol% LA were successfully converted into lactide, revealing that the DEG bound to D-LAOs-DEG does not inhibit the conversion into lactide. However, the lactide yield (4%) was considerably lower than that of synthetic LA homo-oligomers (33%). We presumed that 3HB units in the polymer chain blocked the lactide formation, and therefore, we investigated the LA enrichment in the oligomers. As the results, the combination of an LA-overproducing *Escherichia coli* mutant (Δ*dld* and Δ*pflA*) with the use of xylose as a carbon source exhibited synergistic effect to increase LA fraction in the oligomers up to 89 mol%. The LA-enriched D-LAOs-DEG were converted into lactide with greater yield (18%). These results demonstrated that a greener shortcut route for PLA production can be created by using the microbial D-LAOs secretion system.

[**Key words:** Biosynthesis; Secretory production; Bioprocess; Metal-catalyzed reaction; Polyhydroxyalkanoate; Biomass; Polylactic acid; Oligoester]

Polylactides (PLAs) are one of the most successful biobased polyesters with diverse applications due to physical properties, biocompatibility, and processability (1). Currently, biomass resources such as corn, cane sugar, potato starch, and tapioca starch are used as carbon sources for PLA production (2). The conventional process of PLA production from biomass involves (i) a bioprocess for the production of lactic acid from biomass sugars, and (ii) a multistep chemo-process containing the oligomerization of lactic acid to generate lactate (LA) oligomers, depolymerization of LA oligomers into lactide (cyclic dimer of LA), and the polymerization of lactide into high molecular weight PLA via ring-opening polymerization (ROP) (3,4) (Fig. 1). Although this is the main industrial route, the multistep chemo-bio process is considered complex and expensive relative to petroleum-based polymers (5). Among the steps, the production of lactide from lactic acid, which comprises the oligomerization of lactic acid and the lactide synthesis from LA oligomers, contributes to 30% of total cost for PLA production (6).

Recently, we have discovered the secretory production of D-LA-based oligomers (D-LAOs), which consist of D-LA and D-3-hydroxybutyrate (D-3HB), by recombinant *Escherichia coli* grown on semisynthetic medium using glucose as carbon source (7). This bacterium expresses an engineered polyhydroxyalkanoate (PHA) synthase (PhaC), designated as D-specific LA-polymerizing enzyme (LPE). This enzyme had been formerly developed for the production of LA-based polyesters (8,9). The production of D-LAOs was remarkably increased by supplementing diethylene glycol (DEG) in the bacterial cultivation. The transference of the polymer chain took place from the LPE to DEG via chain transfer (CT) reaction when DEG was added in the culture. The high frequency of DEG-mediated CT reaction drastically decreased the molecular weight of the intracellularly accumulated LA-based polyesters, and also enhanced the production and secretion efficiency of D-LAOs. The oligomers synthesized under DEG supplementation were nearly fully conjugated with DEG at the carboxyl terminal (termed as D-LAOs-DEG).

The aim of the present study is to verify the feasibility of the D-LAOs-DEG on the conversion into lactide, since this is a key reaction to construct a shortcut route in the process of poly(D-lactide) (PDLA) production (Fig. 1). By establishing this new shortcut route, the laborious purification of lactic acid from the microbial culture broth (10) and the lactic acid oligomerization can be eliminated, increasing the sustainability of the material and the cost effectiveness of PDLA production. Moreover, the production of optically

* Corresponding author at: Department of Chemistry for Life Sciences and Agriculture, Faculty of Life Sciences, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan. Tel.: +81 3 5477 2577; fax: +81 3 5477 2627.
E-mail address: staguchi@eng.hokudai.ac.jp (S. Taguchi).

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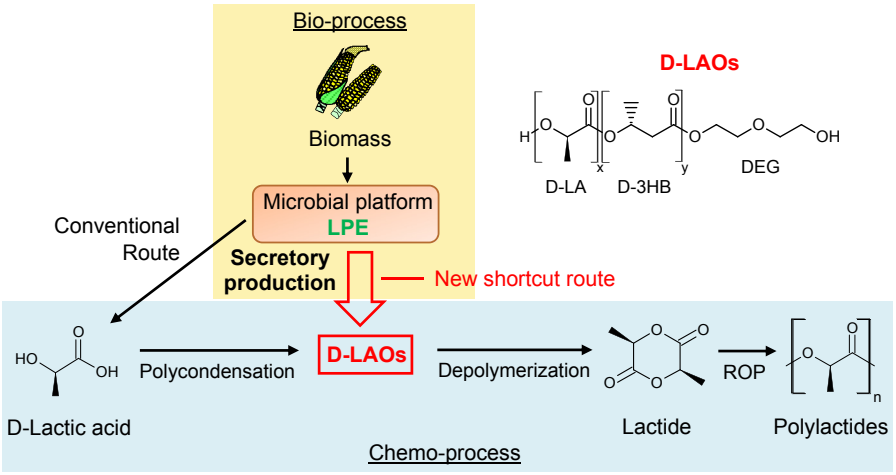

FIG. 1. Scheme of the conventional route and new shortcut route for PDLA production. In the conventional process, D-lactate (LA) oligomers are chemically prepared from purified D-lactic acid, which is generated by bacterial fermentation, via a polycondensation reaction. In the shortcut route proposed in this study, highlighted in red color, D-LA-based oligomers (D-LAOs) are directly secreted by bacteria from renewable biomass. LPE, lactate-polymerizing enzyme; ROP, ring-opening polymerization; DEG, diethylene glycol; D-3HB, D-3-hydroxybutyrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pure D-LAO is beneficial in view of the superior thermal properties of poly(L-lactide) (PLLA)/PDLA stereocomplex (11,12), and the higher production cost of D-LA compared to L-LA. Therefore, D-LAOs-DEG were used as substrates for conversion into lactide via metal-catalyzed reaction. To examine the effect of the terminal DEG in D-LAOs-DEG on the lactide formation, free-form D-LAOs (synthesized without DEG addition) were used for comparison. Moreover, to evaluate the influence of 3HB units in D-LAOs-DEG on the lactide synthesis efficiency, we attempted to synthesize LA-enriched oligomers through metabolic and fermentation engineering approaches.

MATERIALS AND METHODS

Bacterial strains and plasmids *E. coli* BW25113 (13), and the dual-gene knockout mutant (*Δpfla* and *Δdld*) JWMB1 (14) were used as the host strains. The expression vector pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* harboring *pct*, *phaC1*$_{Ps}$(ST/FS/QK), *phaA*, and *phaB* genes (9), was used for the production of D-LAOs.

Culture conditions in test tubes Cultivations for D-LAOs-DEG production were carried out in 10 mL glass test tubes containing Luria–Bertani (LB) medium (1.7 mL) with 20 g/L glucose or xylose and 100 mg/L ampicillin at 30°C for 48 h with reciprocal shaking at 180 rpm. The cultivations were performed with the supplementation of 5% DEG (v/v). For observing the aeration effects on D-LAOs production, the cultivations of the dual-gene knockout mutant with xylose as carbon source, were also performed using 2.5, 3.4, and 5.1 mL medium.

Shake flask cultures Free-form D-LAOs were produced in shake flask cultivation. Seed culture of recombinant *E. coli* was prepared using 2 mL LB medium containing 100 mg/L ampicillin in 10 mL glass test tubes and cultured at 30°C for 12 h with reciprocal shaking at 180 rpm. One milliliter of the seed culture was then transferred into 100 mL LB medium containing 20 g/L glucose, and 100 mg/L ampicillin in a 500 mL shake flask and cultured at 30°C for 48 h with reciprocal shaking at 120 rpm.

Measurement of extracellular D-LAOs The cell-free culture supernatant was analyzed before and after HCl treatment. HCl was added to the supernatant at a final concentration of 2.0 M and incubated at 100°C overnight to hydrolyse the D-LAOs. Afterward, the hydrolysate was neutralized with 2.0 M NaOH. The estimation of D-LAOs in the culture medium was determined by liquid chromatography-mass spectrometry (LC-MS) (LCMS-8030, Shimadzu, Japan) based on the difference of lactic acid and 3-hydroxybutyric acid concentrations in the samples after and before HCl treatment, as described previously (7).

Extraction of D-LAOs from the culture supernatant D-LAOs were concentrated from the cell-free culture supernatant by two-phase extraction using chloroform ($CHCl_3$), with modifications on the previously established method (7). The extraction was performed by adding 1 volume of $CHCl_3$ to 1 volume of supernatant and mixing vigorously. After the separation of $CHCl_3$ and water layers, the $CHCl_3$ phase was transferred to a new test tube. The extraction was performed three times. To remove excess DEG, lactic acid/3-hydroxybutyric acid monomers, and short oligomers, 1 volume of 0.9% NaCl solution pH 8 was added to the resulting $CHCl_3$ fraction. The washing step was repeated twice. The molecular weight distribution of extracted oligomers was determined by electrospray ionization-time-of-flight-mass spectrometry (ESI-TOF-MS), as described in the literature (7).

Lactide synthesis The synthesis of lactide occurs via thermal depolymerization of the LA oligomers via a metal–catalyzed backbiting reaction of the –OH end groups (15). Approximately 40 mg of vacuum dried extracted D-LAOs containing 68, 78, and 89 mol% LA, were individually weighted together with 40 mg zinc oxide (ZnO, Kanto Chemical, Japan). The sample bottle containing D-LAOs and catalyst was placed inside a rotary type Sibata GTO-350RD glass oven (Sibata Scientific Technology, Japan). The reaction system was heated and kept at 180°C while being rotated in a circular motion for 1 h under vacuum. The vaporized lactide was condensed into a bottle cooled on ice, and recovered in chloroform. Water-soluble synthetic L-LA oligomers (Glart, Japan) and free-form D-LAO, were also converted into lactide as experimental controls. The lactide yield (%, 2 × [μmol lactide/μmol initial oligomeric LA]) was calculated based on the amounts of produced lactide and initial oligomeric LA quantified using the ^{1}H NMR analysis.

^{1}H NMR of D-LAOs and lactide ^{1}H NMR of extracted oligomers and generated lactides were recorded in $CDCl_3$ with tetramethylsilane as the internal reference using a JEOL JNM-ECS400 spectrometer (JEOL, Japan) at 400 MHz. Benzoic acid (Wako, Japan) was used as an internal standard to quantify oligomers and lactide based on the integral area of the methyl group ($-CH_3$); 45° excitation pulse was used and relaxation delay was set to 10 s.

RESULTS AND DISCUSSION

D-LAOs-DEG could be converted into lactide via metal-catalyzed backbiting reaction The synthesis of lactide from the extracted D-LAOs-DEG was undertaken to verify the applicability of the biosynthesized D-LAOs-DEG for subsequent PLA production. These oligomers were produced in the test tube by the wild-type strain BW25113 grown on glucose with 5% DEG supplementation. After extraction of the culture supernatant with chloroform, D-LAOs-DEG with 68 mol% LA and degree of polymerization (DP) of approximately trimer to 7 mer were recovered in chloroform phase (designated as extracted D-LAOs-DEG). As controls, extracted free-form D-LAOs (61 mol% LA, DP ~ trimer to 16 mer) and synthetic L-LA homo-oligomers (L-LAOs) (100 mol% LA, DP ~ trimer to 14 mer), were also used as substrates for lactide synthesis.

For generating lactides, the LA oligomers were heated with zinc oxide as catalyst, and the vaporized fraction was recovered by condensation in bottle 3 (Fig. 2A) and subjected to ^{1}H NMR analysis (Fig. 2B). The ^{1}H NMR spectrum (δ in ppm) of the sample generated from D-LAOs-DEG exhibited signals at 5.0 ppm (1H, q, A) and 1.7 ppm (3H, d, B), which were identical to those of the standard D-lactide. The lactide generated from free-form D-LAOs also exhibited