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Title	Secretion Systems and Applications of Microbial Lactate-based Oligomers [an abstract of dissertation and a summary of dissertation review]
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Degree Grantor	北海道大学
Degree Name	博士(工学)
Dissertation Number	甲第12921号
Issue Date	2017-09-25
Doc URL	https://hdl.handle.net/2115/67429
Rights(URL)	https://creativecommons.org/licenses/by-nc-sa/2.1/jp/
Type	doctoral thesis
File Information	Camila_Utsunomia_abstract.pdf, 論文内容の要旨



学 位 論 文 内 容 の 要 旨

博士の専攻分野の名称 博士（工学） 氏名 ウツノミア カミラ

学 位 論 文 題 名

Secretion Systems and Applications of Microbial Lactate-based Oligomers (微生物による乳酸オリゴマー分泌システムの構築とその応用研究)

In the present work, I report the first microbial secretion system of D-lactate (LA)-based oligomers (D-LAOs), which are co-oligomers of D-LA and D-3-hydroxybutyrate (3HB). D-LAOs were unexpectedly found to be produced and spontaneously secreted by engineered *Escherichia coli* from glucose. D-LAOs can be useful compounds to synthesize poly(D-lactic acid) (PDLA) as a precursor of lactide by eliminating two steps of the production process. Aiming the practical applications, the initial low secretory production of D-LAOs could be remarkably increased by the supplementation of diethylene glycol (DEG) into the culture medium. DEG functioned as a chain transfer (CT) agent in the system, leading to the generation of DEG-capped oligomers at the carboxyl terminal (D-LAOs-DEG). The D-LAOs-DEG were successfully converted into lactide via metal-catalyzed depolymerization. This achievement enabled the creation of a green shortcut route in the process of PDLA production via direct secretion of D-LAOs. Furthermore, the newly recognized phenomenon of D-LAOs secretion was investigated in *E. coli*. The obtained results indicated that the passage of D-LAOs across the cell membrane is likely to occur via multiple routes including the courses mediated by membrane protein transporters.

The general introduction including the research theme is described in Chapter 1. Polylactide (PLA) is a biobased plastic entirely derived from renewable feedstocks. In the industrial production of PLA, lactic acid produced by microbial fermentation of sugars is purified and polymerized via polycondensation reaction to form LA oligomers. The LA oligomers are subsequently depolymerized to generate lactide (cyclic dimer of LA), which is then polymerized into PLA via ring-opening polymerization (ROP). These multistep processes are energy consuming and contribute to the high cost of PLA production. Thus, to improve the profitability of PLA, the development of a simplified process is of great interest. In 2008, as an effort to biosynthesize PLA in a one step process, the first microbial factory of LA-based polyesters, was established. This achievement was only possible with the discovery of a D-specific LA-polymerizing enzyme (LPE). To date, the polymers produced with high LA fraction in recombinant bacteria tend to have relatively low molecular weights. This feature of the microbial system of LA-

based polyesters inspired me to pursue the biosynthesis of LA oligomers for creating a green shortcut route in the process of high-molecular weight PLA, which can eliminate the steps of lactic acid purification and polycondensation. To achieve this goal, however, at least two challenges had to be overcome: the biosynthesis of LA oligomers and their conversion into lactide.

In Chapter 2, I present the discovery of the biosynthesis and secretion of D-LAOs by engineered *Escherichia coli*. D-LAOs were unexpectedly found to be synthesized and secreted by the engineered cells expressing the evolved LPE and monomer supplying enzymes from glucose. Presumably, the D-LAOs are produced because LA is not the preferred substrate for LPE, thus causing early chain termination and the production of low-molecular weight compounds. Thus, envisioning the practical application of D-LAOs, I attempted to increase the frequency of CT reaction by adding exogenous alcoholic compounds acting as CT agents, in turn to increase the production of D-LAOs. Four known CT agents were individually supplemented in the *E. coli* cultivation and DEG was determined to be the most efficient CT agent, which enhances extracellular production of D-LAOs. Thus, with the aid of DEG, a microbial secretion system of D-LAOs was successfully created. As a result of extensive analyses, I was able to understand followings; 1) the enhanced extracellular production of D-LAOs was due to secretion rather than to cell lysis, 2) the D-LAOs were almost entirely covalently bound to DEG at the carboxyl terminal (defined as D-LAOs-DEG), and 3) the D-LAOs-DEG have lower molecular weight than the free-form D-LAOs. Understanding the basic features of the produced oligomers was an essential start point to completely explore the new microbial secretion system of D-LAOs.

In Chapter 3, as a proof-of-concept that the biosynthesized oligomers can be used for PLA manufacturing, I aimed to convert D-LAOs-DEG into lactide via metal-catalyzed depolymerization. Indeed, the oligomers could be used in the lactide formation, indicating that the DEG bound to the carboxyl terminal of D-LAOs-DEG does not inhibit the depolymerization reaction. On the other hand, the lactide yield (4%) was significantly lower than that of chemically synthesized LA homo-oligomers (33%). I presumed that the presence of 3HB units in D-LAOs decreases the frequency of LA-LA dyad, and may also block the lactide formation. Thus, as an effort to improve the lactide yield, I attempted to increase the LA fraction in D-LAOs-DEG. As expected, the LA-enriched D-LAOs-DEG (89 mol% LA), a result of metabolic and fermentation engineering approaches, were converted with greater lactide yield (18%). Here, a prototype of the shortcut route in the process of PLA production via microbial secretion of D-LAOs-DEG was successfully demonstrated.

The secretion of D-LAOs by *E. coli* was an unexpected finding of this work. On the other hand, the secretion of 3HB oligomers by natural producers of P(3HB) was previously reported. However, to date, the phenomenon of

oligoesters secretion has never been investigated. Therefore, in Chapter 4, my goal was to address the secretion routes of D-LAOs in *E. coli*. Based on the secretion of compounds with similarities to D-LAOs, I hypothesized that the passage of D-LAOs across the cell membrane would be mediated by membrane-associated proteins. To elucidate the secretion phenomenon, a loss-of-function screening of D-LAOs secretion using gene deletants of membrane proteins related to the transport of organic compounds was performed. The obtained results suggested that porins are likely to mediate the D-LAOs transport through the outer membrane. Additionally, the selection of several inner-membrane associated proteins as candidates indicated the presence of multiple D-LAOs secretion routes across the inner membrane. As a rationale interpretation of this investigation, I proposed a model of D-LAOs secretion routes.

The thesis conclusion is presented in Chapter 5. Here, I discovered the production and spontaneous secretion of D-LAOs by the engineered *E. coli*. The enhanced secretory production of D-LAOs together with the successful conversion of D-LAOs into lactide enabled the creation of a green shortcut route in the manufacture of PDLA and its copolymers. Moreover, the intriguing D-LAOs secretion phenomenon in *E. coli* was assessed, revealing that the secretion is unspecific and occurs via multiple routes. The finding that organic acid oligomers, such as D-LAOs, can be artificially produced and secreted by bacteria is a landmark in this research field and represents a starting point for a myriad of new studies. Thus, in future, it is expected that the extracellular production can be expanded to various organic acid oligomers useful in diverse applications.