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Simple Preparation of Large Amount of Lipopolysaccharide with Receptor Activity for Bacteriophage ϕX174 from *Escherichia coli* C Strain

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Abstract

A convenient method for the preparation of a lipopolysaccharide (LPS) with receptor activity of bacteriophage ϕX174 from large amounts of the dried and defatted cells of *Escherichia coli* C strain was established. An improvement on bacterium cultivation led to yield over 75g of the dried and defatted cells (309g wet cells) from 6L culture medium containing 4% wt/v of D-glucose as a carbon source and 1%wt/v of ammonium sulfate as a nitrogen source. The modified PCP method afforded 2.25g of LPS (3.0% yield from the cells) from 75g of the dried and defatted cells. It was confirmed that the obtained LPS showed *in vitro* an inactivation of bacteriophage ϕX174 from a plaque counting assay.

Key Words: LPS • *E.coli* C strain • receptor • bacteriophage ϕX174

Introduction

Bacteriophage ϕX174 is one of the simplest phages having a circular single stranded DNA as a genetic information, which was first isolated by Sinsheimer.[1] It recognizes lipopolysaccharides (LPSs) of the rough mutants of enterobacteria, such as *Escherichia coli* C, *Salmonella typhimurium* rfb, and *Shigella sonei* etc. as a receptor molecule for the host cell recognition.[2] Feige *et al.* reported from comparative studies using some LPSs lacking terminal sugars prepared from mutants that the terminal pentasaccharide in the LPS from *E.coli* C plays an important role in the recognition.[3] Although their biological approach determined the primary structure of the receptor LPS for the infection to the host cell, so many hidden steps to be clarified in the recognition event still remained as a black box.

We have been paying attention to this interaction between bacteriophage ϕX174 and LPS of *E.coli* C through some approaches such as the chemical synthesis[4] and the conformation analysis[5] of the terminal pentasaccharide because of the simplest models of virus recognition of its host cells. We also envisage the chemical degradation of LPS of *E.coli* C to prepare the terminal penta or polysaccharide as an alternative approach, because the polysaccharide (PS), derived from LPS by weak acid hydrolysis, were found to competitively inhibit the phage inactivation activity of LPS.[6]

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For the purpose of these studies, we need to prepare a large amount of LPS as a starting material of chemical and enzymatic modification. We will report here the establishment of a conventional method for the large scale preparation of LPS of *E.coli* C having receptor activity for bacteriophage ϕX174.

Materials and Methods

General procedure

Turbidity was measured by absorbance at 600 nm using a Shimadzu UV 150-02 spectrometer. The fermentaion of *Escherichia coli* C strain was performed with an ABLE BIOMASTER-D10 jarfermenter (10L) equipped with a pH statting pump. All chemicals were purchased from Nacalai Tesque (Kyoto Japan) and used without further purification. Irradiation of ultrasound for washing or extraction of the bacterial cells was performed with Iuchi ULTRASONIC CLEANER VS-150 apparatus.

Culture conditions

One litter of the final medium contained the following ingredients: 40g D-glucose, 10g $(NH_4)_2SO_4$, 5g yeast extract, 0.6g $MgSO_4$, 0.5g KH_2PO_4, 1g K_2HPO_4, and 3g NaCl. The pH of the solution was adjusted to 7.2 by 2M NaOH and then 1M $CaCl_2$ (6 mL) was added to the solution. Antifoam AF suspension (3.2g paste in 40mL deionized water) and D-glucose solution (240g in 400 mL deionized water) were sterilized separately.

Cultivation was carried out 37 ℃ in laboratory fermenter ABLE BIOMASTER-D10 in 6 L quantities. For inoculation, 200 mL of shaking culture (6 h, 37℃) containing 1 % D-glucose, 0.5 % NaCl, 0.5 % yeast extract, and 1M $CaCl_2$ 10 μL, was used. In fermenter vessel, an aeration rate of 4.2 L per min for 6 L medium (70 % aeration) was used. The pH was kept at 6.8 by using 30 %wt/v NaOH. The bacterial growth was monitored by OD_{600nm}. The cells were harvested by centrifugation (5000 × g, 15 min). The cell paste was washed successively with ethanol, acetone and diethyl ether, and dried under reduced pressure over $CaCl_2$ for 1 week. The dried and defatted weight of *E.coli* C cells were 74.6 g.

Extraction of LPS[7]

The cells (74.6 g) were placed in a centrifuge tubes (stainless steel 60 mm ϕ × 145 mm height) and suspended with 300 mL of the PCP extraction mixture (90 % v/v aqueous phenol: $CHCl_3$: petroleum ether =2 : 5 : 8 v/v/v). The suspension was stirred for 15 min at room temperature under irradiation of ultrasound by IUCHI ULTRASONIC CLEARNER VS-150 (50kHz, 120W). The cells were centrifuged off (5000 ×g, 15 min) and the supernatant was collected in a round flask. The precipitated cells were extracted once more with the same volume of the PCP mixture and centrifuged as above and the supernatant was added to the first extract. The extraction was repeated four times. The combined supernatant was evaporated at 45 ℃ to remove completely chloroform and petroleum ether. The remained phenol solution was transferred into a centrifuge tubes and deionized water (40 mL) was added dropwise until the LPS precipitated. The precipitated LPS was centrifuged (3500 rpm, 20 min), and the supernatant was decanted. The viscous precipitate was washed three times with 80 % v/v aqueous phenol (60 mL) and acetone (60 mL), respectively. The LPS was dissolved in deionized water

and lyophilized for 1 week to afford LPS 2.25 g (3.0 % yield from the dried and defatted cells).

Estimation of receptor activity of LPS

According to the reported procedures,[8,9,10] the phage receptor activity of LPS was measured as a survival ratio of ϕX174 after treatment with various concentration of the extracted LPS (10^{-4}, 10^{-3}, and 0 mg/mL). In the small tube, ϕX174 (3.54×10^{9} p.f.u./mL): 10 μL was incubated with LPS solutions (10^{-3}, 10^{-2}, and 0 mg/mL) : 100 μL, and 0.01 M Tris-HCl buffer [pH 8.1 containing KCl (5 % wt/v), NaCl (1 % wt/v), and $CaCl_2$ (0.01 % wt/v)]: 890 μL for 1 h at 37 ℃. An aliquot (100 μL) was withdrawn, diluted with the same buffer, and assayed for plaque-forming ability on *E.coli* C by the agar overlayer method of Adams.[11] After the agar plates were incubated for 3 h at 37 ℃, the numbers of plaque were counted. The survival ratio of phage was calculated by division of the plaque numbers after 1 h treatment with LPS by the numbers of 0 h control which was spread on a agar plate immediately after addition of an LPS solution.

Results and Discussion

Relative low nutrient media were often applied for the cultivation of gram negative bacteria for the preparation of LPS. The concentration of sugars through 0.1 to 1.6 % wt/v [12-17] have been used as a carbon source. However, such concentration seemed to be disadvantage for the large scale preparation of cells. When we have employed a medium which applied in literatures[8,18] containing 0.2 % wt/v D-glucose, only 5.8 g of the dried and defatted cells of *E.coli* C were obtained from 6 L culture, and ca. 100 mg of LPS was yielded. In order to improve the yield of LPS, an elevation of biomass seemed to be neccessary because LPS is one of an essential component of outer membrane of gram negative bacteria. So, we attempted to cultivate *E.coli* C in a rich medium.

As a preliminary experiment, a medium containing 1% of D-glucose was tested according to the literature for the preparation of LPS of *E.coli* B.[17] The 8 h cultivation afforded 19.2 g of the dried and defatted cells from 6 L culture. Then, a rich medium containing 4 % wt/v of D-glucose and 1 % wt/v of ammonium sulfate was applied. Furthermore, the cultivation was performed by a jarfermenter. The bacterial growth was monitored by OD_{600nm} (Fig. 1). The growth curve showed a plateau reached at 8.5h cultivation. The harvested wet cells (309 g) were defatted by washing successively with ethanol, acetone, and diethyl ether under sonication and dried over $CaCl_2$ under reduced pressure for 1 week to afford 74.6 g of the dried and defatted cells. By increasing D-glucose concentration from 0.2 to 4 % wt/v, the biomass of *E. coli* C gained at least twelve folds.

The LPS was prepared from the dried and defatted cells by the phenol-chloroform-petroleum ether (PCP) extraction method, according to the procedure described by Galanos[7] with some modifications. For conventional operation, irradiation of ultrasound by a laboratory cleaner was used instead of a mechanical homogenizer for suspending the cells with PCP extraction mixture. Because the purpose of this treatment was not meant to break the cells but to obtain them in a fine suspension, a 15 min irradiation of ultrasound (50 kHz, 120 W) by a laboratory cleaner was sufficient. The four times repetition of extractions were the optimal, since the amount of the LPS did not increase when the extractions were repeated more than 5 times. The amount of extracted LPS was 2.25 g, the yield of LPS

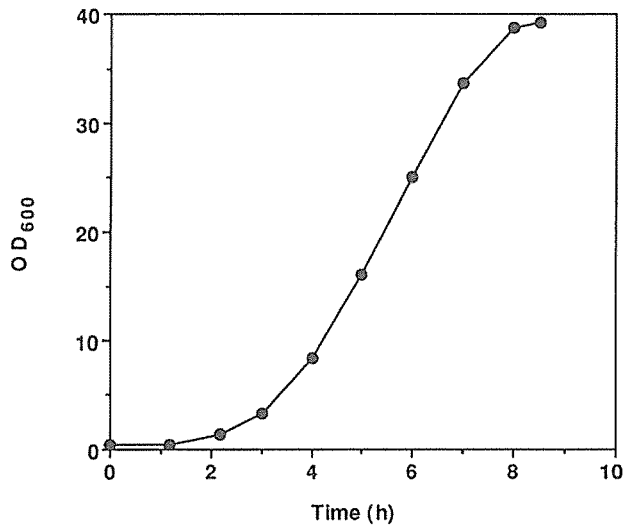

Fig. 1. Growth curve of *E.coli* C.
Culture conditions: One litter medium contained D-glucose (40g), $(NH_4)_2SO_4$ (10g), yeast extract (5g), $MgSO_4$ (0.6g), KH_2PO_4 (0.5g), K_2HPO_4 (1.0g), NaCl (3g), cultivated in 6L quantity, pH 6.8, 37℃ 8.5 h, 70% aeration, 500 rpm stirring.

was 3.0 % wt from the dried and defatted cells. Scherecht *et al.*[19] reported that the yield of LPS did not increase when *Salmonella* sp.s were cultivated in a D-glucose-rich medium (3 % wt/v) for elevation of biomass. The present LPS's yield, 3.0 % wt from the dried and defatted cells, was slightly larger than that of our previous condition described above (2-2.5 % wt).

Some papers reported that the culture conditions, nutrients,[20-22] or aeration,[23-25] altered quantity and quality of LPSs of gram negative bacteria. For example, the LPS derived from the cells grown at high cultivation temperature (37 ℃) had low inactivating activity for some phages than that grown at low temperature (25 ℃),[26,27] and the extracted LPS was next examined whether it has a desired ϕ X174-inactivating activity.

ϕ X174 undergoes an eclipse reaction to release its DNA when it contacts *in vitro* with the isolated receptor, LPS.[8] Once phage released its DNA, it loses the infectivity for the host cells. Hence, the extracted LPS is expected to deprive the infectivity of ϕX174 to the host cell, if the LPS could be recognized by ϕ X174 as a receptor. According to the reported procedures,[8,9,10] the receptor activity of LPS for ϕ X174 was determined by monitoring the loss of phage infectivity after 1 h treatment at 37 ℃ in the presence of various concentraion of LPS (10^{-4}, 10^{-3}, and 0 mg/mL). The Fig. 2. describes the relationship between LPS concentration and the residual infectivity of ϕX174. The survival ratio of phage decreased when the concentration of LPS (from 10^{-4} to 10^{-3} mg/mL) was increased. When the LPS extracted from the cells grown at a D-glucose-rich medium was compared with the LPS from the cells

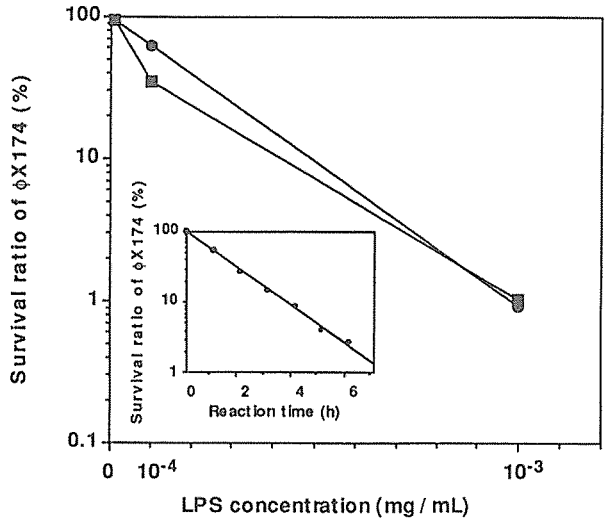

Fig. 2. Receptor activity of the extracted LPS from the cells grown at different media.
● the extracted LPS from the cell grown at a D-glucose-rich medium (4 %wt/v)
■ the extracted LPS from the cell grown at a D-glucose-poor medium (0.2%wt/v)
Conditions: ϕ X174 (3.54×10^9 p. f. u. /mL): 10 μ L, LPS solutions (10^{-3},10^{-2}, and 0 mg/mL) : 100 μ L, 0.01M Tris-HCl buffer [pH 8.1, containing KCl (5 % wt/v), NaCl (1 % wt/v), $CaCl_2$ (0.01 % wt/v)]: 890 μ l, 37℃, 1 h incubation. The survived phages were counted by the agar overlayer method by Adams[11].
Inset: Time dependence of inactivation of ϕX174 by the extracted LPS (10^{-4} mg/mL) from the cell grown at a D-glucose-rich medium.

grown at a D-glucose-poor medium, both LPSs showed the inactivation of the phage in quite similar manner. Inset of Fig. 2. shows the time dependence on inactivation of phages by the extracted LPS (10^{-4} mg/mL) from the cell grown at a D-glucose-rich medium. The degree of inactivation obeys typical first order kinetics.[8] From these results, both LPSs extracted from the cells grown at different media were estimated equivalent with a receptor of ϕX174.

In summary, we established the conventional method for a large scale preparation of LPS from *E.coli* C strain with receptor activity of bacteriophage ϕX174. The present method would be of advantage as a primitive tool of the research of the recognition mechanism between ϕX174 and the receptor LPS.

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バクテリオファージφX174レセプター活性を有する大腸菌C株リポ多糖の簡便大量調製法

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バクテリオファージφX174のレセプターとしての生理活性を有するリポ多糖を大腸菌C株の乾燥脱脂菌体から簡便に大量調製する方法を確立した。培養条件を改良することによって炭素源としてグルコース4%wt／v，窒素源として硫安1%wt／vを含む6Lの培養液から75gの乾燥脱脂菌体（湿菌体で309g）を得ることが出来た。得られた菌体からPCP抽出改良法によってリポ多糖を抽出したところ，2.25gのリポ多糖が得られた（菌体重量の3.0%収率）。得られたリポ多糖がバクテリオファージφX174のレセプターとしての生理活性を有し，*in vitro*でのファージ不活化能力を示すことがプラーク計数法で確認された。