Technical Bulletin

Protocols and Characteristics of Lipopolysaccharide (LPS)

Studies related to LPS

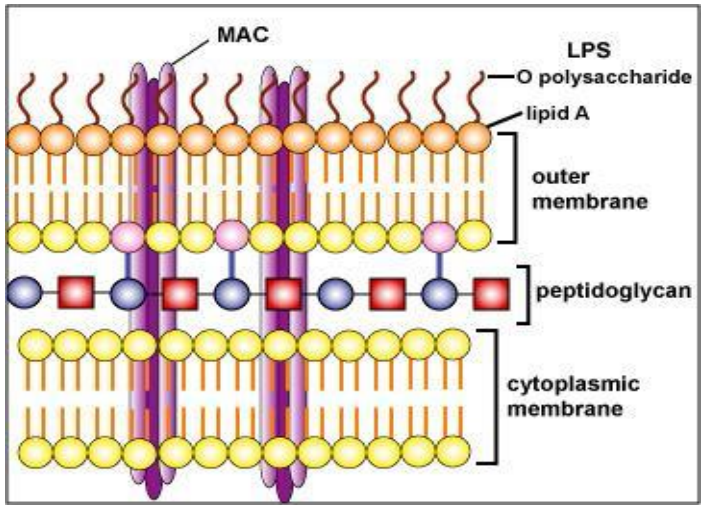
Immune reactions by infection of microorganism are categorized into the innate immunity and the acquired immunity. Although the acquired immunity has high specificity to antigen and can remove the microorganisms infected efficiently, it shows a disadvantage on that it needs much time to operate properly. On the other hand, the innate immunity is able to remove the microorganisms infected by recognizing the pathogen-associated molecular patterns (PAMPs) in the early stage of infection and activate the adaptive immune system through a process known as antigen presentation at the same time. Because the innate immunity can be applied to treatment of genetic diseases, development of medicines, and general foods. Recently NIH has invested 30% of the total amount of research funds and its importance has been increased.

One of the most important mechanisms of the innate immunity is to recognize the pathogens infected into the body. The reason is that it needs to make different reactions inside of the cells in accordance with the kinds of pathogen. These cells present receptors, contained on the surface or within the cell. The immune cell activates the genes required to operate the immune signal delivery system against the pathogen when a specific protein of the pathogen binds to the receptors. These cells undergo activation and release inflammatory mediators responsible for the clinical signs of inflammation.

In this case, which of the genes are activated in immune cells and how can the genes operate the system of signal delivery? We induced an immune response after injection of Lipopolysccharide(LPS) into a drosophila, which is only specifically existing on the cell walls of such bacteria to see how it works and observed the changes of the genes. Microarray technology is used for making the observation of changes in millions of genes that the drosophila has. Finding mutated gene expression and making mutated drosophila, innate immunity of drosophila has been studied in comparison with not mutated drosophila's characteristics. One of the recent studies demonstrated a mechanism of the immunoregulation preventing from septicemia or atopy by controlling overexpression and the principle causing cancer cells from excessive infection.

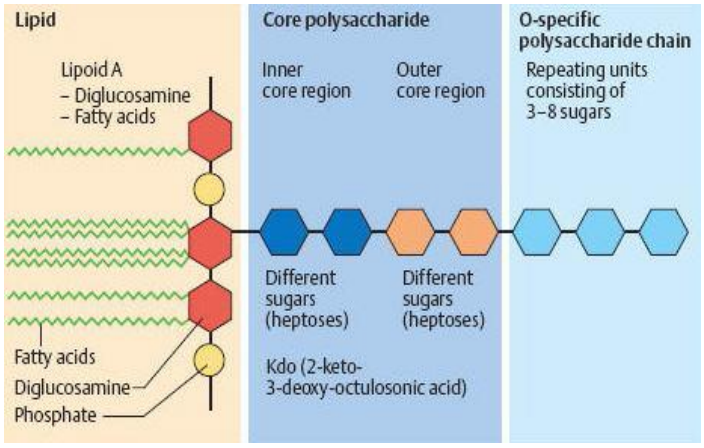
Moreover, when it became public knowledge that LPS plays a vital role in causing Septicemia that may cause a critical damage to various organs to lead up to death of the patient by highly toxic inflammatory factors which are hypersecreted from overexpression of the immune cells in case of serious bacterial infection, the importance to study a clear action mechanism has been emphasized.
Laboratory studies have successfully demonstrated therapeutic effects when administering LPS, or synthetic lipid A molecule, including inhibition of tumor size and growth (Andreani et al. 2007; Chicoine et al. 2001; Kuramitsu et al. 1997; Morita et al. 1996). Morita et al. (1996). Study related to LPS is expected to use for the development of a next-generation medicine with regard to cancer or immunosuppressant drug by using reciprocal control mechanism of the signal delivery system. Also, LPS of Gram-negative bacteria as a functional material in many foods. Orally or dermally administered LPS has positive effects such as being antidiabetic, anti-allergic, anti-dyslipidemia and anti-tumor effects.

LPS Structure



Bacteria have the original prokaryotic characteristics and do not have the structure such as nuclear membrane, mitochondria, or chlorophyll. The cell wall is the most remarkable part of the bacteria's structure in perspective of immunology and the cell wall maintains the size and the form of bacteria and protects rupture of the cell from osmotic pressure. the cell wall's structure of gram negative bacteria is comprised of O-antigen, LPS, periplasm, and peptidoglycan.

The lipopolysaccharide complex



Lipopolysaccharide is comprised of lipid A, core polysaccharide, and O-side chain. Lipid A stabilizes the structure of membrane functions as a poisonous factor. O-side chain is located in the outer area of antigen and Core polysaccharide includes oligosaccharide directly adhered to the lipid.

Purpose of LPS(Lipopolysaccharide) Study

LPS is expected to be a new target of antibiotic. Because Lipopolysaccharide helps bacteria avoid immune system and recognize each other. Understanding of the process of LPS appearance is required to design a medicine inhibiting LPS. The immune response study results of microorganic antigenic carbohydrate obtained from LPS and LTA may be able to contribute to developing new drug, preventing from bacterial infection, and innovating therapeutic methods.

What is LPS?

Lipopolysaccharides (LPS), also known as lipoglycans and endotoxin, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, and elicit strong immune responses in animals. LPS is well-known for its important roles in growth or survival of bacteria and especially for its interactions between the host and the parasite. When bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhea, and possible fatal endotoxic shock.

Functions of LPS

LPS is the major component of the outer membrane of Gram-negative bacteria, contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. LPS also increases the negative charge of the cell membrane and helps stabilize the overall membrane structure. It is of crucial importance to gram-negative bacteria, whose death results if it is mutated or removed. LPS induces a strong response from normal animal immune systems. It has also been implicated in non-pathogenic aspects of bacterial ecology, including surface adhesion, bacteriophage sensitivity, and interactions with predators such as amoebae.

What is Endotoxin?

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxin is invariably associated with Gram-negative bacteria whether the organisms are pathogenic or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, in bacteriology it is properly reserved to refer to the lipopolysaccharide complex associated with the outer membrane of Gram-negative pathogens such as Escherichia coli, Salmonella, Shigella, Pseudomonas, Neisseria, Haemophilus influenzae, Bordetella pertussis and Vibrio cholerae. Endotoxins represent the pyrogens (fever-inducing agents) of gram-negative bacteria, pyrogenicity being probably the first activity of endotoxin recognized. Endotoxins are endowed with a large spectrum of biological activities that may be demonstrated *in vivo* and *in vitro*. Of these, pyrogenicity, leukopenia and leukocytosis, induction of the local, generalized Schwartzman phenomenon, and lethal shock are examples of classical endotoxic activities.

LPS Extraction

Although hot phenol extraction and petroleum/chloroform/phenol extraction methods are widely used in LPS extraction, there are such disadvantages as the complicated protocol or much time taken to be extracted. iNtRON's LPS Extraction Kit enables to extract LPS from bacteria easily and efficiently in base of the phenol-water extraction method.

Major characteristics of the LPS Kit

1) The most efficient and easiest way of LPS extraction
2) Can be done within 60 min.

Protocol

Note:

- The most efficient volume of LPS to maximize its yield is when 2~5ml of cultured cells were used. Culture the bacterial cells with OD600 value between 0.8-1.2.

- Use proteinase K solution in case that much protein was mixed with the extracted LPS

1) Centrifuge 5ml of bacterial cells at 13,000rpm for 30 second

2) Add 1 ml of Lysis Buffer and vortex vigorously.
Note : To improve lysis of bacterial cells, vortex vigorously until the cell clump disappeared.

3) After adding 200ml of chloroform, vortex it vigorously for 10-20 sec. And incubate it at room temperature for 5min.

4) Centrifuge at 13,000rpm for 10min at 4℃. Transfer 400ml of supernatant to new 1.5 ml tube.

5) Add 800ml of Purification Buffer and mix well. Incubate for 10 min at -20℃.

6) After centrifuging the solution at 13,000rpm for 15 min at4℃, remove the upper layer to obtain LPS pellet.

7) Add 1ml of 70% EtOH and wash the LPS pellet by inverting the tube 2-3 times. Centrifuge the mixtures for 3min at 13,000rpm at 4℃. Discard the upper layer and dry the remaining LPS pellet.

Note: This is a washing stage to remove impurities such as salts and etc. Dry the pellet at RT.

8) Add 30–50ml of 10mM Tris-HClbuffer(pH8.0) to LPS pellet and vortex or pipet it. And dissolve completely the LPS by boiling it for 2 min.

Cell culture volume(OD600=1.0)	2㎖(10^9cells)
Yield of LPS	30㎍
Amount of Proteinase K	75㎍(2.5㎕of 30㎎/ ml PK)

Technical data

• The LPS Extraction Yields

The extracted LPS yields were compared with commercial control LPS(1mg/ml),Sigma. In this experiment, LPS were separately extracted from *E.Coli*(2ml, OD_{600}=1.0, Gram negative pellet and LB broth. Approximately 30ug of LPS were extracted from pellet.

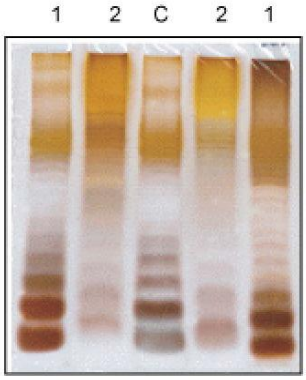


Fig1. Comparison between the extracted LPS and the control LPS (commercial, Sigma)
Lane1: E.coli pellet
Lane2: E.coli incubated LB media
LaneC: Control Sigma LPS(1mg/ml)

• The LPS Patterns among diverse bacteria

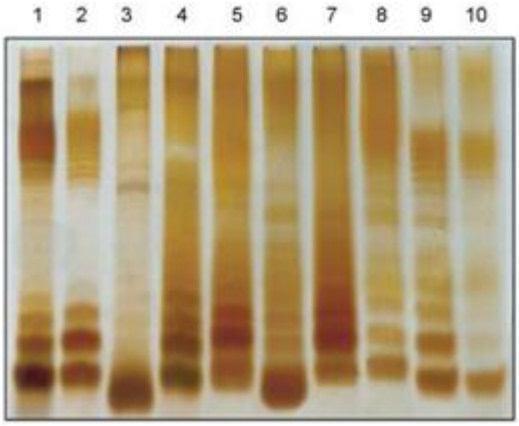


Fig2. LPS Extraction from diverse bacteria species
LPS were extracted from diverse bacteria using LPS Extraction kit, then confirmed with SDS-PAGE after silver staining. LPS Extraction kit is able to use for both the extraction of LPS and LPS pattern among diverse bacteria species.

Lane1: *S. typhimurium*
Lane2: *S. enteritidis*
Lane3: *E.coli (O:055)*
Lane4: *E.coli (O:111)*
Lane5: S.*gallinarum*
Lane6: *S.enteritidis*
Lane7: *S. typhimurium*
Lane8: *E.coli(wild type)*
Lane9: *E.coli (O:1)*
Lane10: *E.coli(O:2)*

Product	Size	Cat.No.
LPS(Lipopolysaccharide)Extraction kit	100rxn	17141

Contents	Size
Lysis buffer	100ml
Purification buffer	80ml

■ iNtRON's silver staining protocol for staining of LPS extracted from bacterial cells

- 10' Stock solution & 1' Working solution

	10' Stock Solution	1'working Solution
Oxidation Solution	Periodic acid: 12g Acetic acid: 100ml DW: 100ml	(200ml) 10'Stock solution: 20ml EtOH: 80ml DW: 100ml
Staining Solution	1N NaOH: 28ml Ammonium Hyhdroxide Solution: 20ml DW: 102ml	(150ml) 10' Stock solution: 15ml 20% Silver Nitrates: 5ml DW: 130ml
Developing Solution	Citric acid: 0.5g DW: 200ml	(200ml) 10'Stock solution: 20ml 37% Formaldehyde: 0.5ml DW: 180ml

- Other solution required

	1'working solution
Fixing Solution	40% ethanol + 5% acetic acid
Staining solution	40% ethanol + 10% acetic acid

Protocol

1. After the gel has been run, fix the protein by incubating the gel slab in the fixing solution for 5 min.

2. Discard the fixing solution, and rinse the gel slab with DW (2 Changes, two minutes per change).

3. Discard DW, and incubate the gel in oxidation solution for 20min at room temperature with gentle agitation.

4. Discard the oxidation solution, and rinse the gel with two changes of DW (10 seconds each: Agitate gently to make sure that the gel slab is covered evenly).

5. Incubate the gel in DW for 30 min at room temperature.

6. Replace DW with the staining solution.

7. Incubate the gel in staining solution for 10 min at room temperature.

8. Discard the staining solution, and rinse the gel several times with DW.

9. Incubate the gel in DW for 15 min at room temperature.

10. Replace DW with developing solution.

11. Incubate until desired contrast is obtained.

12. Quench the reaction by washing the gel with stop solution for a few minutes.

13. Wash the gel several times with DW.

14. Dry or scan the gel.

Et cetera

- Generally 2ml of cultured cells were used to extract LPS. Culture the bacterial cells with OD600 value between 0.8-1.2.
- Use proteinase K solution in case that much protein was mixed with the extracted LPS

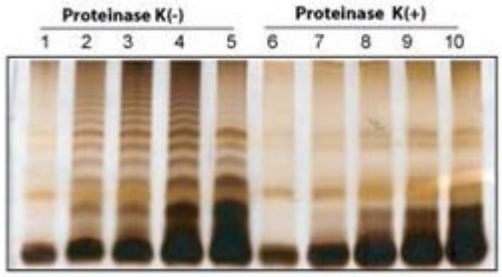


Fig2. Proteinase K treatment after LPS extraction

(1) Generally LPS is composed of lipids, polysaccharides and proteins. The LPS pattern were changed after Proteinase K treatment.
(2) For this experiment, salmonella (SR2N6) were used in the table described below, and Proteinase K were treated with the concentration of 70ug/30ul.
(3) The patterns of extracted LPS from SR2N6 were changed by Proteinase K treatment (The ladder shapes, Proteins, were disappeared.

No.	Proteinase K (-)	No.	Proteinase K (+)
1	1ml	6	1ml
2	2ml	7	2ml
3	3ml	8	3ml
4	4ml	9	4ml
5	5ml	10	5ml

Product	Size	Cat.No.
LPS(Lipopolysaccharide)Extraction kit	100rxn	17141

Limulus amoebocyte lysate (LAL) assay

Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells and it reacts with bacterial endotoxin or lipopolysaccharide (LPS), The limulus amebocyte lysate (LAL) has been widely used for over 30 years for the detection of endotoxin in the quality assurance of injectable drugs and medical devices. The LAL constitutes a cascade of serine proteases which are triggered by trace levels of endotoxin, culminating in a gel clot at the end of the reaction. The Factor C, which normally exists as a zymogen, is the primer of this coagulation cascade. In vivo, Factor C is the perfect biosensor, which alerts the horseshoe crab of the presence of a Gram-negative invader. There are three basic LAL test methodologies: gel-clot, turbidimetric, and chromogenic. The primary application for LAL is the testing of parenteral pharmaceuticals and medical devices that contact blood or cerebrospinal fluid.

Literature for LPS kit

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