*J. Life Science.* (2001), 11(1), 27~29

JOURNAL OF
Life Science
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# Chemical Analysis and Biological Activity of Endotoxin from *Vibrio vulnificus*

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Received: March 22, 2001

**Abstract** *Vibrio vulnificus* endotoxin was extracted, analyzed the chemical composition, tested its biological activity, and compared to those of *Escherichia coli* and *Salmonella typhimurium*. The major fatty acid of three endotoxins were different each other; *V. vulnificus* endotoxin was myristic acid (C14:0), *E. coli* was lauric acid (C12:0), *S. typhimurium* was capric acid (C10:0). The biological activities of *V. vulnificus* endotoxin were similar to those of *E. coli* and *S. typhimurium* in terms of the gelation activity of the *Limulus* amebocyte lysate and the lethal toxicity. But the result of enzyme (AST, ALT, and LDH) analysis showed that the enzyme activity of *V. vulnificus* endotoxin was similar to that of *E. coli*, but smaller than that of *S. typhimurium*.

**Key words**: *endotoxin, chemical composition, biological activity*

## Introduction

Endotoxin is a prominent macromolecular component of the outer membrane of gram-negative bacteria[1,2]. Structural investigation of endotoxin shows that it is composed of a polysaccharide chain with repeating oligosaccharide units of variable length and constitutes the O-antigen region. The latter is connected to the core polysaccharide chain which is relatively invariant within certain classes of bacteria and it is linked to lipid A. Lipid A is composed of several fatty acids and lauric, myristic, palmitic acid are the major ones for almost all of gram-negative bacteria's endotoxin [3,4]. Endotoxin is known to have broad spectra of biochemical and immunological activities(lethal toxicity, pyrogenicity, and antitumor activity, *et al.*) and they depend on the fatty acid composition [2].

*Vibrio vulnificus*, a halophilic bacterium, is perhaps the most invasive of Vibrio species. Unlike other Vibrios, it is associated with a high incidence of septicema correlated with a high degree of mortality [5,6]. The clinical features of *V. vulnificus* are fever, hypotension, abdominal pain, and cutaneous lesion on extremities. Considering the high incidence of hepatic disease and the unrarity of the infection in Korea [7], clinicians should be aware of the disease and microbiologists be able to identify the organism.

In this study *V. vulnificus* endotoxin was isolated by the hot phenol-water method, its fatty acid composition was determined, and its biological activity was tested. Furthermore these results were compared with those of *E. coli* and *S. typhimurium* endotoxin.

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## Materials and Methods

### Bacterial strain and culture condition

*E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 were grown in 10 L of tryptic soy broth (TSB) at 37℃ for 24 h. *V. vulnificus* P-1 was grown in 10L of TSB which contained additional 5 g of NaCl.

### Extraction of endotoxin

Endotoxins of *E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, and *V. vulnificus* P-1 were extracted by the hot phenol-water extraction method as described by Westphal and Jann[8]. Washed and acetone dried cells were extracted with 45% phenol at 70℃. The water phase containing endotoxin was dialyzed against distilled water and freeze-dried.

### Fatty acid analysis of endotoxin

Endotoxin samples were hydrolyzed for 6 h at 100℃ in 2 N HCl. The solution was suspended in chloroform, methanol, and water solvent(4:10:5, v/v). The dried chloroform phase was acidified with 2 N HCl and was added 10% $BF_3$-MeOH. The solution was gently mixed and heated for 30 min in a water bath at 100℃, then was cooled to room temperature. The fatty acid methyl esters were extracted from the aqueous phase by using n-hexane and separated in a GC, equipped with a glass column (3 mm by 3 m) of 10% 1,4-butanediol succinate on Chromosorb W (60-80 mesh) at 185℃ [9].

### *Limulus* amebocyte lysate gelation activity of endotoxin

Equal amount of endotoxin and *Limulus* amebocyte lysate were mixed in test tube and incubated for 1h at 37°C. At the end of the incubation period, gelation was determined by carefully inverting the test tube. Gelation was employed as a sensitive *in vitro* assay for endotoxin. Formation of a hard gel was judged as positive response. Activity was expressed as the lowest concentration of each endotoxin needed to form a hard gel.

### Lethal toxicity of endotoxin

Endotoxin in 0.5ml of pyrogen-free saline was injected intravenously into mouse. Deaths of mouse due to intoxication were recorded daily for 72 h and $LD_{50}$ (the amount of endotoxin required to kill 50% of the experimental animals) was determined [10].

### Response of enzyme by endotoxin

For the determinations of responses of enzymes in blood induced by endotoxin, 2 mg of endotoxin from *V. vulnificus*, *E. coli*, and *S. typhimurium* were injected intravenously into rabbit. After 2, 4, 6, 8, 12, and 24 h, 2.0 ml of blood were collected and analyzed. The activity changes of alanine aminotrasferase (AST), aspartate aminotransferase (ALT), and lactate dehydro-genase (LDH) were observed by the Gilford diagnostics method.

## Results and Discussions

### Fatty acid analysis of endotoxin

The overall fatty acid compositions of endotoxins showed that the major fatty acid component for each endotoxin was myristic acid (C14:0) for *V. vulnificus*, lauric acid (C12:0) for *E. coli* and capric acid (C10:0) for *S. typhimurium* (Table 1). It was noticeable that myristic acid was the major component for *V. vulnificus* endotoxin and palmitic acid was in small amounts (below 2%) in all endotoxins.

**Table 1.** Fatty acid compositions of *V. vulnificus*, *E. coli*, and *S. typhimurium* endotoxin

| fatty acid | % of fatty acid from endotoxin | | |
|---|---|---|---|
| | *V. vulnificus* | *E. coli* | *S. typhimurium* |
| C 8:0 | 7.62 | 9.59 | 6.34 |
| C10:0 | 24.79 | 31.81 | 48.60 |
| C12:0 | 19.77 | 37.03 | 24.99 |
| C14:0 | 41.37 | 6.21 | 2.59 |
| C16:0 | 1.13 | – | 1.84 |
| C17:0 | 4.38 | 11.65 | 9.13 |
| C18:0 | – | 2.92 | 0.26 |
| C18:1 | 0.94 | 0.79 | – |
| C18:2 | – | – | 1.06 |
| C18:3 | – | – | 5.19 |

### *Limulus* gelation activity and lethal toxicity of endotoxin

The *Limulus* gelation activity of *V. vulnificus* endotoxin was found to be comparable to *E. coli* and *S. typhimurium* endotoxin (activity was the same value, 0.1 ng/ml) and none of the *E. coli*, *S. typhimurium*, and *V.vulnificus* endotoxin was lethal in doses of up to 400 $\mu$g per mouse.

### Response of enzyme by endotoxin

The activities of three enzymes (AST, ALT, LDH) in blood were assayed and the results were shown in Table 2. ALT showed a greater activity (increased by 25.21% when compared with that of normal blood) than AST (13.45%) and LDH (12.43%), probably due to liver cell damage by endotoxin. *S. typhimurium* endotoxin induced a greater AST activity (increased by 17.54%) than *E. coli* endotoxin (10.53 %) and *V. vulnificus* endotoxin (12.28%) but the others were similar.

Moreno *et al.* reported that the unique fatty acid compo-

**Table 2.** Changes of enzyme activity by *E. coli*, *S. typhimurium*, and *V. vulnificus* endotoxin

| blood collection | *E. coli* | | | *S. typhimurium* | | | *V. vulnificus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | AST (U/L) | ALT (U/L) | LDH (U/mL) | AST | ALT | LDH | AST | ALT | LDH |
| control[a)] | 19 | 26 | 241 | 19 | 26 | 240 | 19 | 26 | 243 |
| 2h[b)] | 20 | 29 | 245 | 19 | 28 | 250 | 19 | 29 | 256 |
| 4h | 21 | 31 | 258 | 21 | 30 | 256 | 21 | 36 | 260 |
| 6h | 22 | 34 | 280 | 24 | 35 | 286 | 22 | 32 | 283 |
| 8h | 20 | 32 | 273 | 22 | 33 | 270 | 21 | 30 | 276 |
| 12h | 19 | 28 | 251 | 21 | 29 | 250 | 20 | 28 | 252 |
| 24h | 17 | 26 | 242 | 20 | 27 | 240 | 18 | 27 | 241 |
| increase(%)[c)] | 10.53 | 24.36 | 12.17 | 17.54 | 25.64 | 12.77 | 12.28 | 25.64 | 12.34 |

[a)]This was normal blood from rabbit.

[b)]The blood was drawn from rabbit after endotoxin treatment.

[c)]This was calculated by the following equation.

$$\text{Increase } (\%) = \frac{A - B}{B} \times 100$$

A : Enzyme activity or the number of WBC after 2 to 24 h

B : Enzyme activity or the number of WBC at control

sition of the Brucella endotoxin was probably the major factor responsible for some of the distinctive biological activities of Brucella endotoxin in the study of biological activities of Brucella abortus endotoxin [11]. Helander et al. reported that the low biological activity of Agrobacterium sp. endotoxin may be due to its different chemical (fatty acid) composition [12]. Therefore it may be said that the biological activities of endotoxin depend on its fatty acid composition. From the fatty acid analysis of *E. coli, S. typhimurium*, and *V. vulnificus* endotoxin (Table 1), *V. vulnificus* endotoxin was expected to have the strongest activity among three endotoxins because the chain of myristic acid was the longest. But the activities of *V. vulnificus* endotoxin were not the strongest, rather similar to those of *E. coli* and *S. typhimurium* endotoxin in terms of the gelation activity of *Limulus* amebocyte lysate and the lethal toxicity. This indicated that the fatty acid composition did not affect significantly on the activities of three endotoxins.

The results of the responses of enzymes in blood were shown in Table 2. ALT showed a greater activity than AST and LDH. Liver could normally detoxicate a small amount of endotoxin, which was injected into the host, within 2 h (above 90%). But it could not do if excess endotoxin was injected, rather showed various toxic effects to the host. Therefore endotoxin was thought to induce a necrosis in liver. Of course *V. vulnificus* endotoxin had necrotic effect and this was similar to *E. coli* endotoxin but smaller than *S. typhimurium* endotoxin.

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