한국환경과학회지 제5권(제5호), 555~560, 1996
J. of the Korean Environmental Sciences Society

Analysis of Toxic Substance (Endotoxin) by Gas Chromatography

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(Manuscript received 15 June 1996)

The toxic substances (endotoxins) from the bacterial cell walls were extracted by using incubator, centrifuge, UV-Vis spectrophotometer, and their fatty acid compositions were analyzed by Gas Chromatography. The lethal toxicities and pyrogenic activities of toxic substances were tested and the results were compared each other.

The results of fatty acid analyses showed that the major fatty acid of the toxic substance was tetradecanoic acid for *Vibrio vulnificus*, dodecanoic acid for *Escherichia coli*, and decanoic acid for *Salmonella typhimurium*. These three fatty acids were the main fatty acids for three toxic substances (more than 70%). The unique points in the fatty acid compositions were that tetradecanoic acid was composed as important one (37.15%) for *V. vulnificus* and that the amount of hexadecanoic acid was very small (below 2%) for three toxic substances. The lethal toxicity in ICR mice of toxic substance from *V. vulnificus* (LD_{50} was 52.5 mg/kg) was similar to that of *E. coli* (56.5 mg/kg), but weaker than that of *S. typhimurium* (37.5 mg/kg). Toxic substance from *V. vulnificus* was more pyrogenic in rabbit than that from *E. coli*, but less than that from *S. typhimurium*.

Key words : toxic substance (endotoxin), UV-Vis spectrophotometer, fatty acid, Gas Chromatography

1. INTRODUCTION

Mammals infected with gram-negative bacteria often develop a state of shock, which is characterized by hypotension, disseminated intravascular coagulation, renal, hepatic, and cerebral injury. Many of these deleterious consequences of infection can be reproduced in animal by injecting toxic substance (endotoxin), a component of the cell wall of gram-negative bacteria. While the action mechanism of endotoxin remains obscure, it is believed that the toxic effects are mediated by factors produced by host cells. Adoptive transfer experiments with endotoxin-resistant and endotoxin-sensitive congenic mice have implicated cells of hematopoietic origin and in particular monocytes, as the source of these mediators.

The toxic substance, a derivative of phosphorylated glucosamine disaccharide, carries long-chain fatty acids in both ester and amide linkage which appear to play a role in the expression of endotoxic activity. Of the acyl residues present in *Salmonella* approximately one each of dodecanoic, hexadecanoic, 3-hydroxytetradecanoic, and 3-O-(tetradecanoyl)-tetradecanoic acid is involved in ester and two of 3-hydroxytetradecanoic acid in amide linkage. According to our present knowledge, *Salmonella* endotoxin backbone provides three hydroxyl groups which can serve as attachment sites for ester bound fatty acids. The fact, however, that approximately four ester-linked acyl groups are present suggests that at least one of them is not bound to hydroxyl group of the disaccharide backbone. Other possible linkage sites of ester-bound acyl groups are the hydroxyl functional groups of amide-linked hydroxy fatty acids. The fatty acid, an essential component for the expression of toxicity, composition of the toxic substance (endotoxin) in the bacterial cell wall was analyzed by gas chroma-

tography in this study and the lethal toxicity of that was reported in this paper.

2. Materials and Methods

2.1 Instruments and Chemicals

UV-Vis spectrophotometer, incubator, sonic dismembrator model 300, and bench top model 3 freeze dryer with vacuum pump were used for the preparation of toxic substance. Gas chromatography GC-RIA with 10 m×3 mm column of 10% 1,4-butanediol succinate (BDS) on Chromosorb W was used for the analysis of fatty acid composition from toxic substance.

Phenol, tris buffer, RNAse, fatty acid standards, and BF_3 in MeOH were purchased from Sigma Chemical Co. NaCl, K_2HPO_4, EtOH, MeOH, EDTA, diethyl ether, Na_2SO_4, and pentane were special grade reagents.

2.2 Bacterial strains and culture conditions

Cells of *Vibrio vulnificus, Escherichia coli,* and *Salmonella typhimurium* were grown in tryptic soy broth which supplemented with 5 g of NaCl, 17 g of bacto-trypton, 3 g of bacto-soyton, 2.5 g of bacto-dextrose, and 2.5 g of K_2HPO_4/liter at 42℃ for 24 h. Maximal accumulation of toxic substance was occurred when a log phase culture grown at 30℃ to an absorbance at 600 nm of 0.2~0.4 was shifted to 42℃ for 4 h. This procedure was used whether we grew small shaker cultures or large cultures (20 liters) in the incubator. At the time of the harvest, the $A_{600\,nm}$ was 0.8. The cells were harvested by centrifugation. The cell pellet was washed with water and stored at −80℃.

2.3 Preparation of toxic substance

The toxic substance was originally isolated as a contaminant of RNA preparation. Its presence was recognized by the high amount of orcinol-reactive material in proportion to UV absorbance. Until the substance was identified as a cell wall component, it was extracted from whole cells with cold phenol. The bacterial cells were separated from the growth medium by centrifugation and stored frozen. Thawed cells were mixed with an equal volume of 0.05 M Tris buffer, pH 7 to 8. After 3 min of sonic oscillation in a sonic oscillator, the broken cells were centrifuged for 1 h at 4,000× g. The precipitate was discarded and the supernatant solution was centrifuged for 1 h at 20,000× g. The precipitated cell wall fraction was suspended in 0.005 M Tris buffer, pH 8.0. Distilled water was added to make 45% phenol solution. This mixture was shaken at 68~72℃ for 30 min and cooled in ice. Centrifugation of this material in the cold state allowed separation of several phases. The upper aqueous phase was carefully removed with a pipette. Early preparations involved cold phenol extraction of whole cells.

The supernatant solution from the aqueous phenol treatment was acidified at room temperature with 0.1 M acetic acid to pH 5.0 and with 0.1M HCl to pH 2.2. The precipitate, chiefly nucleic acid, was removed from the solution by centrifugation and the solution was neutralized with NaOH. Five volumes of 95% ethanol were added slowly at 0℃ with stirring and the solution was refrigerated for several hours, or preferably overnight. Centrifugation yielded a clear solution which was discarded a precipitate which was dissolved in a minimal amount of 0.05 M Tris buffer at pH 8.0. This viscous solution was kept as a stock in the freezer. Aliquots were subsequently diluted with 0.05 M Tris buffer and incubated with 1 μg of RNAse per ml at 37℃ for 1 h with shaking. This preparation was cooled and dialyzed at 4℃ for a minimal period of 2 days with at least four changes of buffer. The amount of nucleic acid which contaminated various preparations was estimated by assuming that the absorbance at 260 nm measured by UV-spectrophotometer was all due to nucleic acid. The average amount found was 0.0036 g of nucleic acid per g of toxic substance.

2.4 Liberation of fatty acid from toxic substance

Toxic substance was treated with 0.5 ml of 4 N KOH in sealed tubes at 100℃ for 2 h. Kinetic anal-

yses indicated that the release of all fatty acids from the toxic substance was maximal within this time period. The hydrolysates were acidified with 0.5 ml of 6 N HCl and extracted three times with 1.5 ml of diethyl ether. The extracts were then evaporated to dryness under a stream of nitrogen. Hydrolysis was also carried out with 4 N HCl for 2 h at 100℃ in sealed tubes.

2.5 Gas chromatographic analysis of fatty acid from toxic substance

Fatty acids were analyzed as their methyl esters. Methyl esters of fatty acids were prepared by incubating samples in 1.0 ml of BF_3 in methanol (14%, w/v) at 70℃ for 10 min in a sealed tube. The solutions were allowed to cool and 4.0 ml of a saturated solution of NaCl and the samples were mixed throughly. The upper phases were removed and dried over 0.5 g of Na_2SO_4 (2 : 1, w/w). The aqueous phases were then taken to dryness at room temperature under a stream of nitrogen and the derivatives were dissolved in minimal volume of pentane. Analyses were carried out using Gas Chromatography, fitted with 10 m×3 mm column of 10% 1,4-butanediol succinate (BDS) on Chromosorb W (60~80 mesh) using nitrogen as the carrier gas at a flow rate of 10 ml/min.

2.6 Measurement of lethal toxicity for toxic substance

Measurement of lethal toxicity of the toxic substance was performed with specific pathogen-free ICR mouse. Females aged 7 weeks and weighing 22 to 25 g were used. Toxic substance was dissolved in sterile saline and sonicated for 3 min, and serial dilutions were made in saline. Groups of 6 mice each were inoculated intravenously with 0.2 ml of the dilutions, and deaths due to intoxication were recorded daily. All deaths were occurred within 3 days from the injection. Values for 50% lethal doses (LD_{50}) were calculated from mortalities by the maximum likeli-hood method.

2.7 Measurement of pyrogenic activity for toxic substance

The pyrogenic activity of toxic substance was determined by adapting following procedure. Each toxic substance was diluted into 10 ml of rabbit serum and incubated at 37℃ for 20 min. This procedure increased pyrogenic activity by a factor of approximately of 3. The augmented toxic substances were divided into aliquots of 1 ml each and stored at −20℃, until required. Assay injections of each toxic substance were made after dilution of the thawed samples, so that the desired amount of toxic substance was contained in 2 ml of 0.15 M saline. Rabbits for assay were immobilized and their temperatures recorded for 2 h before injections were given. The febrile response was measured as the difference between the mean baseline temperature and the highest temperature reached during the first 2 h after injection.

3. Results and Discussion

3.1 Fatty acid composition of toxic substance

The gas chromatograms of the fatty acid methyl esters of standard mixture, toxic substances of *V. vulnificus, E. coli*, and *S. typhimurium* were shown in Fig. 1, 2, 3, and 4.

The fatty acid compositions of three toxic substances were summarized in Table 1. The major fatty acid was tetradecanoic acid (C 14 : 0) for toxic substance from *V. vulnificus*, dodecanoic acid (C 12 : 0) for *E. coli*, and decanoic acid (C 10 : 0) for *S. typhimurium*. These three fatty acids were the most abundant fatty acids for three toxic substances (more than 70%). The unique points in the fatty acid compositions were that tetradecanoic acid (C 14 : 0) was composed as important one (37.15 %) for *V. vulnificus* and that the amount of hexadecanoic acid (C 16 : 0) was very small (below 2 %) for three toxic substances.

3.2 Lethal toxicity of toxic substance

The lethal toxicity in ICR mice of toxic substance from *V. vulnificus* (LD_{50} was 52.5 mg/kg) was similar to that of E. coli, but weaker than that of *S. typhimurium* (56.5 and 37.5 mg/kg, respectively, Table 2).

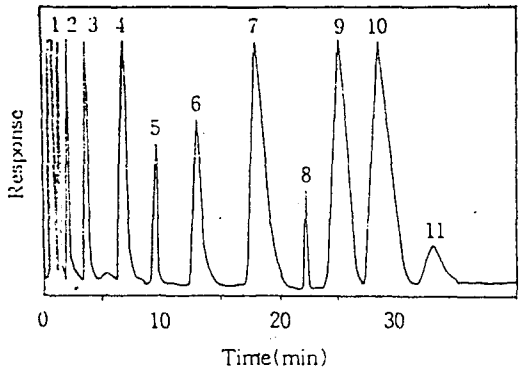

Fig. 1. GC of fatty acid methyl esters of standard mixtures
The methyl esters were separated isothermally at 190℃ on a 10 m×3 mm column packed with 10% BDS on Chromosorb W using nitrogen as the carrier gas at a flow rate of 10 ml/min.
1. C 8 : 0, 2. C 10 : 0, 3. C 12 : 0, 4. C 14 : 0, 5. C 16 : 0, 6. C 17 : 0, 7. C 18 : 0, 8. C 18 : 1, 9. C 18 : 2, 10. C 18 : 3, 11. C 20 : 0 fatty acid.

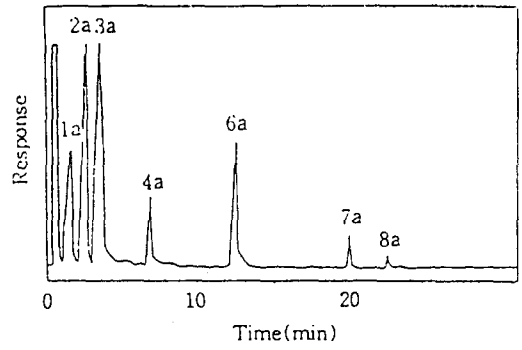

Fig. 3. GC of fatty acid methyl esters of toxic substance from *E. coli*
The methyl esters were separated isothermally at 190℃ on a 10 m×3 mm column packed with 10% BDS on Chromosorb W using nitrogen as the carrier gas at a flow rate of 10 ml/min.
1a. C 8 : 0, 2a. C 10 : 0, 3a. C 12 : 0, 4a. C 14 : 0, 6a. C 17 : 0, 7a. C 18 : 0, 8a. C 18 : 1 fatty acid.

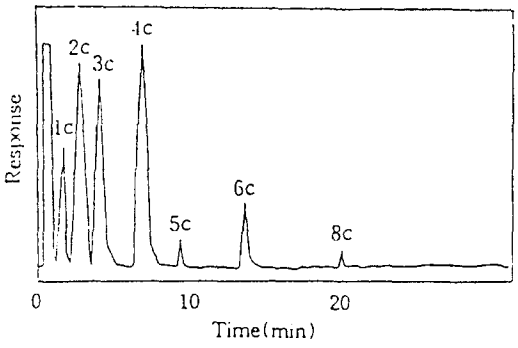

Fig. 2. GC of fatty acid methyl esters of toxic substance from *V. vulnificus*
The methyl esters were separated isothermally at 190℃ on a 10 m×3 mm column packed with 10% BDS on Chromosorb W using nitrogen as the carrier gas at a flow rate of 10 ml/min.
1c. C 8 : 0, 2c. C 10 : 0, 3c. C 12 : 0, 4c. C 14 : 0, 5c. C 16 : 0, 6c. C 17 : 0, 8c. C 18 : 1 fatty acid.

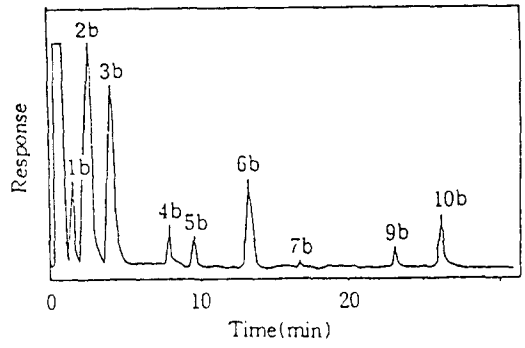

Fig. 4. GC of fatty acid methyl esters of toxic substance from *S. typhimurium*
The methyl esters were separated isothermally at 190℃ on a 10 m×3 mm column packed with 10% BDS on Chromosorb W using nitrogen as the carrier gas at a flow rate of 10 ml/min.
1b. C 8 : 0, 2b. C 10 : 0, 3b. C 12 : 0, 4b. C 14 : 0, 5b. C 16 : 0, 6b. C 17 : 0, 7b. C 18 : 0, 9b. C 18 : 2, 10b. C 18 : 3 fatty acid.

3.3 Pyrogenic activity of toxic substance

All three toxic substances induced a rise in the body temperature in rabbits (Table 3). Toxic substance from *S. typhimurium* was the most active pyrogen of the preparations tested, whereas that from *E. coli* was only slightly pyrogenic. Toxic substance from *V. vulnificus* was pyrogenic, but clearly less than that from *S. typhimurium*.

Table 1. The fatty acid compositions of three toxic substances

fatty acid	% of fatty acid from toxic substance		
	V. vulnificus	*E. coli*	*S. typhimurium*
C 8 : 0	8.65 ± 0.56	9.49 ± 0.76	6.84 ± 0.42
C 10 : 0	25.67 ± 0.92	30.33 ± 1.18	41.10 ± 2.35
C 12 : 0	18.74 ± 0.87	35.51 ± 1.21	29.49 ± 1.38
C 14 : 0	37.15 ± 1.03	9.11 ± 0.57	4.09 ± 0.32
C 16 : 0	1.25 ± 0.08	0.00	1.89 ± 0.09
C 17 : 0	7.60 ± 0.35	11.41 ± 0.92	9.38 ± 0.74
C 18 : 0	0.00	2.86 ± 0.15	0.76 ± 0.04
C 18 : 1	0.94 ± 0.07	1.09 ± 0.08	0.00
C 18 : 2	0.00	0.00	2.19 ± 0.16
C 18 : 3	0.00	0.00	4.26 ± 0.27
C 20 : 0	0.00	0.00	0.00

Table 2. Lethal toxicity of toxic substance from three bacteria

toxic substance from	lethal toxicity (LD_{50}, mg/kg)
V. vulnificus	52.5
E. coli	56.5
S. typhimurium	37.5

Table 3. Pyrogenic activity of toxic substance in rabbit

toxic substance from	sum of body temperature rise in three animals (℃)
V. vulnificus	3.2
E. coli	2.5
S. typhimurium	4.0

REFERENCES

Blake, P. A., R. E. Weaver, and D. G. Hollis, 1980, Ann. Rev. Microbiol., 34, 319.

Grob, R. L. Modern Practice of Gas Chromatography, 2nd ed., New York, Wiley-Interscience, 1985.

Hall, R. C. and D. E. Harris, 1979, J. Chromatogr., 169, 245.

Helander, I., H. Saxen, M. Salikinoja-Salonen, and R. Rylander, 1982, Infect. Immun., 35, 528~532.

Hyde, W., J. Kiesey, P. F. Ross, and H. M. Stahr, Analytical Toxicology Methods Manual, Iowa State University Press, Ames, Iowa, 1977.

Jennings, W. Analytical Gas Chromatography, Orlando, FL, Academic Press, 1987.

Kovats, E. 1915, Helv. Chim. Acta., 1958, 41.

Lee, M. L. Yang, F., and K. Bartle, Open Tubular Gas Chromatography, Theory and Practice, New York, Wiley, 1984.

Luderitz, O., C. Galanos, V. Lehmann, H. Meyer, E. T. Rietschel, and J. Weckesser, 1978, Naturwissenschaften, 65, 578~585.

Lugowski, C. and E. Romanowska, 1978, Eur. J. Biochem., 48, 319.

Miller, L. T., 1982, J. Clin. Microbiol., 16, 548~586.

Progulske, A. and S. C. Holt, 1984, Infect. Immun., 43, 166~177.

Sprouse, J. E. and A. Varano, 1984, Amer. Lab., 54.

Westphal, O. and O. Luderitz, 1954, Angew. Chem., 66, 407.

Willet, J. Gas Chromatography, New York, Wiley, 1987.

기체 크로마토그래피에 의한 독성 물질(내독소)의 분석

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(1996년 6월 15일 접수)

미생물 세포벽의 독성 물질(내독소)을 incubator, centrifuge와 UV-Vis spectrophotometer를 이용하여 추출한 후 지방산 조성을 Gas Chromatography로 분석하였으며 독성 물질의 치사 독성과 발열 활성을 측정, 그 결과를 서로 비교하였다.

지방산 조성 분석 결과 *V. vulnificus*의 독성 물질(내독소)에 대해 tetradecanoic acid가, *E. coli*에 대해서는 dodecanoic acid, 그리고 *S. typhimurium*에 대해서는 decanoic acid가 주 지방산이었으며 이들 세 지방산이 세가지 독성 물질에서 대부분을 차지하였다(70% 이상). 지방산 조성에서 특이한 점은 tetradecanoic acid가 *V. vulnificus*에 대해 주 지방산이라는 점과 세가지 독성 물질 모두 hexadecanoic acid의 양이 매우 적다는 것이다(2% 이하). *V. vulnificus*의 독성 물질의 mice 에서의 치사 독성(LD_{50}이 52.5 mg/kg)은 E. coli (56.5 mg/kg)와 비슷하였으나 *S. typhimurium* (37.5 mg/kg) 보다는 약하였으며 *V. vulnificus*의 독성 물질의 rabbit에서의 발열 활성은 E. coli 보다 강하였으나 *S. typhimurium* 보다는 약하였다.