

# クミンの腸管出血性大腸菌O157によるベロ毒素産生の抑制の解析

誌名	日本食品保蔵科学会誌
ISSN	13441213
著者	武政, 二郎 横井川, 久己男
巻/号	38巻4号
掲載ページ	p. 217-223
発行年月	2012年7月

# Analysis of the Ability of Cumin to Suppress Verocytotoxin by Enterohemorrhagic *Escherichia coli* O157

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We examined the ability of cumin to suppress the production of verocytotoxin (VT) by enterohemorrhagic *Escherichia coli* (EHEC) O157. An extract of cumin seeds was prepared with 70% ethyl alcohol. When EHEC O157 cells were grown at 37°C to the stationary phase in Luria-Bertani medium supplemented with 0.02% cumin extract, the production of both VT1 and VT2 was significantly suppressed. Neither growth inhibition nor a delay in the lag phase was observed under these culture conditions. An active component of the cumin extract was purified by high-pressure liquid chromatography and was identified as 4-isopropylbenzaldehyde (IPBA). When we examined the suppressive effect of IPBA on VT production by EHEC O157, the amounts of both intracellular and extracellular VTs were found to decrease with an increase in IPBA concentration. Our results suggest that IPBA may be potentially useful in reducing the virulence of EHEC O157.

(Received Nov. 30, 2011 ; Accepted Jan. 16, 2012)

**Key words :** verocytotoxin, enterohemorrhagic *Escherichia coli* O157, cumin, spice, 4-isopropylbenzaldehyde  
ベロ毒素, 腸管出血性大腸菌O157, クミン, スパイス, 4-イソプロピル・ベンズアルデヒド

Cumin (*Cuminum cyminum* L.), an annual spice belonging to the parsley family, is used to flavor many ethnic products such as Mexican food, Latin American cuisine, and Indian curries<sup>1)</sup>. The spice has various bioactivities such as bactericidal and antioxidant activities<sup>2),3)</sup>. In India, cumin is also used in the treatment of dysentery<sup>4)</sup>. However, there is no report on the mechanism involved and the active component (s) of cumin that possess the ability to treat dysentery.

Enterohemorrhagic *Escherichia coli* (EHEC) O157 was first recognized as a food-borne pathogen in 1982<sup>5)</sup> and is presently a worldwide threat to public health. EHEC serotype O157 is a member of a large group of verocytotoxin (VT) -producing *E. coli* (VTEC). EHEC O157 produces 2 immunologically distinct VTs (VT1 and VT2), and causes hemorrhagic colitis and hemolytic uremic syndrome. VT1 and VT2 are also referred to as Shiga toxin 1 (StxI) and Shiga toxin II (StxII), respectively. VT is also reported to promote intestinal colonization by EHEC O157 in mice<sup>6)</sup>. Since some of the antibiotics used in the treatment of O157 infection have been reported to enhance the transcription of VT genes

and induce the release of accumulated intracellular VT<sup>7)</sup>, the suppression of VT production by EHEC O157 is very important. VT genes are encoded on bacteriophage genomes that are integrated into the bacterial chromosome<sup>8),9)</sup>, and VT production in EHEC O157 cells is enhanced by various sublethal stresses such as a heat-acid shock<sup>10)</sup> and the presence of antimicrobial agents at subinhibitory concentrations<sup>11)</sup>. High concentrations of iron suppress VT production by EHEC O157<sup>12)</sup>, while low concentrations enhance VT production<sup>13),14)</sup>. Although epigallocatechin gallate and gallic acid were reported to reduce the amount of extracellular VT and increase the amount of intracellular VT, the total amount of VT was not reduced by these catechin derivatives<sup>15)</sup>. Clarithromycin has been reported to suppress VT2 but not VT1 production by EHEC O157<sup>16)</sup>. Thus, an effective method for the complete suppression of VT production has not yet been identified. Although we previously reported that eugenol in allspice reduced the amounts of both intracellular and extracellular VTs<sup>17)</sup>, a relatively high concentration (500 ppm) of eugenol was required for the complete suppression. In this

study, we analyzed the ability of cumin to suppress VT production.

## Materials and Methods

### 1. Strains

Ten strains of EHEC O157 used in this study are described in Table 1. These strains were isolated from human feces at Nara Prefecture, Japan<sup>18)</sup> and were confirmed to be different from each other in terms of their genomic profiles as determined by pulsed-field gel electrophoresis<sup>19)</sup>. Strain no. P98 was genetically identical to the strain derived from the Sakai outbreak in 1996 in Japan, the largest EHEC O157 outbreak in the world<sup>20)</sup>.

### 2. Cumin extract

Cumin seeds were kindly provided by House Foods Co. (Osaka, Japan). One gram of cumin seeds was homogenized at 25°C for 10 min with 5 ml of 70% ethyl alcohol. After centrifugation at 10,000 × *g* for 15 min to remove the insoluble residue, the supernatant solution was used as a 20% cumin extract (1 g cumin/5 ml of 70% ethyl alcohol).

### 3. Culture conditions

EHEC O157 was aerobically cultivated at 37°C for 24 h with agitation in 5 ml of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1 mM NaOH) supplemented with the cumin extract at various final concentrations (0.02%, 0.05%, and 0.1%). The growth of EHEC O157 was analyzed by measuring the absorbance at 660 nm as well as by the standard plate count method with LB agar medium.

### 4. Effect of cumin extract on the growth of *E. coli* O157

EHEC O157 was inoculated at an initial cell density of  $1.5 \times 10^7$  cells/ml into LB medium

supplemented with the cumin extract at different concentrations. After aerobic cultivation at 37°C for 24 h, the number of living cells was determined by the standard plate count method with LB agar medium.

### 5. Analysis of VT production by EHEC O157

The amounts of VT1 and VT2 were determined by a reversed passive latex agglutination (RPLA) assay with 96-well microplates (V-bottom, Greiner Japan, Tokyo, Japan) and a verocytotoxin detection kit (VTEC-RPLA "SEIKEN": Denka Seiken, Tokyo, Japan). The lower detection limit of 1 ng/ml of VTs was confirmed using the VT1 and VT2 standards provided in the kit. As reported previously<sup>17)</sup>, EHEC O157:H 7 at an initial cell density of  $1.5 \times 10^7$  cells/ml was aerobically cultured at 37°C for 24 h with agitation (150 rpm) in 5 ml of LB medium in the presence and absence of the cumin extract. After centrifugation at 10,000 × *g* for 15 min, the supernatant solution was used for assaying extracellular VT. The precipitated cells were washed twice with phosphate-buffered saline (PBS), suspended in 5 ml of PBS, and disrupted by sonication. The cell extract was used for assaying intracellular VT. The supernatant solutions and cell extracts were subjected to 2-fold serial dilution, and each diluted sample (25 μl) was mixed with a suspension of latex beads (25 μl) coated with anti-VTI or anti-VT2 antibody in 96-well microplates, in order to assay extracellular and intracellular VTs, respectively. After incubating the microplates at 30°C overnight, the agglutination of latex beads in each well was examined with the naked eye. The reciprocal of the maximal dilution showing agglutination was expressed as the RPLA titer value for both VTI and VT2 in the original samples

Table 1 *E. coli* strains used

No.	Type of strain (Serotype)	Source <sup>a</sup>	Type of toxin produced
P1	EHEC (O157 : H 7)	FHP	VT1 and 2
P6	EHEC (O157 : H 7)	FHP	VT1 and 2
P15	EHEC (O157 : H 7)	FHP	VT1 and 2
P43	EHEC (O157 : H 7)	FHP	VT1 and 2
P49	EHEC (O157 : H 7)	FHP	VT1 and 2
P60	EHEC (O157 : H 7)	FHP	VT1 and 2
P62	EHEC (O157 : H 7)	FHP	VT1 and 2
P74	EHEC (O157 : H 7)	FHP	VT2
P98	EHEC (O157 : H 7)	FHP	VT1 and 2
P100	EHEC (O157 : H 7)	FHH	VT1 and 2

<sup>a</sup> FHP, feces from a human patient; FHH, feces from a healthy human.

as previously described<sup>17</sup>. Two independent experiments were performed in triplicate, and the results were statistically analyzed.

### 6. Analysis of cumin extract

The active component from cumin extract was isolated using reverse-phase high-pressure liquid chromatography (HPLC) with a Cosmosil 5 C<sub>18</sub>-MS-II column (4.6 × 150 mm; Nakarai Tesque, Kyoto, Japan). Elution was carried out with a linear gradient from 0 to 80% methyl alcohol at a flow rate of 1.0 ml/min, and the absorbance was monitored at 210 nm. To analyze the volatile oil in cumin extract, cumin was boiled in water and the volatile oil was collected. The components of the volatile oil were analyzed using a procedure reported by SMALLFIELD *et al.*<sup>21</sup>, except that a Shimadzu GC 14 A gas chromatograph, a Shimadzu QP 2000 mass spectrometer, and a Hicap-CBP 1-M 25-025 capillary column (Shimadzu, Kyoto, Japan) were used.

### 7. Statistical analysis

The results were analyzed by analysis of variance (ANOVA) using StatView software (SAS Institute, Inc., Cary, NC, USA).

## Results

### 1. Effect of cumin extract on growth and VT production of EHEC O157

We examined the growth of EHEC O157 in the

presence of cumin extract at low concentrations (0.02, 0.05, and 0.1%). Neither growth inhibition nor a delay in the lag phase was observed at these concentrations (Fig. 1A). Fig. 1B shows the amounts of VT1 and VT2 released from the cells into LB medium supplemented with and without the cumin extract. Cumin extract was found to reduce the amounts of both VT1 and VT2. Since the 20% cumin extracts were prepared with 70% ethyl alcohol, the final concentration of ethyl alcohol in the LB medium was 0.07%. Neither growth inhibition nor suppression of VT production was observed with the addition of 0.07% ethyl alcohol into LB medium. Similar results were also obtained with 9 other strains of EHEC O157.

### 2. Isolation and identification of the active component of cumin

The cumin extract was analyzed using reverse-phase HPLC, and the elution profile is shown in Fig. 2. Each peak fraction was collected separately and analyzed. A peak at a retention time of 12.7 min was found to cause a reduction in extracellular VT production by EHEC O157. When this fraction was analyzed by gas chromatography mass spectroscopy, the active component was found to be 4-isopropylbenzaldehyde (IPBA) (data not shown). On separation by reverse phase HPLC, a standard solution of IPBA also showed a single peak at a retention time of 12.7 min with a mass spectrum

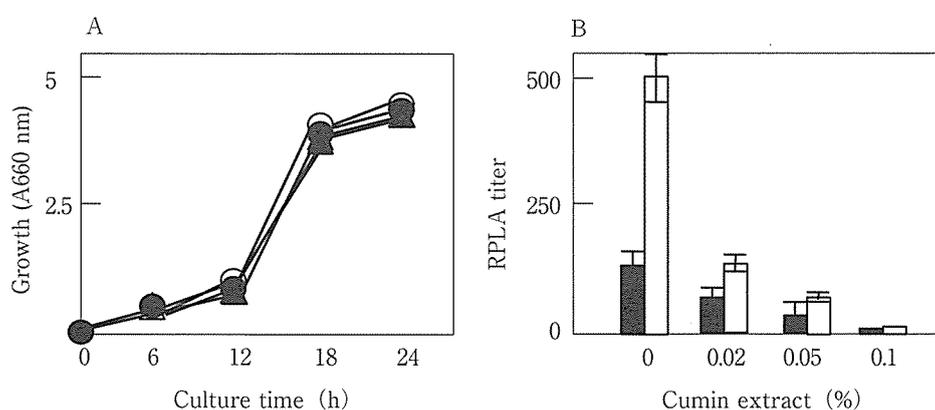
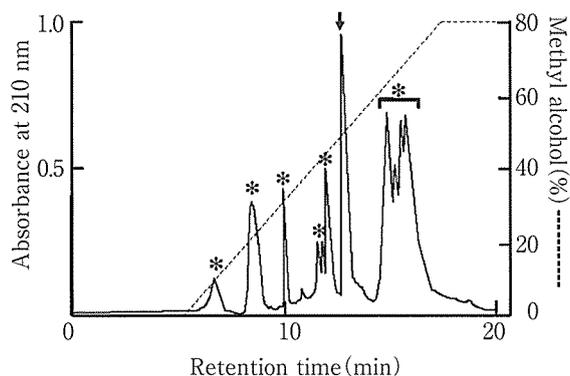


Fig. 1 Effects of cumin extract on the growth (A) and VT production (B) of EHEC O157

Strain P 98 was inoculated at an initial cell density of  $1.5 \times 10^7$  cells/ml into LB medium supplemented with the cumin extract at several concentrations, and was aerobically cultured at 37°C for 24 h. Growth was examined by measuring the absorbance at 660 nm after appropriate dilution. The concentrations of cumin extract added to the medium were 0% (open circles), 0.02% (closed circles), 0.05% (open triangles), and 0.1% (closed triangles). The amount of VT released from the cells into the medium was determined by RPLA assay. Black bars, VT1; white bars, VT2. Two independent experiments were performed in triplicate, and the vertical bars indicate standard deviations.



**Fig. 2** Elution profile of cumín extract on a Cosmosil 5 C 18-MS-II column (4.6×150mm)

The amount of 20% cumín extract injected was 10  $\mu\text{l}$ . Elution was carried out with a linear gradient from 0 to 80% methyl alcohol at a flow rate of 1.0 ml/min, and monitored at 210 nm. The arrow indicates the active peak that reduced the amount of extracellular VT. Asterisks indicate the fractions analyzed for their ability to suppress VT production.

identical to the isolated compound. Thus, IPBA was confirmed to be the active component in the cumín extract that caused suppression of VT production. When we analyzed the essential oils in the 20% cumín extract, the IPBA concentration was found to be 0.24%.

### 3. Effect of IPBA on the growth and VT production of EHEC O157

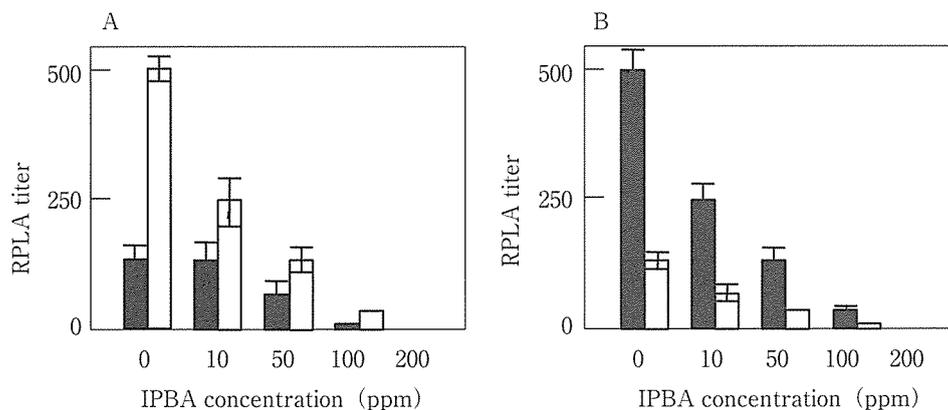
We examined the growth and VT production of EHEC O157 cells grown in LB medium supplemented with varying concentrations of IPBA. The growth was completely suppressed by 200 ppm

IPBA, and partially suppressed by 100 ppm IPBA. However, neither growth inhibition nor a delay in the lag phase was observed at 50 ppm and below. The amounts of both VT1 and VT2 released into the medium showed a decrease with increasing concentrations of IPBA (Fig. 3A). IPBA did not inhibit the RPLA assay at concentrations below 300 ppm. Furthermore, we analyzed the effect of IPBA on the amount of intracellular VT. As shown in Fig. 3B, the amount of intracellular VT was also decreased with an increase in IPBA concentration. Thus, IPBA caused a reduction in both extracellular and intracellular levels of VTs.

We also examined the effect of IPBA on VT production by other EHEC O157 strains. All strains studied grew well without any prolongation of the lag phase in LB medium supplemented with 50 ppm IPBA. Irrespective of the strain used, the levels of both intracellular and extracellular VTs were reduced by the addition of 50 ppm IPBA (Fig. 4).

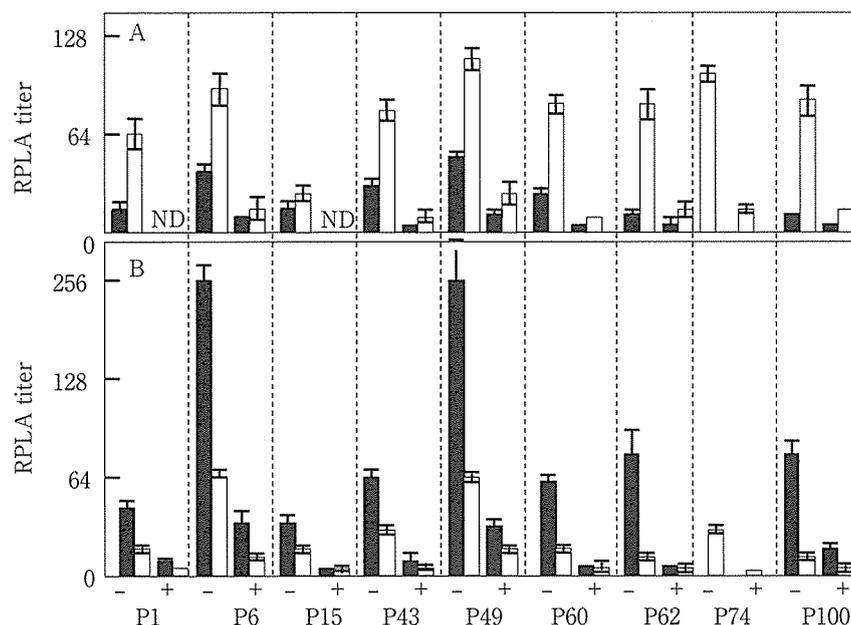
## Discussion

Cumín has antibacterial activities<sup>(1), (22)~(24)</sup>, which may also indirectly result in reduced VT production due to growth inhibition of EHEC O157. Previously, we reported that cumín extract had no antibacterial action against some strains of EHEC O157:H 7 at concentrations 0–0.1%<sup>(19)</sup>. Therefore, we analyzed the effect of cumín extract on VT production at these low concentrations (0–0.1%). Up to now, there has been no report so far on the suppressive effect of cumín on VT production by EHEC O157.



**Fig. 3** Effect of IPBA on the VT production by EHEC O157

Strain P 98 was inoculated at an initial cell density of  $1.5 \times 10^7$  cells/ml into LB medium supplemented with the IPBA at several concentrations, and was aerobically cultured at 37°C for 24 h. The levels of extracellular (A) and intracellular (B) VTs were expressed as RPLA titers. Two independent experiments were performed in triplicate, and the vertical bars indicate standard deviations. Black bars, VT1; white bars, VT2.



**Fig. 4** Effect of IPBA on the VT production of various strains of EHEC O157

The cells were aerobically cultured at 37°C for 24 h with shaking in LB medium supplemented with 50 ppm IPBA (+) and without IPBA (-). The levels of extracellular (A) and intracellular (B) VTs were expressed as the RPLA titer. Two independent experiments were performed in triplicate, and the vertical bars indicate standard deviation. ND, not detected. Black bars, VT1; white bars, VT2.

Our present results showed that cumin reduced the amounts of extracellular VT1 and VT2, and that IPBA was the active component. Since IPBA at 100 ppm reduced VT production by more than 90%, it appears to be superior to eugenol which exhibits a similar effect at 400 ppm<sup>17</sup>. In addition to the suppressive effect on VT production, IPBA showed a higher antibacterial activity against EHEC O157 than eugenol. While the minimum inhibitory concentration (MIC) of eugenol for EHEC O157:H7 was 1,000 ppm<sup>17</sup>, the MIC of IPBA was below 200 ppm. Therefore, IPBA appears to be potentially useful in suppressing the virulence of EHEC O157.

It has been shown that the SOS response induces high-level transcription of the VT genes in EHEC O157<sup>25)~27)</sup>. Since IPBA did not activate the VT production at concentrations of 0–200 ppm, IPBA does not appear to induce the SOS response of EHEC O157:H7.

Many bacterial pathogens, including EHEC, have a conserved membrane histidine sensor kinase (QseC) to activate the expression of virulence factors including VT<sup>28), 29)</sup>. Inhibitors of the QseC-mediated activation, which is referred to as a quorum sensing system, are likely to be useful in antivirulence therapy<sup>30)</sup>. RASKO *et al.* found that an

auto-inducer in the quorum sensing system inhibited the QseC-mediated activation of the *vtx2* gene without inhibiting the growth of EHEC O157<sup>31)</sup>. Therefore, one of the possible mechanisms for the suppression of VT production in EHEC O157 by IPBA might be the inhibition of the QseC-mediated activation of *vtx* genes by binding to QseC or membranes near QseC. Although further studies are required, IPBA may be useful for the prevention and treatment of EHEC O157 infection.

## Conclusions

We analyzed the ability of cumin to suppress VT production by EHEC O157. One of the isolated active components of cumin was IPBA, which reduced the production of both VT1 and VT2 of EHEC O157 strains. In addition to its use as a spice, cumin which contains IPBA may be effective in reducing the virulence of EHEC O157. However, further studies are required to clarify the mechanism by which IPBA suppresses VT production in EHEC O157, as well as to examine the suppressive effect of cumin *in vivo*.

**Acknowledgment** This work was supported in part by Grant-in-Aid for Scientific Research 15300254

from the Ministry of Education, Science and Culture of Japan.

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### クミンの腸管出血性大腸菌O157による ベロ毒素産生の抑制の解析

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腸管出血性大腸菌 (EHEC) O157のベロ毒素 (VT) 産生に対するクミンの抑制能力を調べた。クミン抽出液は70%エチルアルコールを用いて調製した。0.02%クミン抽出液を添加したLB (Luria-Bertani) 培地を用いて、37°Cで定常期までEHECO157を培養したとき、ベロ毒素産生はVT1とVT2共に有意に低下した。また、この培養条件では、増殖抑制や誘導期の遅延は認められなかった。クミン抽出液の活性成分は、HPLC法によって精製され、4-イソプロピルベンズアルデヒド (IPBA) と同定された。EHEC O157のVT産生に対するIPBAの抑圧効果を調べた結果、細胞内および細胞外のベロ毒素量は、IPBA濃度の上昇とともに低下した。この結果は、IPBAがEHEC O157の有害性を低下させることに有用であることを示唆する。

(平成23年11月30日受付, 平成24年1月16日受理)