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原 報

Purification of Anti-*Escherichia coli* O–157 components produced by *Enterococcus faecalis* TH10, an isolate from Malaysian fermentation food, tempeh

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Summary

Enterococcus faecalis TH10, an isolate from a Malaysian fermentation food tempeh, was found to produce a potent antimicrobial activity against enterohemorrhagic bacterium, *Escherichia coli* O–157. In contrast to bacteriocins typically produced by strains of *E. faecalis*, antimicrobial components of *E. faecalis* TH10 did not cause the lysis of blood samples of the rabbit and the human. The antimicrobial activity of *E. faecalis* TH10 showed distinct characteristics from those of typical bacteriocins; it retained activity after treatment with various proteolytic enzymes, and it showed poor antimicrobial activity against closely related lactic acid bacteria. *E. faecalis* TH40, showing the increased production of antimicrobial activity by several folds, was derived from the TH10 strain by ultraviolet light irradiation. A major antimicrobial component of *E. faecalis* TH40 was readily purified by column chromatography, crystallized from ethyl acetate, and identified as succinic acid. Besides succinic acid, *E. faecalis* TH40 produced neutral antimicrobial components but they were only partially purified due to low recovery during the purification steps.

Key words: *Enterococcus faecalis*, bacteriocin, succinic acid, *Escherichia. coli* O–157

Introduction

In recent years, there has been a dramatic increase in the incidence of *Escherichia coli* O–157 outbreaks. Quick, and widespread eruption of enterohemorrhagic strains of *E. coli* can be attributed to the very low infectious dose required for the transmission of oral-fecal routes. Pathogens like *Salmonella typhimurium* typically require 10^5 cells for human infection, whereas only a few cells of enterohemorrhagic *E. coli* are essential to produce illness. Although the threat by such food-associated microorganisms has stimulated continuing efforts in food preservation during production and transportation, there has been a growing concern that the food industry can no longer keep such pathogenic bacteria under sufficient control.

Lactic acid bacteria, a physiologically related group of gram-positive bacteria, produce a variety of extracellularly released peptides and proteins that show a bacteriocidal activity against species closely related to the producer strains[1]. Those antibacterial com-

pounds are specifically termed bacteriocin. It has now become evident that many bacteriocins from lactic acid bacteria show somewhat a broader spectrum of activity, affecting some distantly related species and also closely related microorganisms[2]. In fact, bacteriocin-producing lactic acid bacteria appear to interfere with the growth of the food-born pathogen *Listeria monocytogenes* during the fermentation process[3~5]. Lactic acid bacteria may have a potential application in the prevention of food contamination by infectious diseases. Culture broth of *Enterococcus faecalis* TH10, an isolate from Malaysian tempeh, was found to be effective against the *in vitro* growth of *E. coli* O-157. Some clinical isolates of *E. faecalis* are reported to produce bacteriocin molecules that mediate the lysis of a broad range of gram-positive bacteria cells[6-8]. Our present study revealed that the anti-*E. coli* O-157 activity of the tempeh-derived *E. faecalis* was produced by one major acidic component and several minor neutral substances. The acidic component, readily purified by column chromatography and crystallization from ethyl acetate, was identified as succinic acid. Purification of the minor neutral component was not successful due to a small yield and low recovery during the purification steps.

Materials and methods

1) Materials

Reagents were purchased from the following suppliers; proteinase K, V8 protease, achromopeptidase were from Wako Pure Chemical Industries, Ltd., and trypsin was from Sigma Chemical Company. Other chemicals used were analytical grade reagents. *E. coli* O-157 was clinically isolated in 1996 from a patient Infected by the *E. coli* O-157 outbreak in Sakai, Osaka, by Prof. Dr. Yukiharu Nagata of Azabu University. The strain was isolated on Eosin-methylene blue agar (DAIGO), and characterized by O-157 One Shot PCR Screening Kit (Takara). *E. faecalis* TH10 has been isolated from Malaysian tempeh[9].

2) Mutagenesis

Cell suspension of *E. faecalis* TH10 (10^4/100 μl) was spread on a plate medium (30 ml) containing 1.5% (w/v) polypepton, 0.5% yeast extract, 0.25%NaCl, 1% glucose, 0.025% L-cystine, 0.01%Na_2SO_3, 0.01% Na_2CO_3, 0.05% ascorbic acid, and 1% agar. The plate was irradiated with ultraviolet light (Toshiba GL15 lamp) in a clean bench for 70-80 seconds (3.31×10^{-4} J/cm^2/sec), quickly contained in a dark place, and incubated at 30°C until colonies grew visible. The surviving colonies were cultured on the plate media for longer than three weeks, which is required for the detection of antimicrobial activity. To extract the anti-*E. coli* O-157 component, 2 ml of the culture medium around the *E. faecalis* colony was scraped off, boiled for 10 min with 200 μl of aqueous 20% (v/v) H_2SO_4 solution, and extracted three times with 2 ml of ethyl acetate. The ethyl acetate extracts were combined and concentrated to dryness *in vacuo*, and then dissolved in 50 μl 100 mM potassium phosphate buffer, pH 8.0.

3) Bioassay

Anti-*E. coli* O-157 activity was assayed by a paper-disk method. A paper disk (Φ8 mm × 1.5 mm, Advantec Paper Disk, Toyo Roshi Kaisha, Ltd) was loaded with 60 μl of the sample solution, dried and placed on an agar plate that has been seeded with *E. coli* O-157 in 0.3% soft agar. After incubation at 30°C for 24 h, a zone of growth inhibition

around the disk was presumed to show the presence of active compound. The smallest dosage for antimicrobial activity was assayed using a series of 2-fold dilution.

4) Proteolysis Treatment and Hemolysis Assay

Fermentation broth (200 ml) of *E. faecalis* TH40, a mutant derived from the TH10 strain, was centrifuged at 4,000 × g for 20 min at 4°C to remove the cells. The supernatant solution was adjusted to pH 3.0 with 6N-HCl, extracted three times with an equal volume of ethyl acetate. The ethyl acetate layers were combined, dried over anhydrous Na_2SO_4, and concentrated to dryness *in vacuo*. The ethyl acetate extract (ca. 10 mg in dry weight) was treated overnight at 37°C with 0.1 mg of proteinase K, V8 protease, trypsin, and achromopeptidase, respectively in 100 μl of 20 mM potassium phosphate buffer at pH 7.5, then loaded on paper disks and dried for the bioassay as described above. The amount of the proteolytic enzymes used corresponds to 2.2 units for proteinase K, 1,100 units for trypsin, 2.2 units for V8 protease, and 100 units for achromopeptidase. Hemolysis assay was done with both rabbit and human erythrocytes as described for hemolysin/bacteriocin of *E. faecalis*[10].

5) Purification of an acidic anti-*E. coli* O-157 component.

E. faecalis TH40 was cultured for 8 days in 36 l of a medium containing 1.5% (w/v) polypepton, 0.5% yeast extract, 0.25%NaCl, 1% glucose, 0.025% L-cystine, 0.01%Na_2SO_3, 0.01%Na_2CO_3, and 0.05% ascorbic acid (pH 6.6 before sterilization). The fermentation broth was centrifuged at 4000 xg for 20 min at 4°C to remove cells, and the supernatant solution was extracted with ethyl acetate at pH 3.0. The solvent layer was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*, and the viscous residue (21.9 g) was absorbed on 10 g of dry celite powder before being loaded on an activated charcoal column (1.6 × 30 cm) soaked in ethyl acetate. The column was developed with 120 ml of ethyl acetate-acetone-methanol mixture in (i) 10 : 0 : 0, (ii) 8 : 1 : 1, (iii) 6 : 2 : 2, (iv) 2 : 4 : 4, and (v) 0 : 5 : 5 ratios, successively. Eluates of fractions were concentrated to dryness, dissolved in 50 μl of water, then loaded on paper disks. The fractions from (i) to (iii) were combined and concentrated *in vacuo* and the residue (4.83 g) was absorbed on 2 g of dry celite powder, then loaded on a silica gel column (1.6 × 30 cm) equilibrated in hexane. The column was developed by stepwise elution of 120 ml of hexane-ethyl acetate mixtures from 100 : 0 to 0 : 100 ratio by 10% (v/v) increase of the ethyl acetate content. All the mixtures contained 1% (v/v) acetic acid. The active fractions were combined and concentrated *in vacuo* and the residue (2.14 g) was absorbed on 1 g of dry celite powder. Active component was further purified by preparative HPLC using a silica gel column (7.8 mm × 1.5 m), Shimadzu LC-6A liquid chromatography pump, and a Shimadzu SPD-6A UV spectrophotometric detector. The column was developed with a gradient elution from 1.1 l of hexane-ethyl acetate-acetic acid (100 : 0 : 1) to 1.1 l of hexane-ethyl acetate-acetic acid (0 : 100 : 1) at a flow rate of 10 ml/min and the chromatogram was monitored by absorption at 210 nm. When active fractions were combined, and concentrated *in vacuo* white crystals were formed. Crystals were recrystallized from ethyl acetate and used for characterization.

6) Purification of a neutral anti-*E. coli* O-157 component.

Neutral anti-*E. coli* O-157 components were purified as described above except that samples were neutralized to pH 7 with sodium hydroxide when loaded on to the paper disks. The active components were extracted from 8 l of the culture broth of *E. faecalis* TH40 at pH 3.0. Silica gel column chromatography and charcoal column chromatography were successively done as described above.

Results

1) Anti-*E. coli* O-157 activity of *E. faecalis* TH10

The ethyl acetate extract of *E. faecalis* TH10 showed antimicrobial activity against *E. coli* O-157 (Figure 1) while it showed no inhibitory activity against lactic acid bacteria except for two species, *Streptococcus salivarius* and *Pediococcus acidilactici* (Table 1). *Enterococcus* species were not inhibited by the extract. The antimicrobial spectrum suggests that the extract did not contain molecules that would fall on the category of bacteriocins. The anti-*E. coli* O-157 components failed to mediate the lysis of human and rabbit erythrocytes. Treatment with proteolytic enzymes usually cause a dramatic decrease in the bacteriocidal activity of bacteriocin molecules, but the ethyl acetate extract retained anti-*E. coli* activity after treatment with V8 protease, proteinase K, trypsin, and achromopeptidase. These proteolytic enzymes alone did not affect the growth of *E. coli* O-157. The results suggest that the active component is not a peptide or a protein, but rather it appears to be an acidic low molecular weight substance that is extracted with ethyl acetate at pH 3.

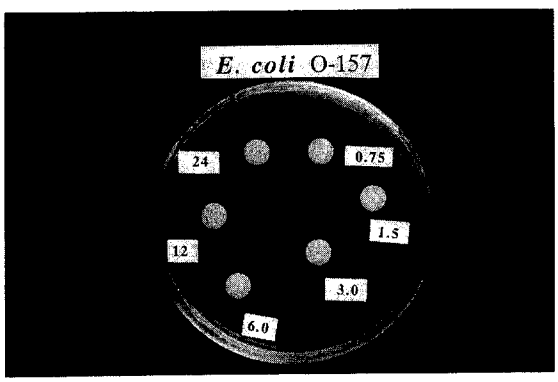

Fig. 1 Growth inhibition of *E. coli* O-157 by ethyl acetate extract of the culture broth of *E. faecalis* TH10. Dry weight of the extract is designated by each paper disk.

2) Mutagenesis

Among the 80 strains that survived the ultraviolet light irradiation at 1% survival ratio, 7 strains were found to produce higher activity than the original strain. Bioassays of a

Table 1 Inhibitory spectrum of ethyl acetate extract of the culture of *E. faecalis* TH10.

Test Organism[b]	Dosage (mg)[a] 24	12	6	3
Escherichia coli O-157	+	+	+	−
Enterococcus faecalis RIMD 3116001	−	−		
Enterococcus faecium	−	−		
Streptococcus salivarius	+	−		
Pediococcus acidilactici	+	−		
Pediococcus pentosaceus	−	−		
Lactococcus lactis subsp. lactis	−	−		
Leuconostoc mesenteroides subsp. mesentroides OR-1	−	−		
Leuconostoc mesenteroides subsp. Dextranicum 7-1-9	−	−		
Lactobacillus plantarum	−	−		
Lactobacillus delbrueckii	−	−		

[a] Dry weight of the ethyl acetate extract loaded on a paper disk. +, Growth inhibition of test organism around paper disk; −, no inhibition.

[b] Test organisms except for *E. coli* O-157 were kind gifts from Prof. Taku Miyamoto of Okayama University.

series of diluted samples indicated that *E. faecalis* TH40 produced the highest antimicrobial activity, which was almost four times higher than the original strain *E. faecalis* TH10. The other 6 strains showed no more or less than 2 times higher activity than the original strain.

3) Purification of acidic anti-*E. coli* O-157 component of *E. faecalis*

In the final step of purification, the acidic anti-*E. coli* O-157 component was recrystallized from ethyl acetate to give 58 mg of white crystals (Table 2a). The smallest dosage required for the inhibitory activity was 200 μg per paper disk. The compound was identified as succinic acid, based on the following physicochemical properties. ^{1}H-NMR (D_2O): δ 2.6 singlet. EI-mass, m/z=45, 55, 74, 100. The compound was also identified by cochromatography with the authentic succinic acid on a capillary gas chromatography using a ULBON HR-20M capillary column (Shinwa Chemical Industries, LTD, Kyoto) installed on a Shimadzu gas chromatography GC-14B. Commercially available succinic acid showed corresponding antimicrobial activity. Succinic acid decreased the antimicrobial activity when it was neutralized, suggesting that the antimicrobial effects depend on the acidity of succinate.

4) Purification of neutral anti-*E. coli* O-157 component of *E. faecalis*

Purification of neutral anti-*E. coli* activity was successively carried out by silica gel column chromatography and charcoal column chromatography. Naturally, a neutral active component was not observed in the same fractions as the acidic component. In the silica gel column chromatography, hexane-ethyl acetate mixtures did not elute the activity, but the anti-*E. coli* O-157 activity was finally eluted by methanol. In charcoal column chromatography, the activity was eluted by methanol after the column was washed with the mixtures of ethyl acetate-acetone-methanol. Neutral antimicrobial compounds

Table 2 Purification of anti-*E. coli* O-157 components from the culture broth of *E. faecalis* TH40.

a. Purification of an acidic component.

	dry weight (g)	total act. (unit)[a]	specific act. (unit/g)	recovery (%)	fold
Ethyl acetate extract	21.9	360	16.4	100	1
Charcoal column chromatography	4.83	100	20.7	27	1.3
Silica gel column chromatography	2.14	1,000	467	270[b]	25
Silca gel column (gradient elution)	1.62	2,150	1,327	597[b]	80
Crystalization from ethyl acetate	0.058	290	5,000	80.6	305

b. Purification of neutral components.

	dry weight (g)	total act. (unit)[a]	specific act. (unit/g)	recovery (%)	fold
Ethyl acetate extract	5,970	235	39.4	100	1
Silica gel column chromatography	3,960	166	42.0	70.6	1.07
Charcoal column chromatography	710	15.6	22.0	6.64	0.558

[a] One unit is defined as the smallest amount required for the antimicrobial activity by the paper disk method.
[b] Recovery of the antimicrobial activity appear to be higher than 100% due to the residual amounts of acetic acid, which is carried over from the elution solvent to the paper disks despite of concentration *in vacuo* to dryness.

were only partially purified, and thus its chemical identity remained unknown (Table 2b). We observed that recovery of the activity was very low when a partially purified sample was further fractionated by preparative HPLC. The result might suggest that the activity was produced by multi-components, and thus the separation of one compound from the others have diminished the anti-*E. coli* O-157 activity.

Discussion

Soyfoods are typically divided into two categories: nonfermented and fermented. Traditional nonfermented soyfoods include fresh green soybeans, whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk and soymilk products, tofu, okara and yuba. Traditional fermented soyfoods include tempeh, miso, soy sauces, natto and fermented tofu and soymilk products. Among fermented soyfoods, tempeh may be one of the most widely accepted and researched mold-modified fermented products in the world. Tempeh is made from soaked and cooked soybeans inoculated with a mold, usually of the genus *Rhizopus*[11]. After fermentation has occurred, the soybeans are bound together into a compact cake by a dense cottony mycelium. An important function of the mold in the fermentation process is to synthesize the enzymes, which hydrolyze the soybean constituents and contribute to the development of a desirable texture, flavor, and aroma of the product. Enzymatic hydrolysis may also decrease or eliminate antinutritional constituents; consequently, the nutritional quality of the fermented product may be improved. A role of the fungal lipase is to liberate oleic and linolenic acids from soybean oil, and these free fatty acids serve as antitryptic factors in tempeh[12]. A recent study also identified a new potent antioxidant in tempeh, and it was identified as 3-hydroxyanthranilic acid[13].

Although *Rhizopus* molds probably play the major role in tempeh fermentation, several accompanying bacteria also appear to contribute to the complex fermentation process by supplementing vitamins and bioactive compounds and by suppressing the growth of malignant contaminants. *Citrobacter freundii* and *Klebsiella pneumoniae* are two bacterial strains responsible for producing vitamin B_{12} during tempeh fermentation[14,15]. It has been reported that a lactic acid bacterium, *Lactobacillus plantarum*, markedly lowered the growth rate of *Bacillus cereus*, whose growth would have resulted in the complete spoilage of the product[16]. In an associative broth culture study, *B. cereus* was completely inhibited by *Lact. plantarum* at pH values of about 5.5[16].

The present paper reports that a strain of *E. faecalis*, isolated from Malaysian tempeh, produces a potent antimicrobial activity against *E. coli* O-157. The active components of *E. faecalis* TH10 obviously differed from bacteriocins in the biochemical characteristics. An acidic component that accounts for the potent activity was purified by three steps of column chromatography and crystallization from ethyl acetate, and the crystal was identified as succinic acid. *E. faecalis* also produced anti-*E. coli* O-157 activity which was effective at neutral pH. Unfortunately, the neutral antimicrobial component was not chemically identified due to the low recovery during the purification steps. Further study would be necessary to purify and characterize the active substance. Our present study suggests that *E. faecalis* may also be considered as an accompanying lactic acid bacterium that is involved in the complex pro-

cess of tempeh fermentation. The antimicrobial products by *E. faecalis* may prevent the contamination of malignant bacteria such as enterohemorrhagic *E. coli* O-157.

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