Synergistic Bactericidal Effect of α-Purothionin and Chelating Agents for Gram-negative Food-poisoning Bacteria

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Synergistic Bactericidal Effect of α-Purothionin and Chelating Agents for Gram-negative Food-poisoning Bacteria

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Key words：ethylenediaminetetraacetic acid （EDTA）, *Escherichia coli*, *Salmonella* spp., *Vivrio parahaemolyticus*, lipopolysaccharide

Contents

Ⅰ Introduction

Gram-negative *Escherichia coli*, *Salmonella* spp. and *Vibrio parahaemolytics* are major food-poisoning bacteria in Japan. Antimicrobial peptides from wheat endosperm, α- and β-purothionins, are inhibitive to fungi[2,7] and some bacteria[2,7,8,9], but less inhibitive to a Gram-negative bacterium *E. coli*[2]. The growth inhibition of *Salmonella* spp. and *V. parahaemolytics* by α- and β-purothionins has not been reported. Since α- and β-purothionins are thought to affect the plasma membrane[3,13], the tolerance of *E. coli* against α- and β-purothionins is possibly caused by low permeability of these peptides across the cell wall.

A chelating agent ethylenediaminetetraacetic acid (EDTA) alone is not toxic[4] but elevates the sensitivity of many kinds of antibiotics to *E. coli*[6]. The sensitization is attributed to the increase in permeability of the antibiotics across bacterial cell wall by the release of lipopolysaccharide (LPS) from the cell wall[6]. An antibacterial peptide from lactic acid bacterium *Lactococcus lactis* subsp. *lac-*

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tis, nisin, is inhibitive to Gram-positive bacteria but less inhibitive to Gram-negative bacteria. Gram-negative bacteria become sensitive to nisin by the mixed treatment of disodium salt of EDTA (Na_2EDTA). Since nisin affects bacterial plasma membrane[1], the permeability of nisin across the bacterial cell wall is probably elevated by Na_2EDTA[10-12]. The molecular weight of nisin (consist of 34 amino acids) is larger than that of the antibiotics such as actinomycin and tetracycline[6] but is smaller than that of α-purothionin (consist of 45 amino acids). If EDTA also elevates the permeability of α-purothionin across the cell wall of Gram-negative bacteria, the mixed treatment of α-purothionin and EDTA may inhibit the growth of the food-poisoning bacteria.

In this study, the growth inhibition of the food-poisoning Gram-negative bacteria by a mixed treatment of α-purothionin and EDTA is observed. The permeability of α-purothionin across Gram-negative bacterial cell wall is discussed.

Ⅱ Materials and Methods

1 Bacterial strains and Chemicals

Bacterial strains used in this study are shown in Table 1. The IFO and JCM type culture strains were purchased from Institute for Fermentation (Osaka, Japan) and the Institute of Physical and Chemical Research (Saitama, Japan), respectively.

α-Purothionin was purchased from Takara Biochemicals (Shiga, Japan), and dissolved with distilled and autoclaved water at the concentration of 1 mg/ml. The stock solution was stored at -20℃. α-Purothionin is further classified to α_1- and α_2-purothionins[14]. The purchased α-purothionin was identified as α_1-purothionin by the assay of amino acids composition[8]. EDTA, Na_2EDTA, tetrasodium salt of EDTA (Na_4EDTA), and calcium and disodium salt of EDTA ($CaNa_2EDTA$) were products of Dojin (Kumamoto, Japan) *S. typhimurium* lipid A (L-5399), ethylene glycol-*O,O'*- bis (2-aminoethyl) -*N,N,N',N'*-tetraacetic acid (EGTA), LPS of *E. coli* (L-4005) and *S. typhimurium* (L-7261) were products of Sigma (USA). Curdlan (β-1,3-glucan) was a product of Wako (Osaka, Japan). EDTA and the related compounds dissolved in distilled water were adjusted to pH 8.0 prior to autoclaving.

2 Assay for antibacterial activity

LB medium (polypepton 1%, yeast extract 0.5%, NaCl 1%, pH 7.2), the stock solutions of α-purothionin and/or chelating agents were dispensed in each well of a 96-well plate. Pre-cultured bacterial broth was diluted with autoclaved 0.9% NaCl (saline), and about 1×10^5 cells was inoculated into each well. The total culture volume in each well was 100 μl. After the incubation at at 37℃, portions of the cultures were diluted with saline and were spread on 1.5% agar plates of Sensitivity Test Broth (Nissui, Tokyo, Japan). The colony-forming unit (CFU) was determined by colony counts of the plates after two days of incubation at 37℃.

The minimal inhibitory concentration (MIC) was defined as the concentration at which no increase of OD_{550} was shown after 24 hr of incubation at 37℃. The minimal bactericidal concentration (MBC) was defined as the concentration at which the CFU decreased to less than 1/100 after 24 hr of incubation at 37℃.

3 Extraction of α-purothionin to lipopolysaccharides

In the previous report[8], 5 μg of α-purothionin bound to 2 mg of curdlan. To estimate the affinity of α-purothionin to LPS, α-purothionin was extracted from the α-purothionin-bound curdlan by a LPS solution. Curdlan (2mg) was pre-washed with 100 μl of 10 mM Tris-HCl (pH 7.5) -buffered saline (TBS), mixed with 50 μl of TBS containing 5 μg of α-purothionin, and incu-

bated at 20℃ for 30 min while 5 sec of vortexing every 5 min. After centrifugation at 2000 *g* for 1 min, the supernatant was discarded. The curdlan was washed three times with 100 μl of TBS and then mixed with 50 μl of TBS containing 50 μg/ml of *E. coli* LPS, *S. typhimurium* LPS or *E. coli* lipid A. The mixture was incubated at 20℃ for 30 min while 5 sec of vortexing every 5 min. After centrifugation at 2000 *g* for 1 min, a portion (10 μl) of each supernatant was loaded on sodium dodecylsulphate (SDS) -polyacrylamido gel electrophoresis (PAGE). SDS-PAGE and the detection of α-purothionin by silver staining were reported previously[8].

Ⅲ Results

1 Bacterial growth inhibition by α-purothionin

The MIC of α-purothionin alone was 20 μg/ml in *V. parahaemolyticus*, 30 μg/ml in *S. typhimurium*, and 100 μg/ml or more in *S. enteritidis* and *E. coli* (Table 1). The MBC of α-purothionin could not be determined because the MBC was very higher than the MIC in these Gram-negative bacteria. Since MIC of α-purothionin for Gram-positive bacteria such as *Bacillus licheniformis*[8] was 5 μg/ml or under, the Gram-negative bacteria examined were relatively tolerant to α-purothionin. The low sensitivity of *E. coli* to α-purothionin is correlated with the results in other study that the concentration of β-purothionin required for 50% growth inhibition of *E. coli* was 250 μg/ml[2] and the antibacterial activity of β-purothionin was nearly equal to that of α-purothionin[7].

Table 1 Effect of α-purothionin on bacterial growth.

Strain		MIC (μg/ml)
Vibrio parahaemolyticus	IFO 12711	20
Salmonella typhimurium	JCM 6977	30
Salmonella enteritidis	IFO 3313	100<
Escherichia coli	JCM1649	100<
Escherichia coli	JCM 5491	100

2 Synergistic bactericidal effect of α-purothionin and chelating agents

The MBC of Na_2EDTA alone was 1 mM in *V. parahaemolyticus* but 10 mM and more in other bacteria (Table 2). The MBC of Na_2EDTA was lowered by the mixed treatment with 10 μg/ml α-purothionin in every bacterial strains (Table 2). *V. parahaemolyticus* is a marine bacterium and indicates optimum growth in the medium containing 3% NaCl. When *V. parahaemolyticus* was grown in LB medium containing 3 % NaCl and 10 μg/ml α-purothionin, the MBC of Na_2EDTA was 10 mM. However, the MBC of Na_2EDTA and the MIC of α-purothionin for *V. parahaemolyticus* were 30 mM and more than 100 μg/ml in the separate treatment.

Table 2 Synergistic bactericidal effect of α-purothionin and Na_2EDTA on Gram-negative bacteria.

Strain		MBC of Na_2EDTA (mM)	
		−PT[a]	+PT[b]
V. parahaemolyticus	IFO 12711	1	0.1
S. typhimurium	JCM 6977	20	1
S. enteritidis	IFO 3313	50<	1
E. coli	JCM 1649	50	10
E. coli	JCM 5491	10	1

[a] The treatment without α-purothionin.
[b] The mixed treatment with 10 μg/ml α-purothionin.

Both *S. typhimurium* and *E. coli* JCM 5491 indicated the same MBC of Na_2EDTA (1 mM) in the presence of 10 μg/ml α-purothionin (Table 2). However, *S. typhimurium* was sensitive to lower concentrations of Na_2EDTA and α-purothionin than *E. coli* JCM 5491 in the mixed treatment (Fig. 1). The CFU of *S. typhimurium* increased from 1.2×10^6/ml to 1.6×10^8/ml in LB

medium after 4 hr of incubation, but the CFU decreased from 1.2×10^6/ml to 3×10^2/ml in LB medium containing 1 mM Na_2EDTA and 5 μg/ml α-purothionin after the same incubation (Fig. 2).

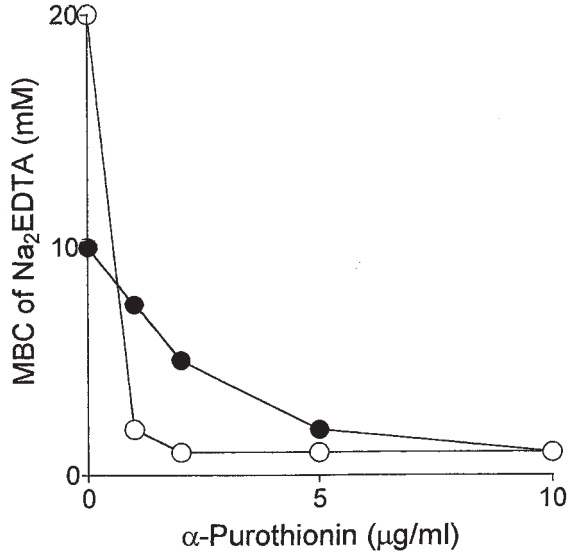

Fig 1 MBC of Na_2EDTA on different concentration of α-purothionin. Open circles: *S. typhimurium* JCM 6977; closed circles: *E. coli* JCM 5491.

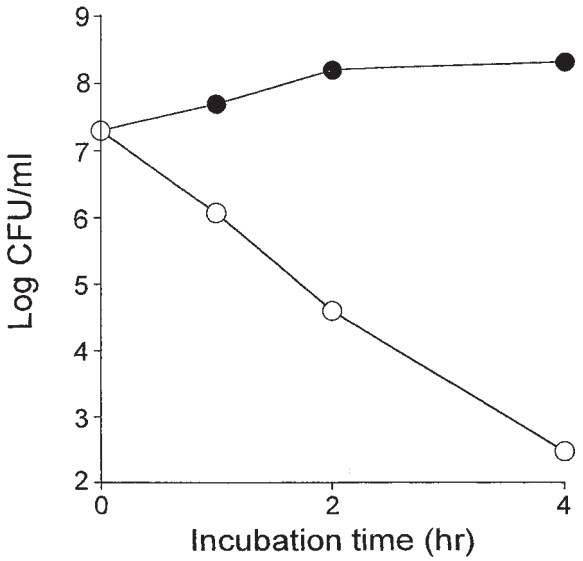

Fig 2 Time course of CFU in *S. typhimurium* JCM 6977. Open circles: addition of 1 mM Na_2EDTA and 5 μg/ml α-purothionin; closed circles: no additive.

The stock solutions of EDTA and Na_2EDTA were adjusted to pH 8.0 by NaOH, whereas EDTA was more inhibitive to *E. coli* and *S. typhimurium* than Na_2EDTA (Table 3). $CaNa_2EDTA$ indicated no antibacterial effect in the separate treatment and a slight synergistic effect with α-purothionin (Table 3). Ca^{2+} suppressed the synergistic effect of EDTA and antibiotics because of stabilization of plasma membrane[6]. The MBC of EGTA was 10 folds higher than that of Na_2EDTA in the mixed treatment with 10 μg/ml α-purothionin, whereas the MBC of EGTA alone was originally higher than that of EDTA alone (Table 3).

Table 3 Effect of chelating agents on bacterial growth.

Chelating agent	MBC (mM)			
	S. typhimurium JCM 6977		*E. coli* JCM 5491	
	-PT[a]	+PT[b]	-PT[a]	+PT[b]
EDTA	5	0.1	5	1
Na_4EDTA	50<	1	10	1
$CaNa_2EDTA$	50<	20	50<	50
EGTA	50<	10	50<	10
Sodium citrate	100<	100<	100<	100<

[a,b] See to Table 2.

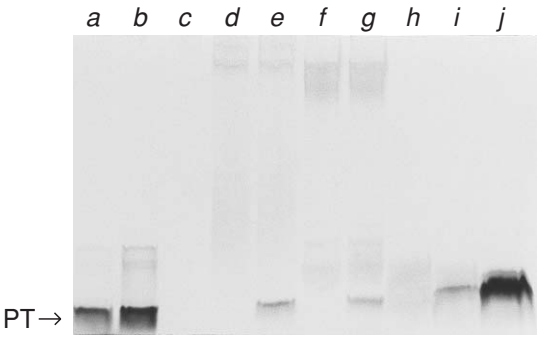

Photo 1 Extraction of α-purothionin (PT) from the PT-bound curdlan by TBS containing LPS or lipid A. *a*: 0.1 μg PT; *b*: 0.2 μg PT; *c,e,g,i*: extracts from PT-bound curdlan; *d,f,h*: extracts from curdlan; c: extracted by TBS; *d,e*: extracted by *S. typhimurium* LPS; f,g: extractedby *E. coli* LPS; *h,i*: extracted by *E. coli* lipid A; *j*: extract *h*+0.2 μg PT.

3 Affinity of α-purothionin to lipopolysaccharide

α-Purothionin was extracted by the LPSs of *S. typhimurium* and *E. coli* from the α-purothionin-bound curdlan (Photo 1, *e* and *g*).

α-Purothionin was also extracted by a component of LPS, lipid A, although the stained bands of α-purothionin and *E. coli* lipid A in the SDS-PAGE gel were overlapped (Photo 1, *h* and *i*). The result indicates that α-purothionin is affinitive to the LPSs and *E. coli* lipid A.

Ⅳ Discussion

The antimicrobial mechanism of α-purothionin is not fully elucidated, whereas the interaction of α-purothionin and plasma membrane seems to be the principal antimicrobial action[13]. In Gram-negative bacteria, the outside of cell wall termed outer membrane contains LPS, and the inside of cell wall is constructed by peptidoglycan. Since α-purothionin was affinitive to LPS (Photo 1), α-purothionin may be trapped at outer membrane of Gram-negative bacteria. EDTA probably elevates the permeability of α-purothionin across cell wall of Gram-negative bacteria, because EDTA releases LPS from the cell wall[6].

The previous study indicated that α-purothionin bound to chitin and β-glucans but not to α-glucans[8]. Chitin is polymeric β-1,4-bound *N*-acetyl-D-glucosamine. LPS is constructed by the three domains of O-polysaccharide, core oligosaccharide, and lipid A. *E. coli* lipid A contains a β-bound polymer of D-glucosamine[6].

α-Purothionin possibly binds to O-polysaccharide because of a heteropolymeric α- and β-bound polysaccharide. Core oligosaccharide contains an α-bound oligosaccharide[6] and unlikely binds to α-purothionin.

α- and β-purothionins are cytotoxic to cultured mammalian cells, but the oral administration of these peptides to guinea pigs (103-229 mg/kg body weight) gives no symptom[9]. The acceptable daily intake of EDTA is 2.5 mg/kg per day[15]. The approved concentration of Na_2EDTA for foods in USA is ranging from 36 to 500 ppm[5], which corresponds to 0.1-1.5 mM in solution. In the mixed treatment with 1 mM Na_2EDTA, MBC of α-purothionin was 2 μg/ml for *S. typhimurium* and 10 μg/ml for *E. coli* JCM 5491 (Fig. 1) The mixture of α-purothionin and Na_2EDTA is possibly applicable for a food preservative, however, α-purothionin and Na_2EDTA are not approved as food preservatives in Japan.

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Summary

The minimal inhibitory concentration of α-purothionin and the minimal bactericidal concentration (MBC) of disodium ethylenediaminetetraacetate (Na_2EDTA) for *Salmonella typhimurium* were 30 μg/ml and 20 mM, respectively. The MBC of Na_2EDTA was lowered to 1 mM by the mixed treatment with 2 μg/ml α-purothionin. The synergistic bactericidal effect was also shown in *Salmonella enteritidis*, *Escherichia coli*, and *Vibrio parahaemolyticus*. α-Purothionin was affinitive to the lipopolysaccharides of *E. coli* and *S. typhimurium* and *E. coli* lipid A.

グラム陰性食中毒細菌に対する小麦α-チオニンとキレート剤の相乗殺菌効果

老　田　　茂*

摘　　要

サルモネラ菌（S*almonella typhimurium*）に対する小麦α-チオニン（α-purothionin）の最少阻害濃度とエチレンジアミン4酢酸2ナトリウム塩（Na_2EDTA）の最少殺菌濃度はそれぞれ30μg/mlと20mMであった．2μg/mlの小麦α-チオニンと混合処理することにより，Na_2EDTAの最少殺菌濃度は1mMに低下した．この相乗殺菌効果は他のサルモネラ菌（*Salmonella enteritidis*）や大腸菌、腸炎ビブリオ菌に対しても認められた．小麦α-チオニンは，大腸菌やサルモネラ菌由来リポ多糖や大腸菌由来リピドAとの親和性が認められた．

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