Microbiol. Biotechnol. Lett. (2017), 45(3), 265–270
http://dx.doi.org/10.4014/mbl.1708.08002
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Evaluation of Urease Inhibition Activity of Zerumbone in vitro

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Received: August 14, 2017 / Accepted: September 19, 2017

A key virulence factor for urinary tract pathogens is the enzyme urease, which catalyzes the hydrolysis of urea into ammonium ions and carbonic acid. Urease activity plays an important role in the pathogenesis of urinary tract infection. In this study, the inhibitory effect of zerumbone against six urease-producing bacteria (*Klebsiella oxytoca*, *K. pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *P. vulgaris*, and *Staphylococcus saprophyticus*) and their urease activities were evaluated. The results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests showed that zerumbone had antibacterial effect against these six urease-producing bacteria. The MIC and MBC of zerumbone ranged from 0.5 to 2 mM and 1 to 4 mM, respectively. In the urease inhibitory assay, zerumbone showed better urease inhibition (56.28 ± 2.45–37.83 ± 3.47%) than the standard urease inhibitor, acetohydroxamic acid (40.46 ± 1.94–22.99 ± 3.53%). However, zerumbone did not affect the levels of the urease subunit. These results clearly indicated that zerumbone has antibacterial potential against urease-producing bacteria and possesses excellent bacterial urease inhibition properties.

Keywords: Zerumbone, urease, anti-urease activity, urease inhibition, natural compound

Introduction

Zerumbone, a monocyclic sesquiterpene, is a major component of *Zingiber zerumbet* Smith and *Zingiber aromaticum,* as shown in Fig. 1 [1]. The rhizomes, which contain large quantity of zerumbone [2], are used as an anti-inflammatory traditional medicine in Southeast Asia. It has been reported that zerumbone has many biological and pharmacological properties, such as anti-inflammatory [3], anti-tumor [4–6], anti-proliferative [7], anti-oxidant effects [8, 9] and anti-platelet aggregation [10].

Various bacteria, fungi, algae and plants possess urease enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide, which is the final step of nitrogen metabolism in living organisms [11]. Excess ammonia production by rapid and spontaneous decomposition of carbamate leads to increased level of body pH and adverse effects on the human health by urease activity [12, 13]. Urease activity plays an important role in the pathogenesis of gastric diseases, formation of kidney stones [14], development of urolithiasis, pyelonephritis and hepatic encephalopathy [15]. Many urease inhibitors have been developed in the past decades, such as imidazoles, phosphorodiamidates and hydroxamic acid derivatives [16]. However, these inhibitors are usu-

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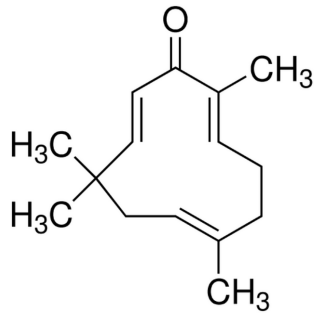

Fig. 1. Chemical structure of zerumbone.

ally associated with high toxicity or unstability. Hence, the development of more effective urease inhibitors from natural sources with safe and more potent efficacy has been the focus of urease inhibition research.

In the present study, we determined the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of zerumbone against various urease producing bacteria and investigated inhibitory effect of zerumbone on bacterial urease activity and expression.

Materials and Methods

Materials

The bacterial strains used in this study were *Klebsiella pneumoniae* ATCC 35657, *Klebsiella oxytoca* ATCC 700324, *Morganella morganii* ATCC 25830, *Proteus mirabilis* ATCC 29906, *Proteus vulgaris* ATCC 49132 and *Staphylococcus saprophyticus* ATCC 15305. All strains were purchased from American Type Cell Collection (ATCC, USA). Blood agar, Mueller Hinton broth, Mueller Hinton agar and MacConkey agar were purchased from Becton-Dickinson (USA). Trizol reagent, random hexamer, and Moloneymurine leukemia virus reverse transcriptase (MMLV-RT) were purchased from Invitrogen (USA). Zerumbone and acetohydroxamic acid were purchased from Sigma Aldrich (USA).

Bacterial culture

Enterobacteriaceae (*K. oxytoca, K. pneumoniae, M. morganii, P. mirabilis, P. vulgaris*) were grown on the MacConkey agar plate and *S. saprophyticus* were grown on the blood agar plate at 37℃ for 18 h. For experiments, the bacterial colonies were collected and suspended in Mueller Hinton broth. The number of bacterial particles in suspensions were set to MacFarland 0.5 and incubated at 37℃ for 18 h.

Broth dilution method to determine MIC and MBC

The antibacterial activities of zerumbone were examined by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the Clinical and Laboratory Standards Institute [17]. To determine the MIC, 1.5×10^8 CFU/ml diluted in Mueller Hinton broth were incubated with zerumbone in 96-well microplates at 37℃ for 18 h. The vehicle control was DMSO. MIC was defined as the lowest concentration of zerumbone that allowed no visible growth after incubation. MBC was determined by subculturing 10 μl of each incubated well that had a same or higher concentration than the MIC on Muller Hinton agar. The MBC was then determined as the lowest concentration of each sample with no visible colony growth on agar plates.

Urease inhibition assay

The bacteria were incubated with indicated concentrations of zerumbone or acetohydroxamic acid (AHA) at 37℃ for 18 h. Then supernatants of the bacterial culture were collected. Five μl of 20% urea (Duksan Pure Chemical, South Korea) was added and further incubated at 37℃ for 10 min. After incubation, the amount of ammonia produced was measured by ammonia assay kit based on indophenol method (Asan Pharmaceutical, South Korea). Briefly, 400 μl of deproteinization solution was added, vortexed and centrifuged at 2500 RPM for 5 min. One hundred μl of supernatant was mixed with 100 μl of phenol (40 g/l), 50 μl of sodium hydroxide (35.6 g/l) and 100 μl of sodium hypochlorite (10%) and incubated at 37℃ for 20 min. The absorbance was measured at 630 nm wavelength using spectrophotometer (TECAN, Switzerland). AHA was used as the standard inhibitor for urease, and the percent inhibition was calculated by using the formula: % Inhibition = 100 – (Optical density of sample / Optical density of control) × 100

RT-PCR (reverse transcription-polymerase chain reaction)

Cultured bacteria were collected and washed twice with PBS. After washing, total RNA was extracted by using Trizol reagent as described in the manufacturer's

Table 1. List of primer sequences used for RT-PCR.

Primer	Relevant sequence (5'-3')	Size of amplicon (bp)	Amplification cycles	Annealing temperature (℃)
KOX-*ure*A-F	CTTTGACGATCCCCCAGTAA	243	30	56
KOX-*ure*A-R	ACATTTCACGTCAGGCCTAT			
KPN-*ure*A-F	GCGGGATCGATACCCATATT	269	30	56
KPN-*ure*A-R	GATCTTCAGGCCAATAACGC			
MOR-*ure*A-F	GTCACTATCCTGGATGCCAA	156	32	50
MOR-*ure*A-R	TTACCTTTCCCGAGCATACC			
PMI-*ure*A-F	ATCGTCCAATGTATGCCTGT	271	32	50
PMI-*ure*A-R	CGTTGAGCCATCGGTAATTC			
PVU-*ure*A-F	ATCGTCCAATGTATGCCTGT	271	30	60
PVU-*ure*A-R	CGTTGAGCCATCGGTAATTC			
SAP-*ure*A-F	AGAATGCTGGTTCCCAAAGA	226	27	54
SAP-*ure*A-R	GGACGTGTCGGAGAAGTAAT			
16S rRNA-F	GACTCCTACGGGAGGCAGC	198	25	58
16S rRNA-R	GTATTACCGCGGCTGCTGG			

instructions. cDNA was synthesized by reverse transcription and subjected to PCR amplification as described in a previous report [18]. The PCR primer sequences and PCR conditions used in this study are described in Table 1.

Statistical analysis

Data in the bar graphs are presented as mean ± standard error of mean (SEM). All the statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software, USA). All the data were analyzed by unpaired Student's *t*-test and $p < 0.05$ was considered to be statistically significant (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Results

Antibacterial activities

In this study, the MIC and the MBC of zerumbone were investigated against the urease producing bacteria and the results are summarized in Table 2. Zerumbone showed the lowest MIC (0.5 mM) against *P. vulgaris* and *S. saprophyticus* and the lowest MBC (1 mM) against *P. mirabilis, P. vulgaris* and *S. saprophyticus*. The MICs of zerumbone, tested against 6 types of urease producing bacteria, were ranged from 0.5 to 2 mM. The lowest MIC recorded in the *P. vulgaris* and *S. saprophyticus* strain followed by *P. mirabilis, M. morganii, K. pneumoniae* and *K. oxytoca*. In the MBC test, the values ranged from being identical or greater than MIC as shown in Table 2.

Table 2. The MIC and MBC of zerumbone against the urease producing bacteria.

Strains	Zerumbone (mM)	
	MIC	MBC
KOX	2	> 4
KPN	2	4
MOR	1	2
PMI	1	1
PVU	0.5	1
SAP	0.5	1

Anti-urease activities

Because urease activity is important for bacterial pathogenesis, we investigated the inhibitory effect of zerumbone on the bacterial urease activity. Six ureases from various bacteria (*K. oxytoca, K. pneumoniae, M. morganii, P. mirabilis, P. vulgaris* and *S. saprophyticus*) were isolated and incubated with 20 μM of zerumbone *in vitro* then evaluated the urease activity. Zerumbone showed great inhibitory effect on the bacterial urease, though the inhibitory effects were varied depend on the bacteria (Fig. 2). Especially, the best anti-urease activity was observed in urease from *Klebsiella* spp. The *in vitro* urease inhibitory activity of zerumbone was compared to that of acetohydroxamic acid (AHA) which is well known

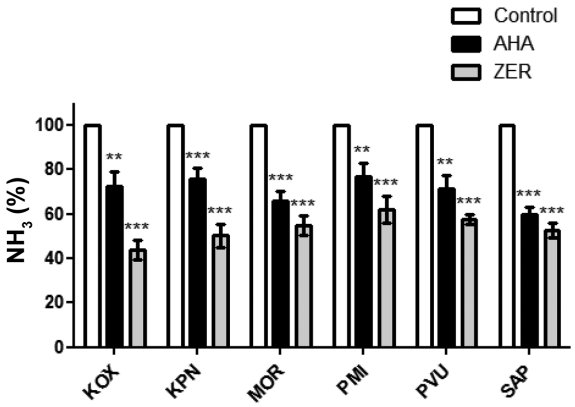

Fig. 2. The anti-urease activity effect of zerumbone and AHA. The effect of urease inhibition was determined by measuring the ammonia production using the indophenol method. The graph was drawn using the data from triplicate experiments and analyzed by unpaired Student's *t*-test (**$p < 0.01$ and ***$p < 0.001$) (AHA: actohydroxamic acid, ZER: zerumbone, KOX: *Klebsiella oxytoca*, KPN: *Klebsiella pneumonia*, MOR: *Morganella morganii*, PMI: *Proteus mirabilis*, PVU: *Proteus vulgaris*, SAP: *Staphylococcus saprophyticus*).

Table 3. The percentage inhibition of zerumbone and AHA in urease activity test.

Strains	% Inhibition	
	ZER	AHA
KOX	56.28 ± 2.45	27.38 ± 3.54
KPN	49.84 ± 2.89	24.28 ± 2.71
MOR	45.27 ± 2.51	34.43 ± 2.76
PMI	37.83 ± 3.47	22.99 ± 3.53
PVU	42.43 ± 1.19	28.42 ± 3.49
SAP	47.37 ± 1.83	40.46 ± 1.94

urease inhibitor. The percent inhibition of urease activity of zerumbone and AHA were listed in Table 3. The result showed that the percent inhibition of urease activity of zerumbone was significantly higher than that of AHA in all bacterial ureases.

Effect of zerumbone on expression of urease gene

To investigate whether zerumbone have an influence on the synthesis of ureases, expression level of urease subunit A was measured. Six species of urease producing bacteria were treated with 20 μM of zerumbone, then incubated at 37℃ for 18 h. Cultured bacteria were collected for cDNA synthesis following RNA extraction. The urease subunit A gene was analyzed by reverse transcriptase PCR. However, the expression level of urease subunit A was not influenced by zerumbone treatment (Fig. 3).

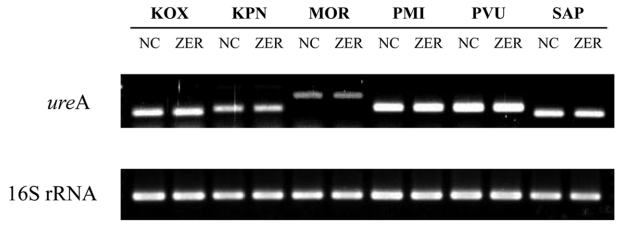

Fig. 3. The expression of *ure*A mRNA in the urease producing bacteria. The expression of *ure*A mRNA and 16S rRNA. The expression level of *ure*A was not shown significant change after incubating with zerumbone. 16S rRNA was used as an internal control.

Discussion

Urease plays a pivotal role for growth of microorganisms by facilitating breakdown of urea into ammonia. However, abnormally releasing ammonia in high quantity causes serious complications [19]. In medicine, bacterial ureases are important virulence factors for the pathogenesis of many diseases [14, 15]. Above all, urinary tract infection (UTIs) is most common disease. UTIs commonly occur in infants, children, adolescent, adult women and patients fitted with catheters [20–23]. The primary etiological agents in Gram-negative bacteria are *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* and in Gram-positive bacteria are *Staphylococcus saprophyticus*, *Enterococcus faecalis* and *Staphylococcus aureus* [24–26]. In the past decades, synthetic compounds such as bismuth complexes, boric and boronic acids, hydroxamic acid derivatives, imidazole derivatives and phosphoramidates were widely studied as potential urease inhibitors [27, 28]. Nevertheless, most of these compounds are too unstable or toxic to use in vivo. Therefore, finding for novel urease inhibitors with promising levels of activity is still goes on.

Our current study has confirmed that zerumbone possessed anti-microbial effect on 6 strains of bacteria that produce urease and determined MIC and MBC of zerumbone on 6 types of urease producing bacteria. In the MIC and MBC test, zerumbone presented the greatest antibacterial effectiveness against *P. vulgaris* and *S. saprophyticus*. Furthermore, we have found that zerum-

bone inhibited the activities of various bacterial urease. Urease inhibitory effect of zerumbone on bacterial urease activity was confirmed by urease inhibition assay based on indophenol method. In the in vitro urease inhibition assay, 20 μM of zerumbone inhibited the activity of urease from 6 strains of bacteria. Zerumbone showed best inhibition for *K. oxytoca* followed by *K. pneumoniae*, *S. saprophyticus*, *M. morganii*, *P. mirabilis* and *P. vulgaris*. The same concentration of acetohydroxamic acid (AHA) was used to compare the ability of urease inhibition. AHA is well known urease inhibitor that had been found useful in treating urinary tract infections by preventing urine alkalization [29, 30]. However, AHA had severe side effects, such as teratogenicity [31], psychoneurologic and musculo-integumentary symptoms [32, 33]. Zerumbone showed better urease inhibition (56.28 ± 2.45–37.83 ± 3.47%) than the standard urease inhibitor AHA (40.46 ± 1.94–22.99 ± 3.53%). We have also confirmed that zerumbone have no influence on the synthesis of ureases. The expression level of urease subunit A genes from 6 types of urease producing bacteria were not changed after zerumbone treatment. Therefore, this study suggests that natural compound zerumbone can be potentially used as an urease inhibitor for prevention and treatment for UTIs.

Acknowledgments

This paper was supported by the Yonsei University Research Fund of 2017.

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국문초록

제럼본의 요소가수분해효소 활성 억제 평가

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요소가수분해효소는 요소를 암모니아와 이산화탄소로 가수분해하는 효소로 요로감염을 일으키는 세균의 주요 병원성 인자이다. 따라서 요소가수분해효소는 세균이 요로감염증을 유발하는데 중요한 역할을 한다. 본 연구에서는 요소가수분해효소를 생성하는 6가지 종류의 세균에 (*K. oxytoca*, *K. pneumoniae*, *M. morganii*, *P. mirabilis*, *P. vulgaris*, *S. saprophyticus*) 대한 제럼본의 억제효과와 요소가수분해효소 활성능을 평가하였다. 최소억제농도와 최소살균농도 실험에서 제럼본은 요소가수분해효소를 생성하는 6가지 종류의 세균에 대해 억제효과를 보였으며, 최소억제농도는 0.5–2 mM, 최소살균농도는 1–4 mM를 나타내었다. 요소가수분해 활성억제 실험에서 제럼본은 요소가수분해효소의 억제제로 사용하는 아세토히드록사민산 보다 뛰어난 요소가수분해 활성억제효과를 보였다. 그러나 제럼본은 요소가수분해효소를 이루는 소단위체의 발현양에는 영향을 주지 않았다. 이러한 결과들을 종합하여 볼 때, 제럼본은 요소가수분해효소를 생성하는 세균에 대한 살균력을 가질 뿐 만 아니라 훌륭한 요소가수분해 활성억제력도 보유하고 있는 것으로 사료된다.

http://dx.doi.org/10.4014/mbl.1708.08002