2012 International Conference on Nutrition and Food Sciences
IPCBEE vol. 39 (2012) © (2012) IACSIT Press, Singapore

Study of Antimicrobial Activity of Aminoreductone Against the Antibiotic Susceptibility and Resistant Pathogenic Bacteria: *Pseudomonas Aeruginosa*, *Escherichia Coli* and *Staphylococcus Aureus*

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Abstract. In this study, antimicrobial activities of aminoreductone (AR), a product formed during the initial stage of the Maillard reaction, were screening against the antibiotic susceptibility/resistant pathogenic bacteria: Pseudomonas aeruginosa (PA), multi-drug resistant Pseudomonas aeruginosa (MDRP), Escherichia coli (EC), methicillin-susceptible Staphylococcus aureus (MSSA) and methicillin-resistant Staphylococcus aureus (MRSA). The inhibitory effects of AR to the 8 pathogenic isolates (PA 1-4, MDRP, EC, MSSA and MRSA) were examined by the disc diffusion method in the comparison with the effects of antibiotics (amikacin (AN), ciprofloxacin (CIP), imipenem (IPM) and levofloxacin (LVX)). The minimum inhibitory concentrations (MIC) were also determined. The killing assay was performed in the presence of the multiply of MIC. The significant growth inhibition of all isolates irrespective to drug-susceptibility by AR was observed. The minimum inhibitory concentration (MIC) of AR ranged from 13 to 20 mM. The bactericidal activity of AR was evaluated by a killing assay with multiples of MIC, demonstrating that the activity was dose-dependent. AR showed the advanced effect of growth inhibition in comparison with antibiotics (AN, CIP, IPM and LVX) frequently used for the treatment of infections caused by a variety of bacteria including pathogenic species. These findings show that AR is a naturally formed antimicrobial agent present in thermally processed foods with potential health benefits in medical practice.

Keywords: Pathogenic bacteria, Aminoreductone, Maillard reaction, Antimicrobial activity, MIC

1. Introduction

Since the discovery of the antibiotics, the therapy with antibiotics provides the main basis for treatment of infectious diseases caused by a variety of microorganisms in the medical practices. Human as well as animals were given a benefit with antibiotics to ameliorate the morbidity life. However, overuse and/or abuse of antibiotics have become the major factor for the emergence and dissemination of multi-drug resistant species among the microorganisms. Multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) etc. are widely distributed in hospitals and are increasingly being isolated from community acquired infections. Thus, the identification of new antimicrobial agents is a top research and development priority among scientists and pharmaceutical companies.

The Maillard reaction, a heat-treatment-induced chemical reaction between amino and carbonyl groups, is significant for foods because it strongly affects the quality and acceptance [1]. The formation of aminoreductone (AR) in the initial stage of the Maillard reaction was first reported by Pischetsrieder *et al*. in a heating solution of lactose and N^{α}-acetyllysine [2]. Because AR can be detected after only short period of heating, it is an important indicator for estimating the extent of the Maillard reaction and heat treatment of food [3-4]. Elucidation of the role and characteristics of AR are, therefore, of great interest to food scientists.

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So far, an antioxidant activity [5], a protective effect on photo-degradation of riboflavin, and an antimicrobial activity against *Helicobacter pylori*, a Gram-negative rod, of AR (1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose) derived from the Maillard reaction of lactose and butylamine were reported [5]. Thus, we hypothesized that AR interferes with certain physiological behaviours such as viability and growth of other pathogenic bacteria. In an attempt to seek alternative agents to antibiotics, and to further explicate the functional properties of AR, this study focused on investigating the effects of AR against the antibiotic susceptibility/resistant pathogenic bacteria: *Pseudomonas aeruginosa* (PA), multi-drug resistant *Pseudomonas aeruginosa* (MDRP), *Escherichia coli* (EC), methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Materials and Methods

2.1. Reagents

Mueller-Hinton broth (MHB) was obtained from Becton, Dickinson and Company. (Cockeysville, MD). Commercially available standard discs (φ = 6 mm) of amikacin (AN: 30 μg $disc^{-1}$), ciprofloxacin (CIP: 5 μg $disc^{-1}$), imipenem (IPM: 10 μg $disc^{-1}$), and levofloxacin (LVX; 5 μg $disc^{-1}$) were also obtained from Becton, Dickinson and Company. Lactose monohydrate was purchased from Nacalai tesque, Inc. (Kyoto, Japan). *n*-Butylamine and agar were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest grade commercially available. Milli-Q water or sterilized water was used in all procedures.

2.2. Preparation of AR and Its Degradation Product

Purified AR was prepared according to our previous reports [3, 5]. Briefly, lactose monohydrate (262 mM) and butylamine (1.16 M) were dissolved in 1.28 M phosphate buffer (pH 7.0). The sample solution (10 ml) was heated at 100°C for 15 min, cooled on ice and extracted three times with double volume of ethyl acetate and the ethyl acetate layer was evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of 20% methanol and filtered through a Sep-Pak Plus C18 cartridge (Waters Corporation, Milford, MA) (activated by 5 ml of ethanol and equilibrated using Milli-Q water) to remove brown components (melanoidin). The clear elute was evaporated again and freeze-dried under reduced pressure to collect the purified AR. The concentration of AR was calculated with the extinction coefficient of AR (17.98 M^{-1} cm^{-1}) at 320 nm [3].

2.3. Bacterial Strains and Culture Conditions

The 8 antibiotic susceptibility and resistant pathogenic species (4 PA, 1 MDRP, 1 EC, 1 MSSA and 1 MRSA isolates) obtained from patients in Kochi Medical School Hospital (Kochi, Japan) were used in this study. All strains were grown on the MHB agar plates supplemented with 1.4% agar and incubated at 37°C under aerobic conditions for 24 h. Whenever appropriate, MHB liquid medium was used in this study.

2.4. Disc Diffusion Susceptibility Methods

Using the filter paper disc diffusion method on the MHB agar plate, the bacterial growth inhibition by AR was assessed [6]. Purified AR was diluted in Milli-Q water and dropped to the disc. Sterilized standard discs (φ = 6 mm) containing 2.5 mg of AR were placed on the MHB agar plates previously spread with 0.1 ml of bacterial suspension (OD_{600} = 0.1) in MHB liquid medium. The plates were incubated for 24 h at 37°C under aerobic conditions. Then the inhibition zones were measured and recorded in millimeter and an average diameter of at least two repetitions was calculated. Four standard commercially available antibiotic discs, such as AN, CIP, IPM and LVX, were also utilized in this assay.

2.5. Determination of Minimum Inhibitory Concentrations (MIC)

MIC of AR against all 8 strains was determined using an agar dilution method described previously [5, 6]. Purified AR was diluted in Milli-Q water. 750 μl of AR solution at given concentrations was separately added to each dish containing 14.25 ml of yet-not-solidified MHB agar. The final concentrations of AR in the agar plates ranged from 0 to 30 mM. Subsequently, each 10 μl of bacterial suspension (OD_{600} = 0.1) was serially 10-fold diluted and inoculated onto the surface of the AR-supplemented agar plates, then incubated

at 37°C for 48 h under aerobic conditions. Sterilized water was used as a control for all experiments. The number of colony forming units (CFU) was determined as a measure of bacterial viability [6]. MIC was defined as the lowest AR concentration to inhibit 5×10^4 CFU compared to that of controls. In addition, the AR-derived Maillard reaction product (MRP) was also used and compared with AR in this study. All tests were performed in duplicate at least.

2.6. Killing Assay

To determine the bactericidal activity of AR against all strains, killing experiments were performed in the presence of 2, 5 or 10 × MIC of AR, according to the methods described previously [6]. In 1.5-ml centrifuge tubes, 0.4 ml of the bacterial suspension (10^8 cfu ml^{-1}) in fresh MHB liquid medium with or without AR (control) were incubated under aerobic conditions with shaking (Bio shaker BR-40LF, Taitec Co., Ltd., Saitama, Japan) at 37°C for 7 h. At 1, 3, 5 and 7 h after incubation, each 10 µl of the suspension was serially 10-fold diluted and inoculated onto the MHB agar plates and cultured for 24 h under aerobic conditions to determine the viability. The ability of AR to kill the microorganism was evaluated by CFU counts and comparison with controls. All examinations were performed in duplicate at least.

3. Results and Discussion

3.1. Inhibitory Effects of AR Against Pathogenic Bacteria

The inhibitory effects of AR against all 8 isolates were examined by the standard disc diffusion and agar dilution methods, which are widely used to study the bioactivity of chemical compounds. The inhibition zone for each isolate by AR (2.5 mg) is shown in Table 1. Inhibition zones ranged from 9.5 mm (MDRP) to 23.3 mm (MRSA) in diameter, suggesting that all isolates exhibited sensitivity to AR. Previously, it was reported that the addition of Cu^{2+} could drastically reduce the concentration of AR because a labile reductone structure in AR could be readily oxidized [5]. Hence, to clarify whether AR was responsible for the antimicrobial ability against the isolates, 5 µg of Cu^{2+} was added to the disc containing 2.5 mg of AR and the effect was evaluated. In the presence of Cu^{2+}, considerable decrease of the inhibition zones was recognized for all isolates (Table 1) because of decreased AR concentration. On the other hand, the disc containing 5 µg of Cu^{2+} alone did not exhibit the inhibitory effect against all strains, indicating that AR *per se* possessed the potential to inhibit the growth of all pathogenic bacteria tested.

Table 1: Inhibitory effects of aminoreductone on microorganisms

No.	Strain	*Inhibition zone (mm)						MIC (mM)
		AR (2.5 mg $disc^{-1}$)	AR (2.5 mg $disc^{-1}$) and Cu^{2+} (5 µg $disc^{-1}$)	LVX (5 µg $disc^{-1}$)	CIP (5 µg $disc^{-1}$)	IPM (10 µg $disc^{-1}$)	AN (30 µg $disc^{-1}$)	
1	MSSA	21.3 ± 1.52	n.d.	33	34	53	27.5	14
2	MRSA	23.3 ± 0.71	n.d	16	13	n.d.	19	13
3	PA1	11.5 ± 0.71	n.d.	n.d.	n.d.	n.d.	9	16
4	PA2	10.0 ± 1.41	n.d.	n.d.	n.d.	n.d.	9	18
5	PA3	11.0 ± 2.65	n.d.	n.d.	n.d.	n.d.	16	16
6	PA4	12.0 ± 0.00	n.d.	n.d.	n.d.	n.d.	11	20
7	MDRP	9.5 ± 0.71	n.d.	n.d.	n.d.	n.d.	n.d.	20
8	EC	10.3 ± 1.52	n.d.	n.d.	n.d.	n.d.	8	18

* Diameter of each disc was 6 mm and values are mean of duplicate ; LVX, Levofloxacin; CIP, Ciprofloxacin; IPM, Imipenem; AN, Amikacin; n.d., not determined, [a,b]: Values in averages of groups are significantly different ($P < 0.05$) unless followed by the same letters; MSSA: methicillin-susceptible *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; PA1-4: *Pseudomonas aeruginosa* (4 individual isolates) ; MDRP: multi-drug resistant *Pseudomonas aeruginosa*; EC: *Escherichia coli*;

Furthermore, the inhibitory effects of AR against all isolates were confirmed by an agar dilution method, demonstrating that all isolates exhibited susceptibility to AR at concentrations lower than 20 mM (Table 1). The MIC values ranging from 13 mM (MRSA) to 20 mM (MDRP and PA 4) were proven in all 8 isolates tested.

Fluoroquinolone antibacterial agents (LVX and CIP), β-lactam antibiotics (IPM) or aminoglycoside antibiotics (AN) are widely used alone or in combination with other antimicrobial agents for the treatment of

several serious infectious diseases. The susceptibility of all isolates to these four antibiotics compared with AR was investigated by using standard commercial discs (Table 1). Interestingly, all isolates including MDRP resists to AN (30 μg) were susceptible to AR (2.5 mg).

As a result, the inhibitory effect of AR affected all isolates irrespective of drug-susceptibility properties. This result suggested that AR could be used both as an alternative agent and as an adjuvant therapy for pathogenic bacteria infection. MRSA and MDRP widely resistant to antibiotics, has adapted to survive treatment with many kinds of antibiotics and is especially troublesome in immunocompromised patient as nosocomial infection because of difficulty and failure to eradicate the infection with antibiotics. The effective antimicrobial activity of AR, a natural product formed during food processing, may provide insight to scientists searching for these alternative remedies.

Amikacin, an aminoglycoside, inhibits protein synthesis by "irreversibly" binding to the 30S ribosomal subunit to prevent the formation of an initiation complex with messenger RNA [6]. Of the 4 antibiotics used, AN showed the highest activity in the growth inhibition of pathogenic strains but showed similarity in effect with AR, suggesting that AN could be used as a good reference during the investigation for the function of antimicrobial activity of AR. AN has been generally recognized to yield some serious adverse effects such as nephrotoxicity, permanent vestibular and/or auditory ototoxicity, hence the use of AR in place of AN could prove advantageous with reduced occurrence of serious adverse effects.

3.2. Killing Ability of AR Against Pathogenic Bacteria

As described above, AR inhibited the bacterial growth in all isolates tested. The *in vitro* activities of AR were evaluated further by determining the bactericidal activity on all isolates with varying drug-susceptibilities. The killing assay was performed in the presence of 2, 5 or 10 × MIC of AR to clarify the bactericidal effects (Table 2). The bactericidal effects were observed on 6 isolates excepting for 2 SA isolates at a concentration of less than 5 × MIC. The most potent bactericidal activity was exhibited for 4 PA isolates at a concentration of 2 × MIC (40 mM) of AR after 3 h of incubation. In the case of MRSA, the bactericidal activity of AR was attained at a much higher concentration than MIC concentration such as 10 × MIC of AR (130 mM). At the AR concentration 5 × MIC, the killing abilities were not found in both Gram positive strains MSSA and MRSA although the number of bacteria was decreased after 7 h of incubation. Almost all strains were killed at an AR concentration of 10 × MIC, whereas only MSSA did not show the killing activity at 10 × MIC concentration of AR. These results indicated that the killing effect of AR differs among individual isolates and is not associated with the drug-susceptibility properties. Furthermore, these results also suggested that MIC of AR achieved antimicrobial activity through bacteriostatic effect, while higher doses were required for bactericidal activity.

Table 2: Killing ability of aminoreductone against microorganisms

No.	Strain /AR concentration	Susceptibility	2× MIC	5 × MIC	Bactericidal concentration (mM)
1	MSSA	Susceptibility	−	−	−
2	MRSA	IPM	−	−	130
3	PA1	LVX, CIP, IPM	+	+	40
4	PA2	LVX, CIP, IPM	+	+	40
5	PA3	LVX, CIP, IPM	+	+	40
6	PA4	LVX, CIP, IPM	+	+	44
7	MDRP	LVX, CIP, IPM, AN	−	+	44
8	EC	LVX, CIP, IPM	−	+	56

Bactericidal (+) and no bactericidal (-) effects observed at 7 h after AR-exposure was shown.
LVX, Levofloxacin; CIP, Ciprofloxacin; IPM, Imipenem; AN, Amikacin.

The exact mechanism by which MRP affects bacterial growth is not yet known. However, it has been suggested that high-molecular-weight compounds like melanoidins with an anionic charges, could develop its antimicrobial activity by binding essential metals, such as iron, copper, zinc, which are key elements in metabolism and essential for the growth and survival of microorganisms including pathogenic bacteria [7-8]. Our findings provide a novel perception that low-molecular weight MRP such as AR (217 Da) can also contribute to exhibit the bacteriostatic and bactericidal activities against pathogenic bacteria. Lanciotti et al.

[12] suggested that the inhibitory effect against *Bacillus stearothermophilus* seemed to be influenced by the reduction of the redox potential in the growth medium as consequence of the addition of MRP with high reducing property. AR from lactose and butylamine showed a high reducing activity [6]. From this discussion, the effect of AR against pathogenic bacteria might be partially caused by its reducing ability. Rufián-Henares et al. (2008) was also investigated the mechanism of action of melanoidins against a Gram-negative pathogenic bacterium (*Escherichia coli*) by cell integrity and outer-inner membrane permeabilization assay [7]. They proposed that antimicrobial activity of melanoidins against Gram-negative pathogenic bacterium by a membrane-damage mechanism [7]. As showed in Table 1, AR represented much more effective activity against Gram-negative strains than Gram-positive strains, suggesting that the effective antimicrobial function of AR is due to thickness of bacterial cell wall as well as membrane permeability. The function of AR might be similar action to melanoidins. To elucidate this issue, we need more investigations with many microorganisms and improved AR or AR-derived compounds. However, these results expect the possibility that the potential of AR possessing an antimicrobial activity is available for our healthy life.

4. Conclusion

Our study clearly showed that AR inhibits the growth and viability of pathogenic bacteria irrespective of drug-susceptibility. Although the mechanism of action of AR has not yet been elucidated, we report for the first time that AR exhibits growth inhibition and killing activity against several pathogenic bacteria here, raising the possibility that AR is a good candidate for preventing the threat caused by pathogenic bacteria infection. Thus, food with the addition of synthesized AR could be used as a functional product for the prevention and treatment of severe infectious diseases caused by a variety of bacteria including drug-resistant.

5. Acknowledgements

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.99-2011.22.

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