EFFECTS OF AMINOREDUCTONE AGAINST THE GROWTH OF FOOD-BORNE BACTERIA IN MEDIUM AND IN MILK

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ABSTRACT

The aim of this study was to determine the antimicrobial potential of aminoreductone (AR), a product formed in the initial stages of the Maillard reaction, against common food-borne bacteria included food-spoilage and food-pathogenic bacteria (*Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecalis* (*E. faecalis*), *Listeria innocua* (*L. innocua*), *Listeria monocytogenes* (*L. monocytogenes*), *Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*)). The result indicated that AR was a strong inhibitor against all bacteria by disc susceptibility assay with inhibition zones ranged from 17 mm (*B. subtilis*) to 24.3 mm (*S. aureus*) in diameter. The minimum inhibitory concentrations (MIC) of AR ranged from 20 to 26 mM. Among test strains, AR possessed bactericidal action to five species (*S. aureus, B. subtilis, L. innocua, L. monocytogenes* and *S. Typhimurium*) at the concentration of less than 10 × MIC. AR also showed strong inhibitory effect against food-borne bacteria even in milk (UHT milk and raw milk). These findings suggest that AR, a naturally formed antimicrobial agent present in thermally processed foods, has a promising potential for food preservation..

Keywords: food-borne bacteria, aminoreductone, Maillard reaction, antimicrobial activity .

1. INTRODUCTION

Generally, microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Spoilage bacteria cause to unpleasant odors, tastes and textures of the foods [1]. Besides, food-borne pathogen lead to outbreaks of food-borne diseases, which are world-widely increasing [2]. It is estimated that the mortality of food-borne diarrheal diseases are about 4 to 6 million per year [3]. Food-borne illnesses and food spoilage associated with *E. coli*, *S. aureus* [4], *L. monocytogenes* [5] and *S.* Typhimurium etc. are caused by consumption of food products contaminated with these bacteria. Many attempts, such as use of synthetic preservatives, have been made to control microbial growth and to reduce the incidence of food poisoning and spoilage with antimicrobial chemicals in decades [6]. However, they are sometimes associated with adverse effects including hypersensitivity, allergic reaction

and immunity suppression [7]. Therefore, there has been a growing interest in research concerning alternative constituents based on the concept of naturally and/or natural products.

Aminoreductone (AR), formed in the initial stage of the Maillard reaction during heating, is a very important indicator for estimating the extent of Maillard reaction or the extent of heat treatment in food [8]. In recent years, the understanding on the role and characteristic of AR has attracted more attention for its use in food preservation. So far, the biological functions of AR such as an antioxidant activity [9], a protective effect on photo-degradation of riboflavin in milk and the antimicrobial activity against pathogenic bacteria (*Helicobacter pylori* and *S. aureus*) were reported [10]. In an attempt to seek alternative food preservatives and to further explicate the functional properties of AR, the purpose of this study is to investigate the antimicrobial activities of AR against food-borne bacteria including of *S. aureus, B. subtilis, E. faecalis*, *L. monocytogenes*, *L. innocua*, *E. coli*, and *S. Typhimurium.*

2. MATERIALS AND METHODS

2.1. Reagents

Mueller-Hinton broth (MHB) and commercially available standard discs (φ = 6 mm) such as amikacin (AN: 30 mg $disc^{-1}$), ciprofloxacin (CIP: 5 mg $disc^{-1}$), imipenem (IPM: 10 mg $disc^{-1}$) and levofloxacin (LVX; 5 mg $disc^{-1}$) were obtained from Becton, Dickinson and Company (New Jersey, USA). 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 commercial grade available. Milli-Q water or sterilized water was used in all procedures. The milks (raw and UHT milk; 130 ^{o}C, 2 s) were supplied by Vinamilk Co. Ltd (Vietnam). Raw milk was transported and stored at 4 ^{o}C after collected from healthy cows by the standard hygiene procedure. Soon after arriving at the laboratory (within 1 h), the milk was used for the experiment.

2.2. Preparation of Aminoreductone

Purified AR was prepared according to our previous reports [8]. Briefly, lactose monohydrate (262 mmol l^{-1}) and butylamine (1.16 mol l^{-1}) were dissolved in 1.28 mol l^{-1} phosphate buffer (pH 7.0). The sample solution (10 ml) was heated at 100 ^{o}C for 15 min, and immediately cooled on ice. The heated sample solution was extracted three times with a 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, USA) (activated by 5 ml of ethanol and equilibrated using Milli-Q water) to remove brown components (melanoidin). The clear eluate was evaporated again and freeze-dried under reduced pressure to collect the purified AR. In a previous study, Shimamura et al. (2004) reported the ^{13}C- and ^{1}H-NMR data on this extracted product, and their signals were assigned to the AR (1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose) [8].

2.3 Bacterial strains and culture conditions

Nine common food-borne bacteria used in this study (*S. aureus ATCC® 25923™*, *B. subtilis ATCC® 11774™*, *E. faecalis ATCC® 29212™*, *L. innocua ATCC® 33090™*,

L. monocytogenes ATCC® 19112™, *E. coli ATCC® 25922™*, and *S.* Typhimurium *ATCC® 14028™*) were obtained from the American Type Culture Collection. All strains were grown on the MHB agar plates supplemented with 1.4 % agar and incubated at 37 ^{o}C for 24 h under aerobic conditions.

2.4. Disc diffusion susceptibility methods

The growth inhibition of 9 food-borne bacteria was assessed using the filter paper disc diffusion method on the MHB agar plate incubated at 37 ^{o}C under aerobic conditions [11]. Purified AR was diluted in Milli-Q water and dropped onto 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 at 37 ^{o}C for 24 h under aerobic conditions. The diameter of inhibition zones including the disc (φ = 6 mm) were measured and recorded in millimeters. The average size of at least two repetitions was calculated. The inhibition zone representing more than 6 mm is defined as anti-bacterial activity.

2.5. Determination of minimum inhibitory concentrations (MIC) of AR

MIC of AR against food-borne strains was determined by agar dilution method as previously described [11]. Purified AR was diluted in Milli-Q water. Aliquots (750 µl) of serially diluted AR solutions were added to each dish containing 14.25 ml of not-yet solidified MHB agar, ranging from 0 to 30 mmol l^{-1} at the final concentrations. 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 ^{o}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 to evaluate the bacterial viability. MIC was defined as the lowest AR concentration to inhibit $1 \times 10^{4\text{-}5}$ CFU ml^{-1} compared to controls. In addition, the AR-derived Maillard reaction product was also used and compared with AR. All tests were performed in duplicate.

2.6. Determination of bactericidal activity of AR

To determine the bactericidal activity of AR against food-borne bacteria, killing experiments were performed by liquid cultures in the presence of 1, 2, 5 or 10 × MIC of AR, according to the previous report [12]. The bacteria cultured in MHB medium for 24 h, corresponding to the late exponential phase, were harvested, washed with MHB medium and then centrifuged at 8,000 *g* for 1 min (KUBOTA 1120, Kubota Corp., Tokyo, Japan) to remove the supernatant. In 1.5 ml centrifuge tubes, 0.4 ml of the bacterial suspension (10^{9} CFU ml^{-1}) in fresh MHB medium with AR or without (controls) were incubated under aerobic conditions under shaking (Bio shaker BR-40LF, Taitec Co., Ltd., Saitama, Japan) at 37 ^{o}C for 7 h. At 1, 3, 5 and 7 h after incubation, each 10 µl of the suspension were serially 10-fold diluted and inoculated onto the MHB agar plates and cultured for 24 h under aerobic conditions to determine the cell viability. The bactericidal ability of AR was evaluated by CFU counts and compared with the controls. All examinations were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Anti-microbial activity of AR against food-borne bacteria

The anti-microbial activity of AR against the food-borne bacteria was assessed by disc diffusion method and the determination of MIC. Seven food-borne bacteria whose biological features were individual such as Gram-staining, morphology, sporulation, and toxin-producing were used. In Table 1, these demonstrated that AR showed strong inhibitory effects on all strains with appearance of inhibition zones at the concentration of 2.5 mg/disc. The inhibition zones ranged from 17 mm (*B. subtilis*) to 24.3 mm (*S. aureus*) in diameter.

Table 1. Growth inhibition of food-borne bacteria by aminoreductone.

No.	Species	AR (2.5 mg $disc^{-1}$)	MIC (mmol l-1)	MBC (mmol l-1)
1	*S. aureus* (+)	24.3 ± 0.4	24	120
2	*B. subtilis* (+)	17.0 ± 0.7	26	260
3	*E. faecalis* (+)	19.8 ± 0.4	26	n.d
4	*L. innocua* (+)	23.3 ± 0.4	20	200
5	*L. monocytogenes* (+)	20.0 ± 0.0	20	40
6	*E. coli* (-)	20.0 ± 0.7	26	n.d
7	*S. Typhimurium* (-)	23.7 ± 0,2	26	130

* Diameter of each disc was 6 mm and the inhibition circle representing more than 6 mm is defined as anti-bacterial activity; Values are the mean of duplicates at least; AR, aminoreductone; (+), Gram +; (-), Gram -; n.d., not determined; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration.

3.2. Minimum inhibitory concentrations (MIC) and Bactericidal activity of AR against food-borne bacteria

The MIC values of AR against food-borne bacteria ranged with 20 - 26 mM (Table 1). Among seven bacteria, *L. innocua* and *L. monocytogenes* were the most sensitive strain (MIC = 20 mM) to AR, followed by *B. cereus* (MIC = 22 mM) and *S. aureus* whereas *B. subtilis*, *E. coli* and *S.* Typhimurium were the most resistant (MIC = 26 mM). *L. monocytogenes* has been recognized to be one of the emerging zoonosis during the last two decades [5]. It should be noted that AR exhibited the strong inhibitory effect against *L. monocytogenes*, an important food-pathogenic bacterium causing to the severe food-borne illness (listeriosis), and *S.* Typhimurium and *B.cereus*, the food-borne bacteria produces toxins.

The growth inhibition of AR against all tested food-borne bacteria was recognized. The inhibitory action was further evaluated by killing assays with 1, 2, 5 or 10 × MIC of AR to determine the minimum bactericidal concentrations (MBC) (Table 1). The MBC was determined in 5 strains and the MBC values were higher than MIC values. The bactericidal effect of AR on 5 isolates (*S. aureus*, *B. subtilis*, *L. innocua*, *L. monocytogenes* and *S.* Typhimurium) was observed at the concentration less than 10 × MIC. The relative high bactericidal activity was exhibited for *L. monocytogenes* at the concentration of 2 × MIC (40 mM), followed by *S. aureus* and *S. Typhimurium* at the concentration of 5 × MIC. On the other hand, no bactericidal effect of AR on *E. faecalis* and *E. coli* with 10 × MIC implied that more than 10 × MIC of AR may be

required to kill these bacteria and/or AR might possesses the bacteriostatic activities to these strains. These results indicated that the killing effect of AR differs among individual isolates irrespective of MIC values and these biological properties.

3.3. Antimicrobial activity of AR in milk

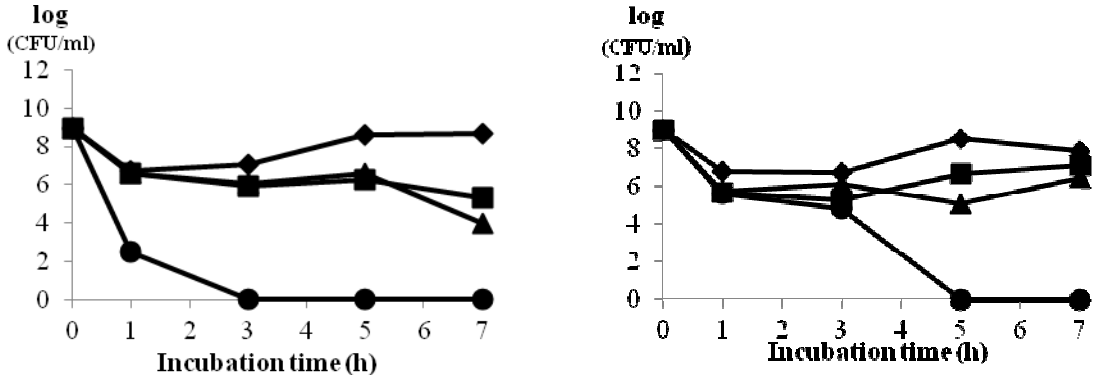
The surface properties of bacteria play an important role to interact their environment [13]. Thus, food containing a lot of components, may affect the resistant of bacteria against antibacterial compounds. In previous studies, many authors had been reported that the food components might affect the activity of antimicrobial compounds in food. The antimicrobial activity of essential oils against food borne bacteria was reduced 1000 times in food due to the dissolution of essential oils in fat. AR is always in the combination with protein in food [12]. It might reduce the activity of AR against bacteria. Although the antimicrobial activity of AR against food-borne bacteria was clarified by *in vitro* assay with nutrient mediums, it was also very important to confirm this activity in the practical samples. Bactericidal effect of AR on three food-borne bacteria (*S. aureus*, *L. innocua* and *S. Typhimurium*) was examined using UHT milk and raw milk with multiplies of MIC. As shown in Figure 1, AR also exerted a significantly bactericidal effect on all three strains tested even in UHT milk. In general, MBC of AR was not much difference between in the MHB broth and in milk. However, the results indicated that AR seemed to quickly kill bacteria in MHB medium than in UHT milk. In the case of *S. aureus*, no colony was found at 3 h after exposure of AR at the concentration of 5 × MIC, but prolonged time (5 hours) was needed in UHT milk. Similar tendency with *S.* Typhimurium was also obtained in between MHB medium (1 h) and UHT milk (3 h) to completely killing by AR.

Milk is liable to be infected by various microorganisms, mainly bacteria including lactic acid bacteria, coliform bacteria, butyric acid bacteria, propionic acid bacteria and putrefaction bacteria [14]. The quality of milk such as contaminations of bacteria and impurities absolutory depends on the clean hygiene of the breeding and industry environments as follows; the pail or milking machine, the strainer, the transport churn or the tank and agitator. Even in milk assigning to top quality, the number of bacteria (approximately 10^6 per ml) can be found in low. As soon as possible, rapid chilling to below 4 ^oC is required for keeping great quality of the milk. Thus, we evaluated the anti-microbial activity of AR using the fresh raw milk whose number of initial bacteria was 10^7 cfu per ml. Interestingly, the decreased number of bacteria was observed at the concentration more than 50 mM of AR (Figure 2). The inhibitory activities of AR seemed to be the time-dependent and dose-dependent manners. In the raw milk with 20 mM of AR, the bacterial growth completely stopped even at 7 h after incubation at 37 ^oC.

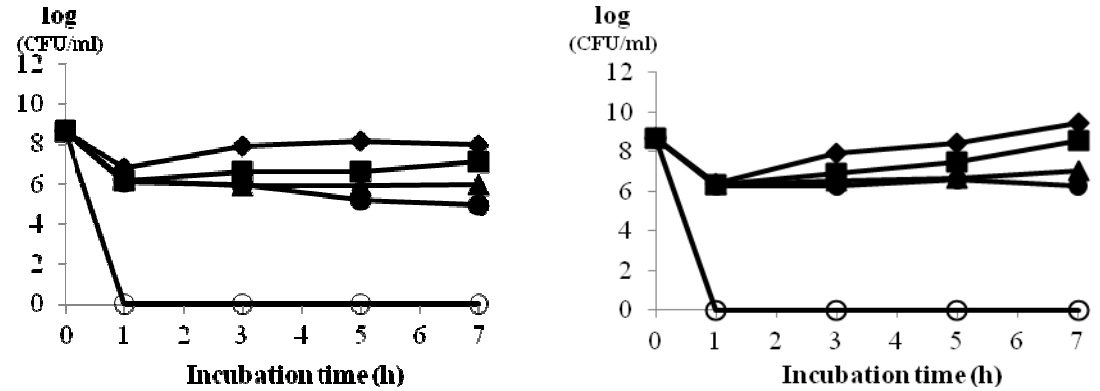
Thus, the present of AR in raw milk might contribute on bacterial growth inhibition and prolongation of the storage time, leading to high quality conservation. In raw milk, the growth of *Bacillus* spp. may limit the shelf life of the milk products caused by thermo-resistant proteases of bacterial origin. Because AR can reduce the number of bacteria in raw milk and presented the antimicrobial activity againts *Bacillus* spp. Thus, AR should be a very important naturally formed active compound for raw milk pre-processing treatment [14]. Furthermore, more than 50 mM of AR would kill bacteria in raw milk.

Various micro-organisms cause food spoilage and such harmful events are considerable concerns in the food industry. Although AR may require high amount and/or long incubation time to exert the antimicrobial effect in food such as milk, the potential ability of AR urge us to consider the application of AR as a naturally formed antibacterial compound against food-borne bacteria.

S. aureus (MIC = 24 mM)



L. innocua (MIC = 20 mM)



S. Typhimurium (MIC = 26 mM)

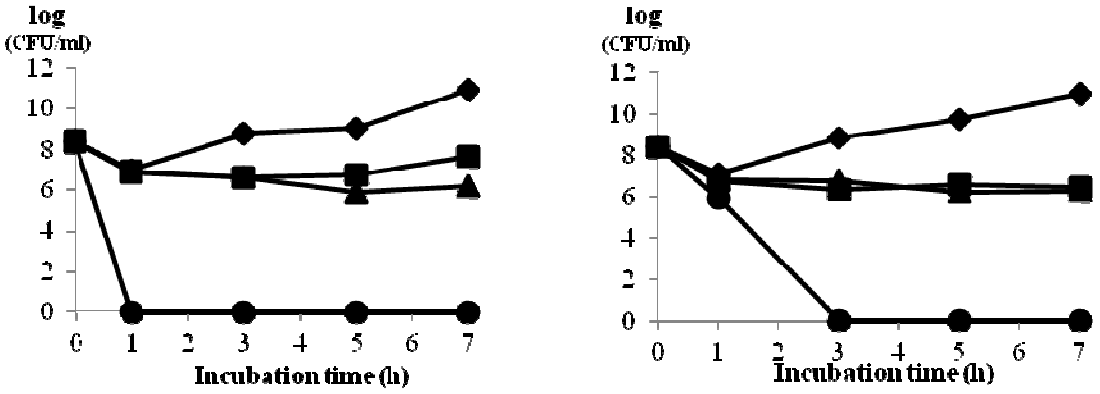


Figure 1. Bactericidal effect of aminoreductone on *S. aureus, L. innocua* and *S.* Typhimurium in MHB (left side) and in UHT milk (right side) with multiplies of MIC.

◆, without AR (control); ■, 1× MIC; ▲, 2× MIC; ●, 5× MIC; ○, 10 × MIC. The killing curves shown are representative curves; experiments were performed in duplicate.

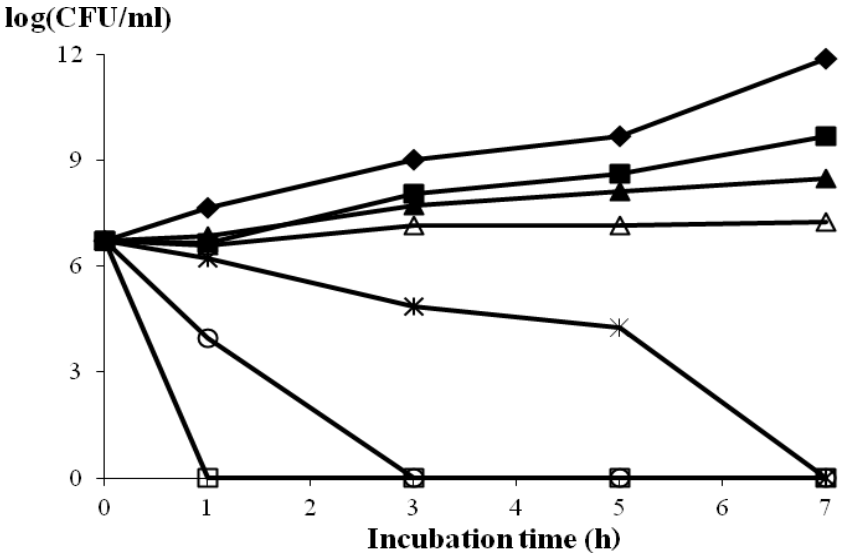


Figure 2. Antimicrobial activity of Aminoreductone against bacteria in raw milk.

Aminoreductone was added in the raw milk at different concentrations

(◆, control; ■, 5 mM; ▲, 10 mM; Δ, 20 mM; ∗, 50 mM; ○, 100mM; ∈, 200 mM).

4. CONCLUSION

AR inhibited the growth and viability of all food-borne bacteria irrespective of their biological properties (Gram-staining, morphology, sporulation, and toxin-producing). In addition, the bactericidal effect of AR was effectively in both nutrient medium and food (UHT milk and raw milk). Although, further investigation will be required to understand the mechanism of antimicrobial action of AR and its safety in food application, AR might be potentially a valuable agent for food preservation. This study provided useful information to consider the technological conditions including AR producing in the Maillard reaction as functional ingredients in food for the food shelf-life.

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TÓM TẮT

ẢNH HƯỞNG CỦA AMINOREDUCTONE TỚI SỰ SINH TRƯỞNG CỦA VI KHUẨN GÂY HƯ HỎNG THỰC PHẨM TRONG MÔI TRƯỜNG NUÔI CẤY VÀ TRONG SỮA

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Nghiên cứu này xác định khả năng kháng vi khuẩn gây bệnh có nguồn gốc thực phẩm và vi khuẩn gây hư hỏng thực phẩm như (*Staphylococcus aureus (S. aureus), Bacillus subtilis (B. subtilis), Enterococcus faecalis (E. faecalis), Listeria innocua (L. innocua), Listeria monocytogenes (L. monocytogenes), Escherichia coli (E. coli), Salmonella Typhimurium (S. Typhimurium)*) của Aminoreductone (AR), sản phẩm hình thành trong giai đoạn đầu của phản

ứng Maillard. AR ức chế sự sinh trưởng của vi khuẩn thực phẩm thử nghiệm với đường kính vòng tròn kháng khuẩn từ 17 mm (*B. subtilis*) tới 24,3 mm (*S. aureus*). Nồng độ ức chế tối thiểu của AR giao động từ 20 - 26 mM. AR thể hiện khả năng diệt khuẩn với 5 trong 7 loại vi khuẩn thử nghiệm như là *S. aureus, B. subtilis, L. innocua, L. monocytogenes and S. Typhimurium* với nồng độ diệt khuẩn nhỏ hơn 10 lần nồng độ ức chế tối thiểu. AR thể hiện hoạt tính kháng khuẩn cao trong sữa (sữa tiệt trùng UHT và sữa nguyên liệu). Nghiên cứu này chỉ ra rằng AR, hợp chất hình thành tự nhiên trong quá trình chế biến thực phẩm có thể đóng vai trò như là một hợp chất bảo quản thực phẩm.

Từ khóa: vi khuẩn gây bệnh qua thực phẩm, aminoreductone, phản ứng Maillard, hoạt tính kháng khuẩn.