Food Control 59 (2016) 352–358

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Antimicrobial effect of lipoic acid against *Cronobacter sakazakii*

Chao Shi [1], Yi Sun [1], Xiaorong Zhang, Zhiwei Zheng, Miaochun Yang, Hao Ben, Kaikuo Song, Yifang Cao, Yifei Chen, Xin Liu, Rui Dong, Xiaodong Xia*

College of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi 712100, China

ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form
6 May 2015
Accepted 7 May 2015
Available online 4 June 2015

Keywords:
Lipoic acid
Cronobacter sakazakii
Cell membrane
Electronic microscopy

ABSTRACT

Lipoic acid (LA) has been reported to be an antioxidant and an essential mitochondrial cofactor, but its inhibitory effect on bacterial pathogens has rarely been studied. In this study, antibacterial activity of LA was tested against several *Cronobacter sakazakii* strains. Minimum Inhibitory Concentration (MIC) of LA against *C. sakazakii* strains was determined using agar dilution method. Changes in intracellular pH (pH_{in}), membrane potential, intracellular ATP concentration were measured to elucidate the possible antibacterial mechanism. Moreover, cell morphology changes were observed under field emission scanning electron microscope. The MICs of LA against *C. sakazakii* strains ranged from 2.5 to 5.0 mg/mL. Addition of LA exerted an immediate and sustained inhibition of *C. sakazakii* proliferation. LA affected the membrane integrity of *C. sakazakii*, as evidenced by intracellular ATP concentration decrease. Moreover, reduction of pH_{in} and cell membrane depolarization were detected in *C. sakazakii* after exposure to LA. Electronic microscopy observations confirmed the cell membrane damage of *C. sakazakii* by LA. Our results demonstrate that LA has moderate antimicrobial activity against *C. sakazakii*. It exerts its antimicrobial action partly through causing cell membrane dysfunction and changes in cellular morphology. Considering its antimicrobial properties, together with its well-known nutritional functions, LA has potential for development as a supplement in infant formula or other foods.

1. Introduction

Cronobacter sakazakii is a gram-negative, rod-shaped, non-spore-forming bacterium (Iversen, Waddington, On, & Forsythe, 2004), and its infection in neonates cause bacteremia, necrotizing enterocolitis (NEC) and infant meningitis (Muytjens & Kollee, 1990), with reported case fatality rates of 50–80% (Healy et al., 2010). In addition to neonates, *C. sakazakii* also can infect immunocompromised adults and the elderly (Lai, 2001). *C. sakazakii* has been isolated from human clinical samples, milk and fruit powders, plants, infant formula and food processing environments (Friedemann, 2007). Although various measures have been taken to reduce its occurrence in infant formula, contamination of this pathogen has still been reported (Muller, Stephan, Fricker-Feer, & Lehner, 2013; Nurjanah, Dewanti-Hariyadi, Estuningsih, & Suhartono, 2014).

Thermal inactivation has been commonly used in controlling *C. sakazakii*, including ultra-high temperature (UHT) processing, ultraviolet light, X-ray irradiation, and high pressure processing (Amalaradjou & Venkitanarayanan, 2011). In addition to physical technologies and chemical inactivation methods, there is currently an increased trend for using natural antimicrobials to control contamination. The antimicrobial properties of several plants extracts against *C. sakazakii* have been reported. Blueberry proanthocyanidins (pH 2.8) and commercial blueberry juice (5 mg/mL) have been shown to cause 1 and 1.50 log CFU/mL reduction on *C. sakazakii* ATCC 29004 and 29544 after 30 min at 37 °C, and both strains of *C. sakazakii* were reduced to undetectable levels from 8.25 ± 0.12 log CFU/mL and 8.48 ± 0.03 log CFU/mL after 1 h at 37 °C (Joshi, Howell, & D'Souza, 2014). Polyphenol-rich cocoa powder (CocoanOX 12%, CCX) and Pulsed Electric Field (PEF) technology were combined to inactivate *C. sakazakii* inoculated into infant formula. Addition of CCX significantly affected the level of *C. sakazakii* inactivation achieved and suppressed subsequent replication of the treated cells over 12 h at 8 °C ($P \leq 0.05$) (Pina-Perez, Martinez-Lopez, & Rodrigo, 2013). Bicarinalin, a linear C-terminus amidated cationic peptide, proved more potent than melittin, ampicillin and tetracycline in *C. sakazakii* inhibition (Tene

* Corresponding author. College of Food Science and Engineering, Northwest A&F University, 28 Xinong Road, Yangling, Shaanxi 712100, China.
E-mail address: foodscixiaodong@yahoo.com (X. Xia).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.foodcont.2015.05.041
0956-7135/

et al., 2014). Moreover, a series of natural organic compounds have been investigated, and their inhibition order was carvacrol = thymol > eugenol > diacetyl > cinnamic acid (Lee & Jin, 2008). In many cases, however, the use of these tested plants or plant products in food is not allowed.

Lipoic acid (LA, $C_8H_{14}O_2S_2$) contains two sulfhydryl groups which may exist in either oxidized or reduced states (Fig. 1). The reduced form is called dihydrolipoic acid (DHLA) while oxidized form is usually referred as lipoic acid (Nikolic et al., 2014). LA is a lipophilic antioxidant and an essential cofactor for mitochondrial respiratory enzymes. Initially, LA was tentatively regarded as a vitamin, found in spinach, broccoli, tomato, garden pea, sprouts, rice bran and animal tissues (Packer, Kraemer, & Rimbach, 2001). Its content in human serum is about 16 mg/L (Baumgartner, Schmalle, & Dubler, 1996). Previous studies have demonstrated that LA is a potent antioxidant capable of chelating metals, eliminating reactive species, and repairing cellular oxidative damage (Biewenga, Haenen, & Bast, 1997). Increasing evidence has confirmed that LA is an important NF-jB inhibitor, a transcription factor that induces the expression of many genes involved in inflammation in a host of diseases (Packer, 1998). It has been recently reported that LA is characterized by high reactivity toward free radicals, increasing tissue glutathione levels, reducing oxidative stress by significantly decreasing formation of lipid peroxides (LPOs) and restoring normal antioxidant enzyme profile (El-Beshbishy, Bahashwan, Aly, & Fakher, 2011). A number of experimental and clinical studies have been carried out which show that LA has potential as a therapeutic agent in conditions related to diabetes, hepatic injury, atherosclerosis and HIV infection (El-Beshbishy, 2007; Packer, Witt, & Tritschler, 1995). Due to aforementioned properties, LA has been used as a dietary supplement in some countries (Smith, Shenvi, Widlansky, Suh, & Hagen, 2004; Zhang et al., 2008).

Although LA has been extensively studies for its nutritional function, data on its antimicrobial activity and its antimicrobial mechanism have not been reported in the literature. The aim of this study was to determine the antimicrobial effects of LA against *C. sakazakii*. In addition, possible antimicrobial mechanisms were investigated by measuring changes in intracellular ATP concentrations, membrane potential, intracellular pH (pH_{in}) and by observing microstructure changes.

2. Materials and methods

2.1. Reagents

LA [HPLC ≥ 98%, CAS: 62-46-4] was purchased from the Chengdu Must Bio-technology Co., Ltd (Chengdu, Sichuan, China) and prepared in solutions with distilled water containing 1% (v/v) of DMSO. Solutions were stored at −20 °C until use. All other chemicals were of analytical grade.

2.2. Bacterial strains and culture conditions

C. sakazakii strains ATCC 29544 and ATCC BAA-894 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Five other *C. sakazakii* strains were taken from our laboratory strain collection, which were originally isolated from various infant formula and infant rice cereal sources in China. A loopful of each strain was inoculated into 30 mL of tryptone soya broth (TSB) and incubated for 18 h at 37 °C.

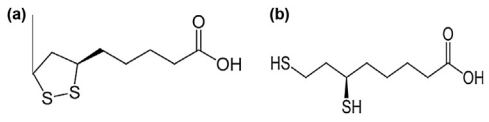

Fig. 1. Oxidized (a) and reduced (b) forms of lipoic acid (LA).

2.3. Minimum inhibitory concentration (MIC) determinations

MIC of LA was determined by agar dilution method as described by the European Committee for Antimicrobial Susceptibility Testing (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical & Infectious, 2000). Ampicillin was used as reference antibiotic. The stock solution of the antibiotic was prepared in sterile water. Those solutions were sterilized through a 0.22 μm Acrodisc filter (Gelman, USA). *C. sakazakii* chromogenic medium (Beijing Land Bridge Technology co., LTD, China) was aseptically transferred into sterile 24-well plates containing either LA or the antibiotic. The content (final volume 500 μL) of each well was gently mixed. The final concentrations of LA samples were 0, 0.3125, 0.625, 1.25, 2.5, 5, and 10 mg/mL, whereas that of ampicillin was 0.1 mg/mL. After hardening, the agar media were spotted with 2 μL (10^4 CFU) of the tested bacterium. The spots were left to dry and then plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration (mg/mL) of LA resulting in no growth of bacteria.

2.4. Growth curves

The growth curves were tested by the method described by Qiu et al. (Qiu et al., 2010), with minor exceptions. Briefly, *C. sakazakii* strain ATCC 29544 was grown to an OD_{600} value of 0.2 in TSB, whereupon 125 μL of the culture was transferred into each well on 96-well microtiter plates. LA, dissolved in 1% DMSO (v/v) at 125 μL to the cultures to obtain final concentrations of 1/64 MIC, 1/32 MIC, 1/16MIC, 1/8MIC, 1/4MIC, 1/2MIC and MIC. Bacteria were further cultured at 37 °C, and cell growth was monitored at 600 nm and 1 h intervals, using a multimode plate reader (Tecan, Infinite™ M200 PRO, Männedorf, Switzerland).

2.5. Measurement of intracellular ATP concentrations

With some modification, the method described by Sanchez et al. (Sanchez, Garcia, & Heredia, 2010) was followed. The overnight culture of *C. sakazakii* strain ATCC 29544 was centrifuged for 5 min at 5000 rpm/min, and the supernatant was removed. The cell pellets were washed 3 times with 0.1 mol/L of sodium phosphate buffer (pH 7.0), then cells were collected by centrifugation under the same conditions. A cell suspension (OD_{600} = 0.5, approximately 10^8 CFU/mL) was recovered with 50 mL of sodium phosphate buffer and 2 mL of cell solution was taken into an Eppendorf tube for treating with LA. The LA was then added to each tube resulting in final concentrations of 0 (control), MIC, and 2MIC, respectively. The samples were maintained at 37 °C for 30 min. To extract the intracellular ATP from cell suspensions, we applied ultrasound to lyse the cell samples on ice. Then the samples were centrifuged for 5 min at 5000 rpm, retrieved the top layer through an Eppendorf tube and stored samples on ice to prevent ATP loss until measurement. Intracellular ATP concentrations were determined using the ATP assay kit (Beyotime Bioengineering Institute, Shanghai, China). After adding 125 μL of ATP assay mix to 125 μL of supernatant in white, opaque 96-well microtiter plates (Nunc, Copenhagen, Denmark), the supernatant ATP concentrations were measured, which represents the intracellular ATP concentration, by applying a microplate reader (Tecan, Infinite™ M200 PRO, Männedorf, Switzerland).