Mechanisms of N^α-lauroyl arginate ethyl ester against *Penicillium digitatum* and *Pectobacterium carotovorum* subsp. *carotovorum*

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Abstract The purpose of this study was to investigate the antimicrobial activity and mechanisms of N^α-lauroyl arginate ethyl ester (LAE) against *Penicillium digitatum* and *Pectobacterium carotovorum* subsp. *carotovorum*. The minim inhibitory concentrations of LAE against *P. digitatum* and *P. carotovorum* were found to be 400 and 25 µg/ml, respectively. Loss of intracellular protein and nucleic acid increased significantly, and membrane permeability reached 76.28, 54.29 and 85.20%, respectively, when 400 µg/ml of LAE was applied to the hyphae and spores of *P. digitatum* and to *P. carotovorum*. Flow cytometry showed that LAE reduced the membrane potential, and the depolarization ratios of *P. digitatum* and *P. carotovorum* were 98.19 and 97.25% ($P < 0.05$), respectively. Transmission electron microscopy photos revealed that LAE caused a rough surface, irregular cellular organelles, protoplast shrinkage, intracytoplasmic coagulation and empty cavities in all three cell types. These results showed that LAE had notable ability to damage the structure of fungal and bacterial cells, making it a possible alternative chemical for use in the preservation of fruits and vegetables.

Keywords LAE · Antimicrobial mechanism · Moulds · Membrane permeability · Flow cytometry

Introduction

Consumption of fruits and vegetables has substantially risen in recent years. However, inappropriate manipulation and storage conditions may cause contamination of fresh products by pathogenic or deteriorative microorganisms, thus increasing the risk of spoilage and economic loss (Ramos et al. 2013; Raybaudi-Massilia et al. 2009). There is an urgent need to continue the development of fruits and vegetables preservatives in the food industry. Currently, synthetic chemicals such as chlorine dioxide, sulfur dioxide and thiabendazole are the primary measures used to control the incidence of postharvest diseases due to their low cost and efficiency (Negi 2012). Nevertheless, the use of some chemical preservatives is restricted because of possible environmental pollution and toxicity (Feng et al. 2008). Biologically based compounds, such as essential oil (Feng et al. 2008; Soyulu and Kose 2015), and antagonistic microorganisms (biological control agents) (Calvo et al. 2017; Yanez-Mendizabal et al. 2011) were suggested as alternative approaches for controlling postharvest diseases. However, these methods remain at the laboratory stage and production costs are high. Therefore, safe preservatives with high efficiency and low cost are in desperate demand.

N^α-lauroyl arginate ethyl ester (LAE), being a cationic surfactant, disperses in water at up to 247 g/kg with chemical stability at pH 3–7 (Pezo et al. 2012). It was shown by several toxicological studies that LAE can be hydrolyzed and quickly broken down into natural components in the human body (Ruckman et al. 2004). In 2005, LAE was classified as Generally Recognised as Safe

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(GRAS) for antimicrobial use. It shows high antimicrobial activity against many food pathogens and spoilage organisms such as *Listeria innocua* and *Salmonella enterica* (Becerril et al. 2013). Previous research into its antibacterial mechanisms indicated that LAE causes leakage of K^+ and structural changes without cell lysis (Rodriguez et al. 2004). Coronel-Leon et al. (2016) also found that LAE could induce the loss of K^+ and disturb the cellular membrane potential. Reports on other aspects of its effects are limited, especially for its antifungal mechanism against moulds.

Many researches have been carried out to evaluate the antimicrobial efficiency of LAE applied on dairy products (Soni et al. 2010; Woodcock et al. 2009) and meat products (Higueras et al. 2013; Loeffler et al. 2014; Luchansky et al. 2005; Schirra et al. 2008). However, limited information is available on the use of LAE in the storage of fruits and vegetables. Also, the antimicrobial activity and mechanisms of LAE against pathogenic microbes causing the spoilage of fruit and vegetables remain unclear.

Penicillium digitatum is the pathogenic fungi that causes green moulds in citrus fruits at high frequency (Raybaudi-Massilia et al. 2009). *Pectobacterium carotovorum* subsp. *carotovorum*, belonging to *Pectobacterium*, causes the bacterial soft rot of pepper, cabbage and other vegetables (Waleron et al. 2014). These strains responsible for the spoilage of fruits and vegetables were used as model organisms to detect the antimicrobial activity of LAE in this study. The underlying mechanisms of action were also investigated through indicators including leakage of protein and nucleic acid and membrane permeability. Our research aims to offer a theoretical foundation for the application of LAE to the storage of fruits and vegetables.

Materials and methods

Materials

P. digitatum CGMCC 3.7771 and *P. carotovorum* CGMCC 1.3614 were purchased from the China General Microbiological Culture Collection Center (CGMCC). The antimicrobial LAE (96.48%) was provided by HWRK Chem (Beijing, China). Propidium iodide (PI) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4 (3)] was obtained from AAT Bioquest (Sunnyvale, CA, USA).

Preparation of the microorganisms

P. digitatum was streaked in potato dextrose agar (PDA) slant medium and cultured in an incubator with constant temperature and humidity at 28 °C for 5 days. Spore

suspension was obtained by washing spores from agar slants in sterilised distilled water and filtered to remove hyphae by sterile cotton. 0.5% (v/v) of Tween 80 was added to the spore suspension to prevent spore clumping, and the final concentration was adjusted to 1.5×10^5 spores/ml. Spores were counted using a haemocytometer (Elena Ayon-Reyna et al. 2017).

One millilitre of the spore suspension was added to 100 ml of potato dextrose broth (PDB) for the culture of hyphae and then included in shaken flasks (150 rpm) at 28 °C for about 5 days.

The cells of *P. carotovorum* in the exponential phase were obtained by centrifugation at $6000 \times g$ at 4 °C for 15 min and resuspended in PBS (pH 7.4). Then, the bacterial suspension was diluted to 10^7 cfu/ml. All suspensions and the hyphae described above were stored at 4 °C until used.

Determination of the minimum inhibitory concentration (MIC)

The agar disk dilution method was used to determine the MIC of LAE against *P. digitatum* (Ruiz-Perez et al. 2016). PDA was mixed with the LAE stock solutions. Then, the medium containing LAE was mixed with 1 ml of the spore suspension by the pour plate method. Moulds were cultured at 28 °C for 48 h. MIC was defined as the lowest concentration at which colony forming was not observed. This procedure was repeated three times.

MIC was measured by the broth microdilution method for LAE against *P. carotovorum* (Xu et al. 2007). Each of the 96-well microplates was loaded with the bacteria and LAE solutions. The cultures were then incubated at 37 °C overnight. The optical density (OD) was detected daily at 600 nm with a Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). The MIC was defined as the concentration sufficient to prevent relative increases in OD_{600nm} ($\Delta OD_{600nm} < 0.05$) over a 24 h period. The medium without LAE was taken as the control, and experiments were repeated three times.

Determination of protein leakage

Intracellular protein leakage was determined according to Neto et al. (2015). Samples of spores, hyphae and bacteria treated with LAE ranging from 50 to 800 µg/ml for 24 h were centrifuged at $6000 \times g$ for 10 min at 4 °C. The culture supernatant collected was kept at 4 °C until used.

The protein content was quantified via the Bradford method (Bradford 1976) and the LAE solution of the concentration series was used for correcting to account for absorbance. Experiments were repeated three times.

Determination of ultraviolet light (UV) absorption

Measurement of UV absorption was performed according to Meng et al. (2016). Samples treated with LAE were centrifuged under the same condition as described in the previous section. A spectrofluorophotometer (model F-7000, Hitachi, Japan) was used to detect the UV absorption of the upper supernatant at 260 nm. Measurements were repeated three times and PBS containing untreated cells were used for correcting to account for absorbance.

Determination of PI uptake by cells

The evaluation of the effect of LAE on cellular membrane permeability was carried out as proposed by Pagan and Mackey (2000) and Klotz et al. (2010). Thirty microlitres of PI (1 µg/ml) was added to 3 ml of spore suspension or bacterial suspension treated with different concentrations of LAE. The mixtures were cultivated at 4 °C for 15 min. Fluorescence was determined by a spectrofluorophotometer using an excitation wavelength of 535 nm and an emission wavelength of 617 nm. The fluorescence of the heat-killed cells (10 min at 90 °C) stained with PI was set as 100%.

Flow cytometry

To evaluate the effect of LAE on the membrane potential, flow cytometry experiments were carried out according to Coronel-Leon et al. (2016) and Cho et al. (2013). Spore suspension and bacterial suspension were treated by LAE at the concentrations of 400 µg/ml and 25 µg/ml. After 24 h, 2 µl of DiBAC4 (3) (250 µg/ml) was added to 1 ml of the treated cells. The mixtures obtained were cultivated at 37 °C for 30 min. The heat-killed cells (10 min at 90 °C) stained with DiBAC4 (3) and the untreated cells were set as the positive control and negative control, respectively.

The fluorescence intensity was measured by a flow cytometer (Becton–Dickinson Inc., Franklin Lakes, NJ, USA) as follows. Samples were excited with a wavelength of 488 nm. The flow rate was set at 400 units/s up to a total of 20,000 units per sample. Data were analysed by Cell-Quest Pro software (Becton–Dickinson Inc.).

Transmission electron microscopy (TEM)

The spores and hyphae of *P. digitatum* were treated with LAE at a concentration of 400 µg/ml, and *P. carotovorum* was treated with LAE at 25 µg/ml. The samples were fixed with 2.5% (v/v) of glutaraldehyde after centrifugation and rinsing. The following procedure was performed as described by Kleyi et al. (2016). After treatment, cells were

observed under a transmission electron microscope (H-7650, Hitachi, Japan).

Statistical analysis

All experiments were independently repeated three times. Results are provided as the mean ± standard deviation ($X \pm SD$). One-way analysis of variance were carried out using the SPSS 18.0 computer program (SPSS Inc., Chicago, IL, USA). Different superscript letters shown in the figures indicate a significant difference ($P < 0.05$).

Results

Antimicrobial effectiveness of LAE against different microorganism

The MIC measured by agar disk dilution method against *P. digitatum* was 400 µg/ml (See supplementary), whereas that for the bacteria was 25 µg/ml against *P. carotovorum*. According to the data, *P. digitatum* was more resistant, with a MIC 16-fold higher than that against *P. carotovorum*.

Effect of LAE on the leakage of intracellular protein from cells

Table 1 shows the total amount of protein released from the cells, which were treated with LAE at different concentrations. It can be seen that the concentrations of LAE causing remarkable leakage of intracellular protein from the cells of the spores, hyphae and *P. carotovorum* were 400, 200 and 200 µg/ml, respectively ($P < 0.05$). After treatment with 400 µg/ml of LAE, the protein leakage increased to 4.90, 8.20 and 4.06 µg/ml in the *P. digitatum* spores, *P. digitatum* hyphae and *P. carotovorum* ($P < 0.05$), respectively.

Effect of LAE on the release of UV-absorbing substance from cells

Nucleic acid absorbs UV strongly at 260 nm. The relationship between A_{260nm} and the different concentrations of LAE for the three cell types is shown in Table 2. LAE at concentrations of 400, 200 and 100 µg/ml led to the significant release of nucleic acid from the cells of spores, hyphae and *P. carotovorum*. At the same test concentration, *P. carotovorum* showed a higher susceptibility to LAE.

Table 1 Protein leakage from LAE-treated cells

Concentration of LAE (µg/ml)	Protein leakage (µg/mL)		
	Spores of <i>P. digitatum</i>	Hyphae of <i>P. digitatum</i>	<i>P. carotovorum</i>
0	1.70 ± 0.20 ^a	3.01 ± 0.40 ^a	0.30 ± 0.09 ^a
25	1.60 ± 0.30 ^a	3.02 ± 0.33 ^a	0.76 ± 0.25 ^{ab}
50	1.73 ± 0.11 ^a	3.34 ± 0.34 ^a	0.92 ± 0.18 ^{ab}
100	1.85 ± 0.14 ^a	4.10 ± 0.34 ^a	1.26 ± 0.27 ^{ab}
200	2.24 ± 0.19 ^a	6.52 ± 0.79 ^b	2.89 ± 0.19 ^b
400	4.90 ± 0.17 ^b	8.20 ± 1.17 ^c	4.06 ± 0.12 ^c
800	5.94 ± 0.42 ^c	9.00 ± 1.42 ^c	7.52 ± 0.55 ^d

Triplicate measurements were obtained; the values are means ± standard deviations

Means in the same row labelled with different letters (a–d) are significantly different ($P < 0.05$)

Table 2 Release of UV-absorbing substance from LAE-treated cells

Concentration of LAE (µg/ml)	$A_{260\text{nm}}$		
	Spores of <i>P. digitatum</i>	Hyphae of <i>P. digitatum</i>	<i>P. carotovorum</i>
0	0.043 ± 0.01 ^a	0.078 ± 0.01 ^a	0.054 ± 0.01 ^a
25	0.071 ± 0.02 ^a	0.124 ± 0.03 ^a	0.133 ± 0.02 ^{ab}
50	0.112 ± 0.04 ^a	0.227 ± 0.04 ^{ab}	0.151 ± 0.02 ^{ab}
100	0.150 ± 0.07 ^{ab}	0.313 ± 0.04 ^{ab}	0.306 ± 0.03 ^b
200	0.211 ± 0.05 ^{ab}	0.489 ± 0.01 ^b	0.563 ± 0.01 ^c
400	0.407 ± 0.04 ^b	0.632 ± 0.07 ^c	0.774 ± 0.05 ^d
800	0.580 ± 0.05 ^{bc}	0.756 ± 0.06 ^d	0.883 ± 0.04 ^d
Heat treatment	0.600 ± 0.04 ^c	0.819 ± 0.05 ^d	0.915 ± 0.02 ^d

Triplicate measurements were obtained; the values are means ± standard deviations

Means in the same row labelled with different letters (a–c) are significantly different ($P < 0.05$)

Effect of LAE on the membrane permeability of cells

PI can bind to DNA, thus exhibiting an increased fluorescence intensity after entering the membrane of the damaged cells. Accordingly, the fluorescence intensity produced by different cells may reflect the changes in membrane permeability. As shown in Table 3, when the

concentrations of LAE were 400, 200 and 100 µg/ml, the membrane permeability of the three cell types reached 50%, which was a significant increase over that of the control group ($P < 0.05$).

Table 3 Membrane permeability of LAE-treated cells

Concentration of LAE (µg/ml)	Membrane permeability (%)		
	Spores of <i>P. digitatum</i>	Hyphae of <i>P. digitatum</i>	<i>P. carotovorum</i>
0	11.08 ± 0.72 ^a	22.08 ± 2.34 ^a	17.31 ± 1.11 ^a
25	11.00 ± 0.50 ^a	23.04 ± 3.40 ^a	23.00 ± 3.23 ^{ab}
50	11.78 ± 0.80 ^a	23.98 ± 2.90 ^a	33.65 ± 4.99 ^{ab}
100	22.41 ± 4.47 ^{ab}	25.41 ± 8.47 ^{ab}	46.05 ± 3.24 ^b
200	33.86 ± 8.38 ^{ab}	47.86 ± 2.78 ^b	71.25 ± 5.99 ^c
400	54.29 ± 4.25 ^b	76.29 ± 7.25 ^c	85.20 ± 7.08 ^{cd}
800	75.17 ± 5.90 ^c	90.17 ± 3.90 ^d	89.12 ± 8.19 ^d
Heat treatment	100 ^d	100 ^d	100 ^d

Triplicate measurements were obtained; the values are means ± standard deviations

Means in the same row labelled with different letters (a–d) are significantly different ($P < 0.05$)

Effect of LAE on the membrane potential of cells

DiBAC4 (3), which is an anionic lipophilic dye, can enter depolarised cells because of its sensitivity to the membrane potential. Increased fluorescence intensity can indicate a decrease in membrane potential.

As shown in Fig. 1, after 24 h of exposure to 400 $\mu\text{g/ml}$ of LAE, the peak of fluorescence intensity of *P. digitatum* showed a significant shift from 10.12 to 98.19% ($P < 0.05$). For *P. carotovorum*, the depolarisation ratio was significantly increased to 97.25% after treatment with 25 $\mu\text{g/ml}$ of LAE ($P < 0.05$).

TEM

To assess the influence of LAE (400 and 25 $\mu\text{g/ml}$) on the ultrastructure of microbial cells, TEM was used to observe any morphological or structural changes. In the control group (Fig. 2a1, b1), the non-treated spores and hyphae grew normally with intact structures. The cells were all clear with smooth surfaces, and the cellular organelles were visible and appeared to be arranged in an orderly fashion. In contrast, the cell wall of LAE-treated spores and hyphae became rough with cavities (Fig. 2a2, b2). The protoplast appeared to be shrinking, and plasmolysis was present in the spore cells. Cellular organelles in both the spores and hyphae were irregular, and intracytoplasmic coagulation

was observed. Even large vacuities appeared inside the cells, suggesting the leakage of intracellular materials. In addition, the particle size of the LAE-treated spores was obviously smaller than that of the non-treated spores.

An intact membrane and normally distributed cytoplasm were observed in the control cells of *P. carotovorum* (Fig. 2c1). After treatment with the LAE, although there was no serious cell lysis, the protoplast appeared to shrink and the cell wall became thinner (Fig. 2c2). There also was clear zone and cytoplasmic aggregates within the cells.

Discussion

In the present study, we observed the potent ability of LAE to inhibit *P. digitatum* and *P. carotovorum*, typical pathogenic microorganisms causing spoilage in postharvest fruit or vegetables (Supplementary). In previous studies, research of Asker et al. (2009) showed that LAE inhibited the growth of two fungi, *Aspergillus niger* and *Penicillium chrysogenum*, and Becerril et al. (2013) reported that LAE was a potent bacteriostatic agent against *Escherichia coli*, *S. enterica* and *L. innocua*, which were consistent with the findings of our study.

Disturbing the synthesis of intracellular protein or damaging the cellular structure are common antiseptic mechanisms (Frost et al. 2009; Neto et al. 2015; Wang

Fig. 1 Percentage of stained cells measured by flow cytometry analysis. The untreated cells (**a1**: *P. digitatum*; **b1**: *P. carotovorum*); the cells treated at 90 °C/10 min (**a2**: *P. digitatum*; **b2**: *P.*

carotovorum); the cells of *P. digitatum* treated with 400 $\mu\text{g/ml}$ (**a3**) and the cells of *P. carotovorum* treated with 25 $\mu\text{g/ml}$ (**b3**)

Fig. 2 Transmission electron microscopy of control and LAE-treated cells. The untreated cells (**a1**: *P. digitatum* spores; **b1**: *P. digitatum* hyphae; **c1**: *P. carotovorum*); the cells treated with 400 µg/ml of LAE

(**a2**: *P. digitatum* spores; **b2**: *P. digitatum* hyphae) and the cells treated with 25 µg/ml of LAE (**c2**: *P. carotovorum*)

et al. 2015). Our results consistently showed that LAE caused protein leakage in *P. digitatum* and *P. carotovorum* (Table 1). Comparing spores with hyphae, the concentration of LAE resulting in remarkable protein leakage from *P. digitatum* spores was twice that for *P. digitatum* hyphae. The spores of *P. digitatum* were more resistant to LAE than the hyphae, which might be due to their thicker cell envelope, strong propagation ability and hardness in the environment (Tournas 1994). Comparing fungi with bacteria, *P. digitatum* hyphae had susceptibility similar to that of *P. carotovorum* because the same concentration (200 µg/ml) of LAE led to significant protein leakage in both.

Leakage of nucleic acid is one of the causes of cell death according to Meng et al. (2016). Our research showed that the uptake of UV absorption indicated a significant release of nucleic acid from cells of the spores, hyphae and *P. carotovorum* as the concentration of LAE increased

(Table 2). The leakage of both protein and nucleic acid indicated that the damage to the cellular structure was caused by LAE. At the same concentration, *P. digitatum* spores were more resistant to LAE than were the hyphae, which was consistent with the protein leakage results. Apparently, *P. carotovorum* had the highest susceptibility to LAE of the three cell types.

Membrane permeability rose significantly as the concentration of LAE increased ($P > 0.05$) (Table 3). In agreement with Tables 1 and 2, LAE damaged the membrane structure, which could then influence cell membrane permeability and cause the leakage of protein and nucleic acid. The reason may be that the positive charge on the surface of the LAE molecule interacts with the opposite charge on the outer membrane surface of bacteria or fungi, and then the hydrophobic region (lauroyl group) of LAE may osmosis the hydrophobic core of the outer membrane (Pattanayaiying et al. 2014), which results in the

deconstruction of the outer membrane and the increase of membrane penetrability. Besides, the susceptibility of the three cell types to LAE revealed by the PI staining was in accord with the results of the protein and nucleic acid leakage. It was noteworthy that the concentration of LAE (100–200 µg/ml) causing significant leakage of protein and nucleic acid from *P. carotovorum* was 4–8 times higher than the MIC value (25 µg/ml), indicating that 25 µg/ml of LAE had an inhibitory effect on *P. carotovorum*, whereas this amount of LAE had not yet significantly influenced the membrane penetrability. In such a case, the bacteria might be in a state of viability but nonculture (Oliver 2010).

Our study also suggested that LAE significantly disturbed the membrane potential and led to the depolarisation of cells (Fig. 1). Coronel-Leon et al. (2016) revealed that LAE changed the membrane potential of *Yersinia enterocolitica* and *Lactobacillus plantarum* resulting in the leakage of intracellular K⁺, which is a key ion essential for maintaining osmotic pressure and membrane potential.

There are few reports about the morphological structure of moulds treated with LAE. As for bacteria, the microchange of *P. carotovorum* after exposure to a low concentration of LAE was similar to that reported in the existing literature (Coronel-Leon et al. 2016; Rodriguez et al. 2004). It was reported that the hydrochloride might cause the coagulation of DNA and cellular protein (Zhou et al. 2010). Pattanayaiying et al. (2014) also observed the degeneration and coagulation of cellular materials in *E. coli* treated with LAE, which was also reflected in the present research. Our TEM results revealed that LAE caused a rough cellular surface, irregular cellular organelles, protoplast shrinkage, intracytoplasmic coagulation and empty cavities in all three cell types (Fig. 2). Taking the results of protein and nucleic acid leakage together with the TEM observations, we showed that the sensitivity to LAE was ordered *P. carotovorum* > hyphae of *P. digitatum* > spores of *P. digitatum*.

Conclusion

LAE exhibited potent antimicrobial effects on *P. digitatum* and *P. carotovorum*, typical pathogenic microorganisms of postharvest fruit or vegetables. The results indicated that LAE at low concentration disturbed the membrane potential to inhibit growth of the microorganism. Subsequently, the membrane structure and cellular organelles were damaged when the concentration of LAE was increased. As a consequence, the permeability of the cell membrane was dramatically increased, and cellular material was released from the inactivated microorganism. This study suggested that LAE has great application potential for preventing the decay of fruit and vegetables.

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