Antimicrobial activity of Lauroyl Arginate Ethyl (LAE), against selected food-borne bacteria

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Abstract

The antibacterial activity of the novel antimicrobial substance, Lauroyl Arginate Ethyl (LAE) was examined against five food-borne bacteria (Staphylococcus aureus, Listeria innocua, Escherichia coli, Pseudomonas aeruginosa and Salmonella enterica). Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determined by a broth microdilution method showed that LAE exhibit a strong antimicrobial activity against all strains tested. This antimicrobial activity was independent of the inoculum size and remained after two heating treatments. The results obtained in the survival study demonstrated that LAE showed a fast bactericidal effect. Images of scanning electron microscope suggested that cells membranes are the principal target of LAE.

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1. Introduction

In the last decades, food industry has intensified effort to modify their products to the current market. The demand for minimally processed, easily prepared and ready-to-eat ‘fresh’ food products, besides the globalization of food trade and distribution from centralized processing, have involved the development of new technologies to extend the shelf-life of food while maintaining its quality, freshness and safety (Appendini & Hotchkiss, 2002; Suppakul, Miltz, Sonneveld, & Bigger, 2003).

Some of these techniques have been designed to prevent the microorganism proliferation avoiding the food degradation and reducing the risk of pathogens. These include active packaging, in which antimicrobial agents are incorporated into the packaging material creating a protective layer, either by vapor phase or by direct contact with the food (Appendini & Hotchkiss, 2002; López, Sánchez, Batlle, & Nerín, 2007).

However, to get an efficient antimicrobial material is not an easy task. In recent years, antimicrobial materials containing Essential Oils (EOs) as antimicrobial agent have been developed, and several effective antimicrobial packaging have been obtained (Gutiérrez, Escudero, Batlle, & Nerín, 2009; Gutiérrez, Sánchez, Batlle, & Nerín, 2009; Manso, Cacho-Nerin, Becerril, & Nerín, 2013; Rodríguez, Nerín, & Batlle, 2008).

Nevertheless, these substances usually have strong odor and thus, they can modify the organoleptic properties of the packaged foodstuff being incompatible with the foodstuff. Consequently, active materials containing a particular EO can be used only for a small variety of food. Antimicrobial packaging material intended for using in a wide variety of food, need to consider other non-aromatic active substances with very strong antimicrobial properties, which must be completely safe for the consumer.

There are other antimicrobial compounds that could be used for this purpose and one good example is the use of certain surfactants. Due to its chemical structure, surfactants are capable of disintegrate cell membranes at very low concentrations, producing the alteration of membrane potential, cell permeability and consequently the bacterial death (Rodríguez, Seguer, Rocabayera, & Manresa, 2004). Lauroyl Arginate Ethyl (LAE) is a new surfactant derived from lauric acid and arginine whose antimicrobial properties have been shortly described in literature (Luchansky et al., 2005; Rodríguez et al., 2004; Woodcock, Hammond, Ralyea, & Boor, 2009).

Toxicological studies have determined that LAE was rapidly metabolized by humans to the naturally occurring dietary components lauric acid and arginine (Hawkins, Rocabayera, Ruckman, Segret, & Shaw, 2009) and thus, it is considered as a safe product. Besides, LAE has been Generally Recognized as Safe (GRAS) by the FDA in 2005. Therefore, LAE represents a potential...
non odorous alternative to essential oils for the development of new food preservation alternatives including antimicrobial active packaging.

The aim of this study is to evaluate the antimicrobial activity of LAE against foodborne pathogens as alternative of EOs in active food packaging. For this purpose, the antimicrobial activity, inactivation kinetics and growth kinetics were determined. Furthermore, morphological alteration in Escherichia coli due to treatment with the antimicrobial substance has been studied by scanning electron microscope. The results obtained are shown and discussed.

2. Material and methods

2.1. Antimicrobial substances

Lauroyl Arginate Ethyl (LAE: Chemical Abstracts Service (CAS) Registry number 60372-77-2) was supplied by VEDEQSA S.A. (Barcelona, Spain).

2-fold serial dilutions of LAE were prepared in distilled sterile water.

2.2. Bacterial strains

The following foodborne microbial strains were selected for use in the tests: Gram-positive bacteria Staphylococcus aureus (American Type Culture Collection (ATCC) 29213) and Listeria innocua (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) 20649); Gram-negative bacteria E. coli (American Type Culture Collection (ATCC) 25922), Pseudomonas aeruginosa (American Type Culture Collection (ATCC) 27853) and Salmonella enterica subspecie enterica (Colección Española de Cultivos Tipo (CECT) 556).

The strains were stored at −18 °C in sterilized skimmed milk and subcultured as follows. Bacteria were subcultured in Mueller–Hinton agar (MH) at 37 °C for 24 h except for L. innocua, which was subcultured in Soya Triptone agar (TSA), and subsequently suspended in physiological saline solution to provide adequate optical density at 625 nm.

2.3. Antimicrobial susceptibility test

To study the inhibitory concentrations, a broth dilution method was used (Becerril, Gómez-Lus, Gohi, López, & Nerín, 2007). Briefly, 10 μL of LAE solution was mixed with 990 μL of the appropriate broth medium containing 10^5 Colony Forming unit (CFU)/mL of tested bacteria to final concentration ranging from 6.25 to 200 mg/L. The cultures were incubated at 37 °C overnight with shaking. After the incubation, the bacterial growth was determined by measuring the optical density at 625 nm. The minimal inhibitory concentration (MIC) was defined as the lowest concentration in which bacterial growth was not detected. To determine the minimal bactericidal concentration (MBC) the corresponding decimal dilutions were made and plated in TSA. MBC was defined as the lowest concentration of the active substance that produces at least a 3 log reduction in the number of surviving bacteria.

MICs and MBCs were expressed as milligrams of antimicrobial agent per liter (mg/L).

Controls samples were carried out without LAE.

In order to determine the influence of inoculum size in the antimicrobial activity, the test was repeated against E. coli using different inoculum concentrations, 10^2, 10^3 and 10^4 CFU/mL. Some technologies of producing active packaging need high temperatures, so MICs and MBCs were determined after a heat treatment of the antimicrobial substance. Heat treatments consisted of heating LAE to 90 °C or 120 °C for 15 min in a closed vial.

2.4. Inactivation study

50 ml of broth medium containing 10^6 CFU/ml of the bacteria under study was mixed with the appropriate dilution of LAE to obtain the concentration equal to MBC and incubated at 37 °C. Immediately after being prepared, that means at “time 0”, a 100 μL aliquot was removed, appropriated diluted and 100 μL were spread on the solid media. After the incubation period (24 h at 37 °C), the number of survivors (CFU/ml) was determined by counting the colonies. This process was repeated at different times depending on the active agent evaluated.

Controls without antimicrobial substances were carried out in each experiment.

Survival curves were obtained by plotting the logarithm of the surviving fraction versus incubation time in the presence of LAE. The survival fraction is calculated as N/N0, where N0 is the initial number of viable cells (CFU/ml) and N is the number of cell survivors (CFU/ml).

2.5. Scanning electron microscopy

Using the method described in the antimicrobial susceptibility test, E. coli was treated in liquid media with LAE at MIC concentrations for 3 min and 5 min. Control without adding the antimicrobial substance were also carried out. After the treatment, E. coli were harvested by centrifugation, re-suspended in saline solution (NaCl 0.9%) and washed twice. The pellet obtained was fixed in 2.5% glutaraldehyde and 0.3% sucrose in 0.1 M sodium cacodilate solution, and then dehydrated in graded alcohols (30%, 50%, 70%, 90%, and 100%). The obtained suspension was filtered on a 0.2 μm Isopore™ Membrane (Millipore), air-dried, and then, sputter-coated with gold under vacuum. Morphology of the E. coli cells was observed on an Inspet F scanning electronic microscope (FEI) working at 5–30 kV and reaching a resolution of 1.5 nm.

3. Results

3.1. Antimicrobial susceptibility test

Table 1 shows MICs and MBCs obtained in the antimicrobial test. As can be seen, LAE is a stronger antimicrobial against all bacteria tested since MIC values were between 100 and 12.5 mg/L.

P. aeruginosa was the most resistant bacteria with MICs and MBCs from 4- to 8-fold higher than for the other bacteria. E. coli, L. innocua and S. enterica showed a similar susceptibility with a maximum difference of 2-fold dilutions.

According to the data from Table 1, LAE exhibit a bactericidal effect (MBC/MIC = 1) for all bacteria except against S. aureus, since the ratio MBC/MIC was higher than one.

In order to determine the influence of inoculum size in the antimicrobial activity, MICs and MBCs were determined against the strains of E. coli, L. innocua and S. enterica. As can be seen, LAE is a stronger antimicrobial for this bacteria than for P. aeruginosa. Moreover, LAE has an equal or longer bactericidal effect (MBC/MIC = 1) for all bacteria except against S. aureus.
E. coli using different inoculum concentrations. As it is shown in Table 2, the antimicrobial activity of LAE was not affected by the inoculum size.

Some technologies of producing active packaging involve processes at high temperature. To assure that the antimicrobial agents maintain their antimicrobial properties after the treatment with high temperatures, MICs and MBCs for E. coli were determined after heating LAE at 90 °C and 120 °C for 15 min. The results (Table 3) showed that MICs and MBCs were the same as those obtained without treatment.

3.2. Inactivation kinetics study

Fig. 1 show the experimental inactivation curves obtained from each antimicrobial and bacteria. These data indicate that LAE behave as fast bactericidal, reducing 3.5 log bacteria between 2 and 6 min.

3.3. Scanning electron microscope (SEM) observation

In order to check for morphological changes resulting from the antimicrobial treatment, SEM was performed on bacteria exposed to LAE at MIC concentrations. Microphotographs show that treated bacteria displayed considerable morphological alterations in comparison to the control bacteria (Fig. 2A). After 3 min of exposure (Fig. 2B), the cells, exhibit external modifications as irregular shape, significant rough surface, pore formation and cell debris. After 5 min of exposure (Fig. 2C), alterations are more noticeable and a high number of collapsed bacteria are detected. All these alterations on the outer bacteria envelope may indicate that cell membranes are the main target of this substance.

4. Discussion

Due to their antimicrobial properties, Essential Oils have been studied as food preservatives in different applications, including active packaging technologies, with good results (Manso et al., 2013; Rodríguez et al., 2008). However, their strong odor can modify the organoleptic properties of the packaged food, thus preventing their use in some types of food. For this reason, it is necessary to investigate other non-odorous compounds that can be used as alternative in these cases. In this study, the antimicrobial activity of the non-odorous compound LAE is evaluated in order to investigate the potential activity of this compound in their use in food preservation.

The data obtained in the antimicrobial susceptibility test have demonstrated that the LAE showed a strong antimicrobial activity against the foodborne bacteria studied. This activity is ten times higher than that obtained for the essential oils of oregano and cinnamon in a previous work (Becerril, Nerín, & Gómez-Lus, 2012), thus presenting an interesting potential as antimicrobial substance in food industry. As expected, P. aeruginosa was the most resistant bacteria to LAE. This bacteria contains a layer of extracellular polysaccharide in its cell wall, similar to a capsule, which confers to this cell a stronger resistance to antimicrobials (Joklik, Willett, & Amos, 1992).

LAE is not a natural compound like Essential Oils, however, it has been demonstrated that it is a safe molecule for the consumer. Hawkins et al. (2009) proved that LAE is hydrolyze in the human body into lauric acid, a fatty acid naturally present in the organism, and arginine, an amino acid considered essential in childhood. These results indicated that LAE is rapidly metabolized to natural endogenous compounds.

Besides its strong antimicrobial properties, LAE has several characteristics that offer advantages in the development of new alternatives in food preservation for a general application, some of them have been demonstrated in this study.

First, it has been proved that LAE keeps its antimicrobial properties after heat treatments. This advantage would allow the use of LAE in applications that require high temperature, such as some types of active packaging. Besides, in the tested conditions it has been demonstrated that the activity of LAE is neither affected by using a higher inoculum size. This means that this substance may be useful with high contaminated food. Finally, this study has demonstrated that LAE showed a fast antimicrobial activity according to the inactivation curves, meaning that it is necessary little time of exposure to LAE to reduce the viability of bacteria.

In addition to these advantages, it is important to point out that other studies demonstrated that the antimicrobial properties of LAE remain constant from pH 3 to pH 7 (EFSA-Q-2006-035, 2007), suggesting that this substance may be useful as antimicrobial agent for a wide range of food.

To investigate the mechanism of action of LAE, SEM was employed. The images have demonstrated that LAE led to dramatic changes in cell envelope, indicating that membranes are the main target of this substance. This hypothesis is in consonance with the results obtained by Rodríguez et al. (2004), which demonstrate that LAE interact with the lipids from the bacterial membranes, producing disturbance in membrane potential and structural changes in Salmonella typhimurium and S. aureus. Images obtained in the present study have demonstrated that LAE also disrupt the membrane of E. coli, producing leakage of cytosol components and consequently the cell death. However, cell lysis was not detected by Rodríguez et al. (2004) in S. typhimurium or S. aureus.
The results obtained in the survival study are in agreement with this hypothesis, since LAE showed fast bactericidal activity that is in concordance with a fast mechanism of action, as membrane disruption.

Similar alterations were found in a previous work (Becerril et al., 2007) for the essential oil of oregano indicating that both substances could act by disrupting membrane integrity. Moreover, inactivation kinetics of OR (data not published) also have indicated that OR have a fast bactericidal activity according to this mechanism of action.

5. Conclusions

This work has shown that LAE possess strong and fast antimicrobial properties against foodborne pathogens in direct contact with them. This activity is even higher than that obtained for OR that it is supposed to have the same mechanism of action. Furthermore, LAE maintains its high antimicrobial activity in different situations, and therefore, it could be a potential alternative as antimicrobial agent in food industry in different applications, including the promising antimicrobial packaging. Additional experiments must be done in order to develop active packaging containing LAE and to study their efficiency.

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