Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia Food Science 1 (2011) 173 – 177

Procedia Food Science

11th International Congress on Engineering and Food (ICEF11)

Improving water solubility of natural antibacterials to inhibit important bacteria in meat products

Lidia Dorantes[a*], Gerardo Aparicio[b], Arturo Ramirez[a]

[a] *Biochemical Engineering,ENCB, Instituto Politécnico Nacional, Mexico DF. Project ICYTDF PICS08-15*
[b] *Microbiology Department,ENCB, Instituto Politécnico Nacional, Mexico DF,*

Abstract

The use of natural antimicrobial compounds, such as cinnamic acid and its phenolic derivatives, may help prevent survival of *Escherichia coli* O157:H7 and other pathogenic bacteria in foods. The limitation of the widespread use of these acids is their low solubility in water systems. Therefore the objective of this work was to evaluate the impact of the concentration of soluble salts of hydroxycinnamic acids on the viability of *E. coli* O157:H7 and *S.* Gallinarum. The solubility enhancement of the compounds at pH 7 could bring important consequences, since, for instance, the majority of outbreaks involving *E. coli* O157:H7 have been caused by the ingestion of meat and lacteous derivatives that present nearly neutral pH. Also, experimental errors in the evaluation of inhibitory concentrations may be prevented, avoiding lack of solubility of phenolic acids and their tendency to precipitate during incubation of media. Morphological changes on ultrastructure of *E. coli* O157:H7 was analyzed using transmission electron microscopy. Sodium salts of ferulic and *p*-coumaric acids were prepared and tested against *E. coli* O157:H7 in concentrations of 0.2, 0.4, 0.6, 0.8 and 1% in trypticase soy medium at pH 7. A bacteriostatic and/or bactericidal effect was also concentration-dependent. A bactericidal effect was found at concentrations of 0.8 and 1%, a bacteriostatic effect at the intermediate concentration of 0.6%, and an almost normal growth at the lowest concentrations of 0.2 and 0.4% showing their potential to be used as pathogen inhibitors.

Selection and/or peer-review under responsibility of 11th International Congress on Engineering and Food (ICEF 11) Executive Committee.

Keywords: water solubility; natural antimicrobials, meat

1. Introduction

A variety of foods have been implicated in outbreaks of illness attributed to pathogenic bacteria, such as *E. coli* O157:H7. Food industry has re-focused attention on *E. coli* O157:H7 as a cause of significant morbidity and mortality in outbreaks of food-borne illness. Among foods implicated in outbreaks due to

[a]Corresponding author: Graduados en Alimentos, Escuela Nacional de Ciencias Biológicas, IPN. Carpio y Plan de Ayala s/n Santo Tomás. Mexico 11340, DF. AP 42-186. Tel.: +52-55-57296000 X 62467 ldoran@ipn.mx

2211 601X
Selection and/or peer-review under responsibility of 11th International Congress on Engineering and Food (ICEF 11) Executive Committee.

doi:10.1016/j.profoo.2011.09.027

E. coli O157:H7 are: milk and milk derivatives such as cheeses, un-pasteurized cream; meat products such as hamburgers, undercooked beef patties, cooked meats, and canned salmon [1]. Foods with lower pH have also been implicated, such as apple juice. Indeed, inadequate thermal treatments of foods and lack of good manufacturing practices are associated with the outbreaks. However, the presence of antimicrobial compounds may constitute an additional barrier to the survival of pathogenic bacteria.

On this regard, the antimicrobial activity of phenolic compounds has been challenged against several bacteria, recognizing that Gram-positive are generally more sensitive to them. Among these compounds, the substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids present in foods from vegetable origin [2]. As an example, the antibacterial activity of Capsicum extracts against several pathogenic bacteria has been demonstrated by our working group [3]. In most cases, the extracts of Capsicum have been prepared using isopropanol and the active compounds using small quantities of ethanol, since most of the active compounds present poor solubility in water. Nevertheless, the presence of isopropanol in foods is not allowed, and an exhaustive evaporation of the solvent should be done following the extraction.

Considering the above the objective of this work was to improve the solubility of two natural antimicrobials, ferulic and coumaric acids, in water, and to test its antibacterial activity against two bacteria important in meat products.

2. Materials & Methods

The sodium salts of the phenolic acids were prepared mixing equimolecular amounts of the phenolic acid and sodium hydroxide. The solubility of ferulic and coumaric acid was successfully increased and a challenge test was performed with the salts. Two media were used for the challenge test: one was trypticase soy broth (TSB) and the other was a meat soup. This was prepared in such a way as to have 9% of meat solids, 1% of salt, and 90% of water. Several groups of beakers were prepared: the first froup (controls) contained 50 mL of soy broth; the second group besides the broth contained 0.2, 0.4, 0.6,0.8 and 1% ferulate; and the third group besides the broth contained 0.2,0.4,0.6, 0.8 and 1% coumarate. Each beaker was inoculated with 10^4 CFU/mL of *Escherichia coli* O157 and incubated at 37ºC for 24 and 48 hours. Afterwards, a bacterial count was performed using the technique of Miles and Mishra [4]. Additionally a challenge test was prepared with the meat soup using 1% ferulate and 1% coumarate and challenged as described above with the bacteria.

The ultra-structural analysis was made on *E. coli* O157:H7 treated with two different concentrations of coumarate, one with bactericidal effect (1%) and another with bacteriostatic effect (0.6%). Two millilitres of bacterial suspensions were grown overnight and added to 18 mL of TSB containing the corresponding concentration of coumarate, and then incubated at 37ºC for 24 h. Aliquots of 1.5 mL were taken every 2 h until a time of 10 h had elapsed, and then a final one after 24 h. Each sample was centrifuged at 6000 rpm for 5 min to be fixed with 2.5% glutaraldehyde (Electronic Microscopy Science-EMS; Washington, USA) in phosphate regulator solution (pH 7.3) for 1 h. It was washed three times with the regulator solution and then post fixed with 1% osmium tetraoxide (EMS; Washington, USA) in the same solution for 1 h. Afterwards, each sample was washed with ethanol-water solutions of increasing concentrations (40-90%) for 10 min and then 100% ethanol (three changes of 10 min each). The samples were mixed with propylene Epson-oxide resin (EMS; Washington, USA) 1:2, 1:1 and 3:1 for 2 h. Finally, they were included in 100% Epson resin with two changes of 2 h each, then identified and incubated at 60ºC for 24 h to achieve resin polymerization. The polymerized samples were cut with a Leica ultra- microtome model Ultracut UCT, contrasted with uranyl acetate and lead citrate, before they were examined with the electron transmission microscope Jeol model JEM 1010 at an acceleration voltage of 60KV, Akishima, Japan [5].

3. Results & Discussion

The lack of solubility of antibacterial compounds in aqueous systems, often found in food supply, markedly reduces their activity. Therefore, sodium salts of ferulic and coumaric acids were prepared in increasing concentrations of 0.2 to 1%. The percentages of dissociated hydroxycarbonyl groups as well as the non-dissociated hydroxyl groups for ferulic and coumaric acids were calculated from the pKa values. Hydroxycarbonyl groups are almost completely in the anionic form (≥99%) in both cases, while hydroxyl groups are practically non-dissociated (≥96%) at pH 7. For the purpose of this text, the above compounds will be referred as ferulate and coumarate.

The solubility enhancement of the compounds at pH 7 could bring important consequences, since, for instance, the majority of outbreaks involving *E. coli* O157:H7 have been caused by the ingestion of meat and lacteous derivatives that present nearly neutral pH. Also, experimental errors in the evaluation of inhibitory concentrations may be prevented, avoiding lack of solubility of phenolic acids and their tendency to precipitate during incubation as reported by Wen *et al*. [6].

In order to show the growing, survival and inactivation of the bacteria, the media of the log of the number of survival bacteria was calculated from the experimental data, and plotted versus the time of incubation.

At the beginning of each experiment, every culture was maintained during the log phase, and adjusted to obtain a final population of about 10^4 CFU/mL. The number of survival units was evaluated by triplicate after 0, 4, 8, 12, 24, 36 and 48 h in samples with different anti-microbial concentrations, and the average of these evaluations was used to draw the figures. Controls did not present antimicrobial compounds and the populations increased from 10^4 to 10^9/10^{10} CFU/mL, without detecting a lag phase. The results showed that *E. coli* O157:H7 was inhibited by coumarate and ferulate in soy broth (Figures 1 and 2). Similar results were obtained for S. Gallinarum (data not shown).

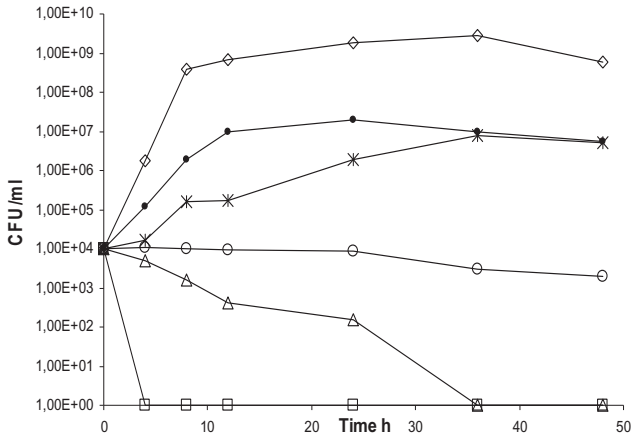

Fig. 1. Survival kinetics of *Escherichia coli* O157:H7 exposed to different concentrations of sodium coumarate (1 % ; 0.8 % ; 0.6 % ; 0.4% ; 0.2 % ; control)

The impact of ferulate and coumarate over the survival kinetics of *E. coli* O157:H7 and the *Salmonella* was very similar and also concentration-dependent. A bactericidal effect was found at concentrations of 0.8 and 1%, a bacteriostatic effect at the intermediate concentration of 0.6%, and an almost normal growth at the lowest concentrations of 0.2 and 0.4%, as shown in Figures 1 and 2. Previous reports on the antimicrobial activity of phenolic acids are often contradictory due to differences in experimental

methodologies, medium composition and limitations due to poor solubility of phenolic acids [6]. When using agar plates, little or no activity may be observed because of the low solubility and diffusion of the active compounds. In the present work no problems of solubility were observed, since the corresponding salts were used instead.

Figure 2 show the effect of different concentrations of ferulate on the viability of *E. coli* O157:H7 and showing a significant difference at a level of $p \leq 0.05$, when concentrations of ferulate and coumarate were compared with the controls. A complete inactivation of E.coli was obtained in the meat soup added with 1% of coumarate or ferulate. This supports the idea of using these phenolic acids as another factor in the multiple barrier technology for preserving foods, particularly in the meat products chain.

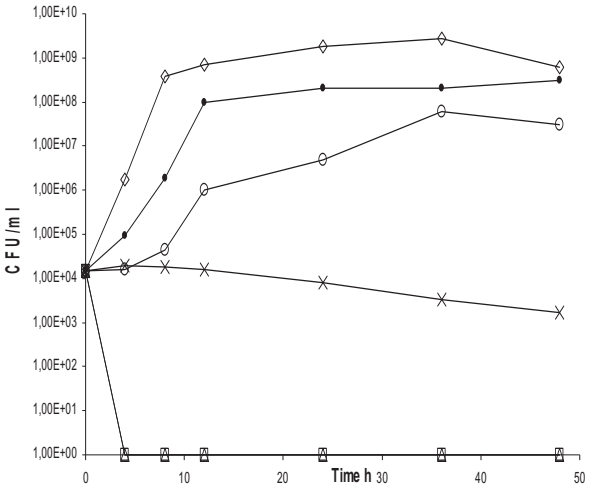

Fig. 2 . Survival kinetics of *Escherichia coli* O157, exposed to different concentrations of sodium ferulate (1 % ; 0.8 % ; 0.6 % ; 0.4% ; 0.2 % ; control)

Morphological alterations on *E. coli* O157:H7 could be observed on the micrographs (not shown) obtained with transmission electron microscopy. These alterations took place after exposing both bacteria to a coumarate concentration of 0.6% (bacteriostatic) or 1% (bactericidal).

The micrographs of *E. coli* O157:H7 shows that the control cells after 24 h showed an intact internal and external membranes structure, with continuity and a homogeneous cytoplasm. However, after 2 h of exposure to 0.6% coumarate or bacteriostatic concentration, there was an increase in the electronic density of the cytoplasm, and membranes were evident. From 6 h to 24 h elongated cells appeared, which measured up to 12 μm; this was an indication of a mechanism of adaptation.

A bactericidal effect was observed when *E. coli* O157:H7 was exposed to a 1% concentration. Micrographs taken after incubation times of 2 h, and 24 h showed a contraction of the inner membrane and its content, creating a large inter-membrane space, mainly on the far extremes of the bacteria, which increased with time. Bacterial cells with vacuole formation on the cytoplasm were also observed. On this condition, no modification of the tri-laminar structure typical of Gram negative bacteria was apparent. The cytoplasmic content appeared to lose density, which suggested the presence of a degradation process. This phenomenon was observed in both the cells with vacuole formation and on the cells with contracted membranes. Several membrane fragments were observed, which indicated cellular death. The

morphological change of elongation of *E. coli* O157:H7 at a bacteriostatic concentration was observed by Hajmeer *et al*. [7] when they tested the effect of a 10% sodium chloride concentration. In both cases, irregularities in cell morphology such as elongation, partial disintegration of the membrane, and loss of integrity due to exposure to stressing conditions were observed and were all concentration-dependent. According to the TEM observations, bacterial death by effect of coumarate at 1% matched the loss of cell integrity due to salt exposure.

4. Conclusion

Solubility of hydroxycinnamic acids was increased in aqueous media when using the salt forms. An inactivation of the *Escherichia coli* O157 was observed in the presence of sodium coumarate and ferulate. Similar results were obtained for *S.* Gallinarum. This increases their potential to be used as bacteria inhibitors. It has been suggested that hydroxyl groups located on the benzene ring play an important role on bacterial death. Both bacterial strains were equally sensible to ferulate and coumarate.

References

[1] Bell, C., 2002a. Pathogenic *Escherichia coli.* In: Blackburn, C.W., McClure P.J. (Eds.), Foodborne Pathogens. Woodhead Publishing Limited, Florida, USA, pp. 279-306.

[2] Shahidi, F., Naczk, M., 1995. Phenolic compounds in fruits and vegetables. In: Food Phenolics. Technomic Publishing Company. Lancaster, Pennsylvania, USA, pp. 75-107.

[3] Careaga, M., Fernández, E., Dorantes, L., Mota, L., Jaramillo, M., Hernández H., 2003. Antibacterial activity of Capsicum extract against *Salmonella typhimurium* and *Pseudomonas aeruginosa* inoculated in raw beef meat. International Journal of Food Microbiology 83, 331-335.

[4] Miles, A.A., Misra, S.S., 1938. The estimation of the bactericidal power of the blood, Journal Hygiene 38,732-749.

[5] Bozzola, J.J., Russel, L.D., 1991. Electron microscopy. Principles and techniques for biologists. Jones and Barlett. Sudbury MA, USA, pp.18-19.

[6] Wen, A., Delaquis, P., Stanich, K., Toivonen, P., 2003. Antilisterial activity of selected phenolic acids. Food Microbiology 20, 305-311.

[7] Hajmeer, M., Ceylan, E., Marsden, J.L., Fung D.Y.C., 2006. Impact of sodium chloride on *Escherichia coli* O157:H7 and *Staphylococcus aureus* analyzed using transmission electron microscopy. Food Microbiology 23, 446-452.

Presented at ICEF11 (May 22-26, 2011 - Athens, Greece) as paper FMS576.