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Comparison of the Antibacterial Activity of Chelating Agents Using the Agar Diffusion Method

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Abstract: The agar diffusion assay was used to examine antibacterial activity of 2 metal chelators. Concentrations of 0-40 mM of ethylenediaminetetraacetic acid (EDTA) and ethylenediamine-N,N'-disuccinic acid (EDDS) were prepared in 1.0 M potassium hydroxide (KOH). The pH of the solutions was adjusted to 11.0 with citric acid and wells in agar media seeded with bacterial isolates were filled with the solutions. Agar plates were incubated at 35°C for 18-24 h and zones of inhibition around the agar wells were measured. Results indicated that 10 mM EDTA produced significant ($p<0.05$) zones of inhibition of *Acinetobacter calcoaceticus*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus simulans* growth, while 20 mM of EDTA produced significant ($p<0.05$) zones of inhibition of *Salmonella typhimurium*. Increases in the concentration of EDTA added to agar wells generally produced significantly increases in the size of zones of inhibition. EDDS only inhibited growth of *A. calcoaceticus* and *P. aeruginosa*. Significant ($p<0.05$) zones of inhibition of both isolates were produced by 10 mM of EDDS and significantly larger zones were produced by higher concentrations of EDDS, although intrazonal growth of *A. calcoaceticus* was present in all zones of inhibition of this isolate. The addition of these chelators to formulations of sanitizers used in poultry processing may improve the ability of sanitizers to wash away microorganisms on processed carcasses, but findings from this study indicate that these chelators also possess antimicrobial activity that may aid in reducing contamination of carcasses.

Key words: Chelators, bactericidal, agar diffusion assay, EDTA, EDDS

INTRODUCTION

Despite the use of several commercially available chemical sanitizers, poultry meat contaminated by pathogenic bacteria continues to be widely cited as a major cause of human foodborne diseases (Friedman *et al*., 2004). Therefore, alkaline salts of fatty acids are currently being examined as possible sanitizers for poultry processing operations (Hinton *et al*., 2009). *In vitro* tests utilizing the agar diffusion assay have shown that alkaline salts of fatty acids prepared by dissolving caproic, caprylic, capric, lauric, or myristic acids in potassium hydroxide (KOH) possess antibacterial activity towards several bacteria associated with poultry processing (Hinton and Ingram, manuscript submitted for publication). Alkaline salts of these fatty acids are Generally Recognized as Safe (GRAS) (Kabara *et al*., 1977) surfactants that can be used to wash broiler carcasses during poultry processing (Hinton *et al*., 2009). Effective formulations of these bactericidal surfactants may serve as alternatives to chemical sanitizers (Oyarzabal, 2005) that are currently used in commercial poultry processing.

The cleansing ability of alkaline salts of fatty acids and other surfactants may be improved by including detergent builders (Nagarajan and Paine, 1984) in the formulation of these sanitizers. Minerals, such as calcium and magnesium, dissolved in water can increase water hardness (Hinton and Holser, 2009), thereby decreasing cleansing activity of surfactants. Detergent builders reduce water hardness by sequestration, precipitation, or ion-exchange of minerals dissolved in hard water. Ethylenediaminetetraacetic acid (EDTA) and (S, S)- ethylenediamine-N,N'-disuccinic acid (EDDS) are two chelating agents that can be used as detergent builders in surfactant formulations. These compounds are sequestrants that can bind minerals dissolved in water, thereby inhibiting the ability of these water hardness minerals to reduce the cleansing efficiency of surfactants.

Previous research has indicated that due to its relative resistance to biodegradation (Means *et al*., 1980), EDTA may persist in the environment when it is introduced into streams and rivers via wastewater. Therefore, biodegradable chelators such as EDDS have recently

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been developed (Meers *et al*., 2005). Earlier research has indicated that EDTA may possess bactericidal activity (Estrada *et al*., 2010) and a recent study (Hinton and Ingram, manuscript submitted for publication) illustrated that the agar diffusion method can be used to screen the antibacterial activity of various formulations of carcass sanitizers. Since EDTA and EDDS may be included in formulations of these sanitizers, the objective of this study was to utilize the agar diffusion assay to examine the antibacterial activity of EDTA and EDDS.

MATERIALS AND METHODS

Bacterial cultures: Fresh cultures of *Acinetobacter calcoaceticus*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus simulans* (Hinton and Ingram, 2005) isolates were grown in Difco Tryptic Soy Broth (Becton Dickinson and Co., Sparks, MD, USA). Cultures were incubated at 35°C for 18-24 h at 100 rpm in a Model D76 gyrotory water bath shaker (New Brunswick Scientific, Edison, NJ, USA). Cultures were harvested and suspended in a solution of 0.1% Difco Bacto Peptone (Becton Dickinson and Co.) water as previously described (Hinton and Ingram, 2005) to produce bacterial suspensions containing 10^7 cfu/ml.

Difco Trypticase Soy Agar (TSA) (Becton Dickinson and Co.) containing 90% of required water was prepared, sterilized at 121°C for 15 min and tempered to 50°C in a water bath. Tempered agar media was seeded with bacterial cultures by adding a volume of bacterial suspensions equivalent to the remaining 10% of the required agar volume to produce an inoculated agar media containing 10^8 cfu/ml. Bacterial suspensions were mixed in tempered agar and 25 ml aliquots of agar were added to Petri plates to produce an agar layer approximately 6.5 mm thick. Solidified agar plates were used immediately or stored at 4°C for up to 2 days before use.

Chelators: Concentrations of 0, 10, 20, 30 and 40 mM of ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) (Sigma Chemical Co; St. Louis, MO, USA) or (S, S)- ethylenediamine-N,N'-disuccinic acid, trisodium salt solution (EDDS) (Sigma) were dissolved in a solution of 1.0 M potassium hydroxide (KOH) (Sigma). The pH of the solutions was adjusted to 11.0 by adding dilute concentrations of citric acid (Sigma). Solutions were filter sterilized by passing through 0.2 µm filters (Nalge Nunc International, Rochester, NY, USA). A suction device (Bell and Grundy, 1968) fitted with metal tubing was used to make 8 mm wells in the solidified, seeded agar that was prepared earlier. Agar wells were filled with 0.1 ml of EDTA or EDDS solution and plates were incubated aerobically at 35°C for 18-24 h. After incubation, zones of inhibition of bacterial growth around the agar wells were measured with a Traceable® Carbon Fiber Digital Calipers (Fisher Scientific, Inc., Pittsburg, PA, USA).

Statistics: Statistical analyses of differences in the size of zones of inhibition produced by EDTA and EDDS were performed with the GraphPad InStat® version 3.05 for Windows 95 (GraphPad Software, San Diego, CA, USA). One-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparison tests was performed to determine significant differences in group means. The P value for all ANOVA tests was <0.05.

RESULTS AND DISCUSSION

Results of the present study indicate that EDTA possesses bactericidal activity towards several bacteria associated with poultry processing (Table 1). Significant increases in the size of zones of inhibition of *A. calcoaceticus*, *E. faecalis*, *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. simulans* were produced by 10 mM of EDTA; however, 20 mM of EDTA was required to produce significant zones of inhibition of *Salmonella typhimurium*. Further increases in the concentration of EDTA added to the agar wells generally produced significant increases in the size of the zones of inhibition of Gram positive and Gram negative bacteria. EDTA is a synthetic chelator produced by the chemical reaction of ethylenediamine, formaldehyde and sodium cyanide (Genik-Sas-Berezowsky and Spinner, 1970). Chelators, such as EDTA, have been reported to possess antibacterial activity because of the ability of these chelating agents to disrupt bacterial cell membranes by removing essential divalent cations required to link lipopolysaccharide molecules in the outer membrane (Vaara, 1992). Furthermore, strong chelators may inhibit microbial metabolism by binding trace minerals required for cellular reproduction, growth and survival (Boziaris and Adams, 1999).

There was less inhibition of bacterial growth around agar wells filled with EDDS when compared to zones of inhibition produced by EDTA. Concentrations of 10, 20, 30, or 40 mM EDDS produced no significant zones of inhibition of *E. faecalis*, *E. coli*, *L. monocytogenes*, *P. aeruginosa*, *Salmonella typhimurium*, or *S. simulans* (data not shown); however, zones of inhibition of *A. calcoaceticus* and *P. aeruginosa* were produced around wells filled with 10 mM of EDDS (Table 2). There was some intrazonal growth by *A. calcoaceticus* around the wells filled with EDDS. Growth of reduced populations of microorganisms within detectable zones of inhibition may be interpreted as only partial susceptibility of microorganisms by microbicidal agents, such as EDDS, which exhibit some antimicrobial activity (Arikan *et al*., 2003). In contrast to EDTA, EDDS is a naturally occurring chelating agent that is produced by several microorganisms (Takahashi *et al*., 1999). Bacteria such as *Sphingomonas*, *Brevundimonas*, *Pseudomonas* and *Acidovorax* have been used in production of this chelator. Since EDDS is a naturally occurring chelator, many microorganisms have probably developed mechanisms to degrade this compound; therefore, it

Table 1: Size of zones of inhibition of bacterial isolates produced by ethylenediaminetetraacetic acid (EDTA) dissolved in potassium hydroxide[1,2]

EDTA Conc. (mM)	*Acinetobacter calcoaceticus*[3]	*Enterococcus faecalis*	*Escherichia coli*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Staphylococcus simulans*
0	0.00[a]±0.00	0.00[a]±0.00	0.00±0.00	0.00[a]±0.00	0.00[a]±0.00	0.00±0.00	0.00±0.00
10	4.76[b]±0.63	1.61[b]±0.41	2.41[b]±0.45	0.58[b]±0.45	1.11[b]±0.40	0.22[a]±0.58	2.23[b]±0.47
20	5.38[bc]±0.98	2.50[c]±0.52	2.87[b]±0.47	1.29[c]±0.37	1.44[b]±0.33	1.84[b]±0.76	2.73[c]±0.35
30	6.05[cd]±0.82	2.75[cd]±0.48	3.46[c]±0.65	3.42[d]±0.54	1.81[c]±0.32	3.07[c]±0.61	3.26[d]±0.61
40	6.57[d]±0.76	3.16[d]±0.54	3.75[c]±0.53	4.22[e]±0.36	2.53[d]±0.42	4.09[d]±0.90	3.89[e]±0.66

[1]Solution pH adjusted to 11.0 with citric acid.

[2]Values are averages ± standard deviation, n = 15.

[a-e]Within columns, different letters indicate significant ($p<0.05$) differences. Conc. = Concentration

Table 2: Size of zones of inhibition of bacterial isolates produced by (*S*, *S*)- ethylenediamine-N,N'-disuccinic acid (EDDS) dissolved in potassium hydroxide[1,2]

EDDS Conc. (mM)	*Acinetobacter calcoaceticus*[3]	*Pseudomonas aeruginosa*
0	0.00[a]±0.00	0.00[a]±0.00
10	4.29[b]±1.47	1.63[b]±0.56
20	6.08[c]±1.07	2.35[c]±0.58
30	8.00[d]±1.20	2.95[d]±0.42
40	9.09[e]±0.90	3.96[e]±0.44

[1]Solution pH adjusted to 11.0 with citric acid.

[2]Values are averages ± standard deviation, n = 15.

[3]Intrazonal growth present.

[a-e]Within columns, different letters indicate significant ($p<0.05$) differences. Conc. = Concentration

may exhibit less toxicity and antibacterial activity than EDTA. EDDS has been shown to increase populations of soil bacteria when it is added to contaminated soil to aid in the removal of metal contaminants; therefore, it is possible that this organic compound may be utilized as a metabolic nutrient that stimulates the growth of some microorganisms (Cao *et al*., 2007).

Chelators are routinely added to surfactant formulations to serve as detergent builders (Kovach, 2007). Chelating agents decrease water hardness by binding minerals dissolved in water, thereby increasing the ability of the surfactants to remove dirt and debris during washing operations. Formulations of alkaline salts of fatty acids are currently being examined as microbicidal surfactants that can potentially be used as sanitizers in poultry processing operations (Hinton *et al*., 2009). The addition of chelators to these formulations may increase the ability of these sanitizers to reduce contamination of broiler carcasses by pathogenic, spoilage and indicator microorganisms. Findings of this study indicate that EDTA and EDDS differ in their ability to inhibit the growth of bacteria associated with poultry processing. However, since capacity of chelators to increase the cleansing activity of surfactants is primarily due to the ability of these compounds to reduce water hardness, each of these chelators may still be considered for use as builders to improve the efficacy of alkaline salts of fatty acids as sanitizers that can be used to reduce microbial contamination of broiler meat.

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