Evaluation by Differential Scanning Calorimetry of the Effect of Acid, Ethanol, and NaCl on *Escherichia coli*

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ABSTRACT

The influence of acid, ethanol, and NaCl on the cellular components and inactivation of *Escherichia coli* were evaluated using differential scanning calorimetry. Cell viability was assessed using plate counting. The thermal stability for ribosomal subunit denaturation and the total apparent enthalpy decreased with increasing ethanol, salt, and acid concentrations. The reduction of the ribosomal subunit denaturation peak was the primary contributor to the decrease in the total apparent enthalpy. Thermograms indicated that even at concentrations at which less than a 0.4-log reduction of cell viability with a concomitant minimal reduction of total apparent enthalpy occurred, a decrease in onset temperature of ribosomal transition was evident. Acid treatments at pH 3 induced by HCl and by 0.4 M acetic acid caused the DNA denaturation temperature in vivo to decrease. Application of chemical treatment prior to heat treatment noticeably reduced the viability of *E. coli* cells at all the heat treatment temperatures (60, 62.5, and 65°C) compared with that of heat treatment alone, suggesting an increased sensitivity of bacteria to heat treatment. Differential scanning calorimetry in vivo can be used to assess the effectiveness of hurdles when thermal processing technologies with hurdles are designed.

Thermal processing is the most common food preservation method for inactivation of pathogenic and spoilage bacteria, producing a safe product with enhanced shelf life. However, high temperatures employed during thermal processing can adversely affect texture, flavor, and the nutrient value of food products. Therefore, mild heating in conjunction with antimicrobial agents has been utilized to preserve nutritional and textural qualities while maintaining extended shelf life. This approach is known as hurdle technology (1, 23, 25). Hurdle technology is based on the reduced thermal resistance observed for bacteria chemically or physically treated before or during heat treatment (20, 24). The effectiveness of hurdle technology can be enhanced when hurdles target different cellular components. The most commonly employed hurdles to reduce the intensity of heat treatment include controlling water activity (a_w), increasing acidity, and using preservatives (2, 8, 9, 28). Several researchers have demonstrated that homeostatic mechanisms of microorganisms are affected by ethanol (15, 17), salt (10, 35), and acids (1, 7), leaving the bacteria in a dormant state or causing death.

The accumulation of ethanol during fermentation inhibits microorganisms. Cell growth and survival in the presence of ethanol varies among bacteria. The D-values of *Salmonella Typhimurium*, *Lactobacillus delbrueckii* (9), and spoilage *Lactobacillus* species (2) decreased with increasing ethanol concentration. Results of studies on mesophilic microorganisms suggest that membrane damage is the primary effect of ethanol on bacteria. Ethanol replaces water molecules in the cell membrane and weakens hydrophobic interactions, leading to disruption of the hydrophobic core that maintains membrane integrity (16). An increase in leakage of small ions such as those of magnesium, potassium, and hydrogen and molecules such as nucleotides accompanies the ethanol inhibition of bacteria (15, 39). At ethanol concentrations above 5%, protein leakage was observed from *Zymomonas mobilis* cells (34).

An increase in external salt concentration changes the osmotic pressure difference across the bacteria membrane. A decrease in cell volume and turgor pressure due to the loss of water from the cell was observed when external salt concentration increased from 1 to 335 mM (31).

Bacteria can keep the intracellular pH at a value close to neutrality in a low-pH environment through homeostasis (12). Under highly acidic conditions (pH of <3), the penetration of H⁺ across the cell membrane exceeds removal of H⁺ (5). When a culture is acidified with an organic acid to a pH lower than the pKₐ of that organic acid, the protonated form of the organic acid molecule diffuses through cellular membrane into the cell. The cells are inactivated by the release of anions and protons from the organic molecule (7). Among weak organic acids, acetic acid (CH₃COOH; pKₐ 4.76) and lactic acid (CH₃CHOHCOOH; pKₐ 3.86) are particularly effective for inactivation of bacteria because their relatively small molecular weight allows them to diffuse easily into the cell (13).

Differential scanning calorimetry (DSC) has been used to characterize the conformational transitions of cellular components of bacteria during heat treatment in vivo (18, 26). The thermal stability of a cellular component can be determined from the peak temperature of the corresponding transition from DSC thermograms of whole cells (4, 6, 18, 26).
DSC. A differential scanning calorimeter (DSC 111, Setaram, Lyon, France) was used to record the thermograms of the untreated and treated E. coli cells. All DSC measurements were conducted using fluid-tight stainless steel crucibles. A DSC run was performed with an unsealed empty sample and reference crucibles to obtain the instrument baseline measurement. Pellets of cells were weighed (56 ± 0.3 mg wet weight) and transferred into the sample crucible. The water content of the pellets was determined using a freeze dryer (Freezone 4.5 freeze dry system, model 77510, Labconco, Kansas City, Mo.) as 80% on a wet basis. For each DSC run, a reference crucible was filled with approximately 45 µl (approximately 80% of sample weight) of pure distilled water. Both crucibles were sealed using aluminum rings and covered with screw caps. The sealed crucibles were refrigerated at 4°C until used for DSC. The sample and reference crucibles were placed in the calorimeter and equilibrated at 1°C using liquid nitrogen.

Samples were heated in the calorimeter at 3°C min⁻¹ from 1 to 150°C. After heating, samples were rapidly cooled with liquid nitrogen and rescanned to observe the reversibility of thermograms. Samples were reweighed after measurements to check for loss of mass during heating, and samples showing signs of leakage were discarded.

Viability after DSC heat treatment following chemical treatment. Weighed cell pellets (approximately 70 mg wet weight) of chemically treated and control cultures were transferred into sterile empty sample crucibles using sterile loops. Each crucible was capped (not sealed) using an aluminum ring and screwed cap. An empty reference crucible was filled with approximately 56 µl (approximately 80% of sample weight) of pure distilled water. The capped crucibles were kept in a refrigerator (4°C) until used for DSC. Pellets in the crucible were heat treated to 60, 62.5, or 65°C with a 3°C min⁻¹ heating rate using the calorimeter. After cooling, 50 µg of the heated pellet from each crucible was transferred with a sterile loop to a 1.5-ml sterile polyethylene tube. Sterile peptone water was added at 1:20 (wt/vol) to make a final volume of 1 ml of suspended cells. Cell suspensions were serially diluted and plated onto TSA. The plates were incubated for 36 h at 37°C, and viable counts were determined.

DSC data analysis. DSC thermograms were corrected for differences in the empty crucibles by subtracting the empty crucible baseline. Total heat corresponding to the endothermic peaks of whole cells (enthalpy, J g⁻¹) between approximately 40 and 130°C were determined by integrating the curve of temperature versus heat flow using software provided by the instrument manufacturer. A curved baseline, taking into account the variation in heat capacity before and after the transition passing through three designated points on the thermogram, was used to calculate the apparent enthalpy of whole cells. Data points at three temperatures were selected to determine the baselines for all DSC curves. The initial temperature point was on the pretransition baseline (40°C). The midpoint was selected at a temperature below the onset of the final peak, which corresponds to transitions in the cell envelope (approximately 108°C). The final point was on the postransition baseline (130°C).

Statistical methods. Differences between control cells and chemically treated cells were compared by equal-variance t tests with the MINITAB statistical program (Minitab Inc., State College, Pa.). Differences were considered significant at P < 0.05.

RESULTS

Effect of ethanol on thermal transitions and viability of E. coli. Figure 1 shows the DSC thermogram for...
**Effect of HCl on thermal transitions and viability of E. coli.** Addition of HCl affected cellular components, as indicated by changes in peak temperatures and areas of transitions in the DSC thermograms (Fig. 3). A substantial decrease in the onset temperature and ribosomal peak (a2) temperature of the cells was observed as the pH of the culture medium decreased. The temperature of a2 decreased by 4°C at pH 4 and by 9°C at pH 3. The acid treatment resulted in the shift of DNA peaks b and c to lower temperatures (Fig. 3 and Table 1). Reductions in the total apparent enthalpy accompanied the acid treatment at pH 4 (approximately 24%) and at pH 3 (approximately 38%). Loss of viability subsequent to acid treatment at pH 4 and pH 3 was minimal (Table 1).

**Effect of acetic acid on thermal transitions and viability of E. coli.** Peak a2 appeared to include two overlapping endothermic transitions for cells treated with acetic acid (Fig. 4 and Table 1). The onset temperature, peak temperature, and enthalpy of the transition a2 decreased as acetic acid concentration increased. At 0.4 N acetic acid in the culture medium, the ribosomal denaturation peak was completely obliterated. The transition b and c shifted to lower temperatures at and above 0.1 N acetic acid (Fig. 4). The total apparent enthalpy decreased with increasing acetic acid concentration. The viability loss before DSC measurement in cells treated with acetic acid was less than 0.4 log units up to 0.2 N acetic acid. A 0.4 N acetic acid concentration in culture medium caused pH to drop to 2.8 and the viability of cells to decrease by 5.3 log units (Table 1).

**Effect of heat treatment on viability of chemically treated E. coli cells.** E. coli culture media containing 12% ethanol, 1.1 M NaCl, 0.1 or 0.2 N acetic acid, or HCl to obtain pH 4 or pH 3 were kept for 1 h and then heated in the calorimeter to 60, 62.5, and 65°C. Viability loss of the E. coli cells before DSC treatment varied between 0.2 and 0.4 log units. The viability of E. coli cells after combined chemical and heat treatment was significantly lower (P < 0.05) than that of the cells after heat treatment alone (Fig. 5). Application of chemical treatment such as HCl (pH 3) or 0.2 N acetic acid prior to heat treatment noticeably reduced the viability of E. coli cells (3.7 log units for HCl and 3.8 log units for acetic acid) at the lowest temperature of 60°C compared with that for cells treated with heat alone (0.1 log units), ethanol and heat (1.0 log units), or NaCl and heat (0.5 log units). For cells in 0.2 N acetic acid medium, an approximately 8-log reduction after heat treatment at 62.5°C was achieved. Viable cells were not observed after 65°C treatment for cells treated with 0.2 N acetic acid or HCl at pH 3. After heat treatment at 65°C, log unit reductions of 7.9 for 0.1 N acetic acid treatment, 6.5 for HCl treatment at pH 4, 6.0 for 12% ethanol treatment, and 5.7 for 1.1 M NaCl treatment were observed,

untreated and ethanol-treated E. coli pellets. Individual endothermic peaks (a through d) were associated with components such as ribosomal subunits (peaks a1, a2, and a3), DNA (peak b), DNA with cell wall (peak c), and the outer membrane of gram-negative organisms (peak d) (22, 26). Ribosomal subunits, which are composed of RNA and ribosomal proteins, were affected by ethanol treatment, but other transitions remained unchanged up to 10% ethanol (Fig. 1). The onset and denaturation temperatures for ribosome transition decreased as ethanol concentration increased (Table 1). The total apparent enthalpy also decreased as a function of ethanol concentration in the culture medium mainly because of the reduction of the ribosomal subunit denaturation transition. After treatment with 12% ethanol, the decrease in peak temperature of the transition associated with outer membrane components (peak d) was significant (P < 0.05).

**Effect of NaCl on thermal transitions and viability of E. coli.** NaCl was added to the medium to assess the effect of increasing concentration on cellular components of E. coli cells and on cell viability. Comparison of thermograms for control and treated cells revealed that only the transitions associated with ribosomal denaturation were affected by 1.1 M NaCl (Fig. 2). NaCl treatments resulted in an absence of peak a1 and changes in peak temperatures of peak a2 and a3. The total apparent enthalpy remained unchanged (P > 0.05) (Fig. 2 and Table 1). After 1.1 M NaCl treatment, the peak temperatures of ribosomal transition was lowered by 2°C for peak a2 and by 1°C for peak a3. A decrease in onset temperature (approximately 8°C) of peak a2 is visible in thermograms of the treated cells. Treatment at 1.9 M NaCl caused the DNA denaturation peak temperature to increase by 1°C (Fig. 2, thermogram C and Table 1). Viability of cells was reduced by approximately 1 log unit at 1.9 M NaCl in the medium (Table 1).
TABLE 1. Effects of chemicals on viability and DSC transitions of E. coli<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Viability loss [−log(N/N&lt;sub&gt;0&lt;/sub&gt;)]</th>
<th>Total apparent enthalpy (J/g)</th>
<th>Onset temperature (°C)</th>
<th>Transition temperature (°C) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Peak a&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Peak a&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>4.19 ± 0.07</td>
<td>54.6 ± 0.5</td>
<td>69.8 ± 0.2</td>
<td>77.9 ± 0.2</td>
</tr>
<tr>
<td>Ethanol (vol/vol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6%</td>
<td>0.01 ± 0.02</td>
<td>4.12 ± 0.14</td>
<td>47.1 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.4 ± 0.2</td>
</tr>
<tr>
<td>10%</td>
<td>0.09 ± 0.01</td>
<td>3.80 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12%</td>
<td>0.35 ± 0.09</td>
<td>3.76 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.2 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.0 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>15%</td>
<td>4.79 ± 0.48</td>
<td>2.85 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.4 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.1 M</td>
<td>0.24 ± 0.13</td>
<td>4.21 ± 0.12</td>
<td>46.6 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.9 M</td>
<td>1.15 ± 0.11</td>
<td>4.20 ± 0.20</td>
<td>46.1 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>pH 4.0</td>
<td>0.15 ± 0.13</td>
<td>3.20 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.8 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>0.22 ± 0.12</td>
<td>2.61 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.2 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.04 N (pH 4.9)</td>
<td>0.09 ± 0.10</td>
<td>4.15 ± 0.11</td>
<td>42.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.8 ± 0.1, 71.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 N (pH 4.2)</td>
<td>0.21 ± 0.04</td>
<td>3.89 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.1 ± 0.1, 70.9 ± 0.3</td>
</tr>
<tr>
<td>0.2 N (pH 3.9)</td>
<td>0.38 ± 0.07</td>
<td>3.01 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.3 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.2 ± 0.3, 68.2 ± 0.1</td>
</tr>
<tr>
<td>0.4 N (pH 2.8)</td>
<td>5.31 ± 0.65</td>
<td>0.82 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the mean ± standard deviation viability loss and DSC transitions independently determined from at least two trials.

<sup>b</sup> Significant differences between control and chemically treated cells at 95% confidence interval.

<sup>c</sup> ND, not detectable.

<sup>d</sup> Peak a<sub>2</sub> appeared to include two overlapping thermal transitions.
wheras the viability of cells treated with heat alone decreased 4.7 log units.

**DISCUSSION**

In previous DSC studies, ribosomes have been suggested as a potential key target for bacterial inactivation by heat (22, 29) or pressure (33). Except for some chelating agents such as EDTA and hydrogen peroxide and some antibiotics such as erythromycin and tetracycline, ribosomal subunits have not been considered as major target sites for most chemicals (14, 27, 32). In the present study, comparison of the DSC thermograms for control cells with those of cells treated with ethanol or acetic acid revealed a major reduction in size and peak temperature of ribosomal subunit transitions in the presence of 15% ethanol or 2% acetic acid with a concomitant 5-log reduction in viability.

Even at concentrations at which minimal reductions in viability and enthalpy of ribosomal transitions were observed, a decrease in onset temperature of ribosomal transition was evident. The decrease in thermal stability of a transition is interpreted as an increase in sensitivity of the state of the material prior to the transition to heat treatment. The results of the present study suggest that the sensitivity of E. coli cells to heat treatment increases following treatment with ethanol, HCl, NaCl, and acetic acid. The increase in sensitivity of bacterial cell to heat treatment or the decrease in thermal stability of ribosomal subunits is chemical specific and a function of the concentration of the chemical.

The reduction in the temperature of ribosomal denaturation transition subsequent to chemical treatment is due mainly to the disappearance of peak a1, which is proposed to be the denaturation of the 30S ribosomal subunit (26). The absence of peak a1 suggests that the smaller flexible ribosomal subunit is susceptible to chemical treatment. The absence of peak a1 observed for *Lactobacillus plantarum* may be attributed to its concealment by shifting of peaks a2 and a3 to lower temperatures due to low pH (22) or to denaturation of the 30S ribosomal subunit as a result of Mg2+ loss (40). The onset temperature decreased slightly, and transition temperatures of peaks a2 and a3 did not change above 1.1 M NaCl in spite of the decreased viability (Table 1).

The change of DNA thermal stability depended on chemical additives. Although addition of ethanol to the me-
The denaturation temperature also is affected by base pair composition. At acidic pH, the stability of DNA with higher GC content is more strongly dependent on pH because of greater protonation of GC pairs than AT pairs (47). Although the denaturation temperature decrease of approximately 10°C induced by 0.4 N acetic acid was greater than the decrease induced by HCl at pH 3 in this study, neither acid decreased the denaturation temperature to a value as low as that attained at comparable in vitro studies, suggesting that the internal pH of the bacteria is closer to the neutral pH. Thus, the DNA peak temperature monitored in DSC studies in vivo may be used to probe the internal pH of bacteria.

The survival of the chemically treated cells after heat treatments was lower than that of untreated cells (Fig. 5). Thus, there is an association between the reduction in the ribosomal subunit transition in the DSC thermogram and an increase in the heat sensitivity of chemically treated cells. This finding supports those of previous DSC studies in that heat resistance of bacteria is related to the onset temperature and the thermal stability of the main ribosomal subunit peak (22, 29, 33). In the present study, DSC revealed that the reductions in the onset temperatures (by 8 to 12°C) and transition temperatures (peak a2, by 3 to 10°C) of the major ribosomal subunit occurred in the thermograms of chemically treated cells. Among chemically treated cells, the onset temperature and thermal stability of the ribosome subunit were lower in cells treated with HCl or acetic acid than in cells treated with ethanol or NaCl.

The reduction of total apparent enthalpy was greater in the acid-treated cells because of greater apparent enthalpy reductions in the ribosomal and DNA transitions of their thermograms. The DSC results suggest that both lower temperatures and lower thermal energy are necessary for bacterial inactivation subsequent to chemical treatment. The 1 log unit viability loss observed with the addition of 1.9 M NaCl did not change the total apparent enthalpy requirement for cell death in subsequent heat treatments in spite of a thermal stability reduction of 2°C in ribosomal subunits. These results may indicate that NaCl-induced changes may be entropy driven rather than enthalpy driven.

DSC thermograms of E. coli revealed stability changes in cellular components after chemical treatment. Mild chemical treatment affects the thermal stability of ribosomal subunits in the cell, thereby increasing the sensitivity of bacteria to heat treatment. The heat sensitivity is greater for acid-treated cells; more cellular components were irreversibly affected after the treatments. These hurdle effects should be considered when current thermal processing technologies are modified, and results of the DSC studies in vivo can be used to assess the effectiveness of hurdles.

REFERENCES