Bioluminescent ATP Assay for Rapid Estimation of Microbial Numbers in Fresh Meat

KENNETH J. LITTEL*, SYLVIA PIKELIS and ARNOLD SPURGASH

Food Applications, Packard Instrument Company, 2200 Warrenville Road, Downers Grove, Illinois 60515

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ABSTRACT

The utility of a bioluminescence adenosine triphosphate (ATP) procedure to estimate bacterial levels in fresh meat products was investigated. A double filtration (DF) sampling procedure was used. In this system two filters were fitted in tandem. A prefilter was used to trap food particles which contained contaminating ATP while the second filter retained the microbial population. The second filter was treated with an enzyme reagent to hydrolyze nonmicrobial ATP that was present on the bacterial filter. Using standard curves, that related bacterial ATP (B-ATP) and plate counts, the bacterial ATP levels in fresh beef and chicken samples were transformed into estimated bacterial levels in the products. The ATP procedure was able to predict bacterial levels within \( \pm 0.5 \log_{10} \) of the actual plate count for greater than 90% of the fresh beef and chicken samples tested. Mean femtogram (fg) ATP/CFU levels in fresh beef and chicken mixed bacterial flora were 0.88 and 0.94, respectively. Minimal sensitivity of the double filtration/enzyme method was approximately \( 5 \times 10^4 \) CFU/g of meat sample.

Fresh meat quality is normally evaluated by determining the numbers of spoilage bacteria present in the product (5). If too many are present, the useful shelf-life of fresh meat is significantly reduced. The aerobic plate count is the most widely used method to enumerate bacteria, but it takes 2 to 3 d to complete. To maximize the shelf-life potential of fresh meat, a more rapid quality control method is required.

Research during the past decade has produced a wealth of new rapid microbiological techniques (20). These new methods can be grouped into two general categories, based on the time required to obtain a result. Real time procedures return results in less than 1 h while retrospective assays require incubation periods of up to 72 h. Methods that fall into the real time category are: firefly bioluminescent/adenosine triphosphate (ATP) assay (1,2,7), direct epifluorescence filter technique (DEFT) (12), and Limulus lysate endotoxin assay (6). Retrospective assays include radiometric (13) and electrical impedance (3) and microcalorimetry (4) measurements. In general, real-time methods measure cellular components while retrospective methods measure metabolic by-products or actual growth. The traditional plate count would be considered a retrospective assay, because microbial numbers are estimated by counting colony forming units after an incubation period.

The bioluminescent ATP assay is based on the purified firefly bioluminescent reaction, which is highly specific for ATP (10). ATP is found in bacterial cells in relatively constant amounts, and as a result can be used as an index to estimate bacterial numbers (1,2,7). Since firefly luciferin/luciferase will react with ATP regardless of its source, nonmicrobial (somatic) ATP must be eliminated to ensure the selective measurement of microbial ATP. Early research efforts using ATP as an estimate of the bacteriological content in foods failed because somatic ATP was not effectively removed from the sample (15). There are two general approaches used to eliminate interference caused by somatic ATP in food samples. One is the enzymatic destruction of the somatic ATP before measurement of the bacterial ATP, as used by Kennedy and Oblinger (8) with fresh beef samples. In this study, an apyrase was used as an ATPase. A second approach is to physically separate the bacterial population from the somatic ATP. Stannard and Wood (18) used a three-stage separation technique. A short centrifugation step was used to remove large food debris. A cation exchange resin was used to absorb the smaller food particles, and was subsequently removed by gravity. Bacteria remaining in the supernatant liquid were concentrated onto a membrane filter before bacterial ATP extraction. Patel and Wood (11) used a similar approach, replacing filtration with a centrifugation step to concentrate the bacterial flora.

This paper describes a simple and practical filtration/ enzyme treatment that concentrates the bacterial population and eliminates nonmicrobial sources of ATP in meat samples, thus allowing bacterial ATP to be selectively quantitated. Fresh beef and chicken samples were prepared for the bioluminescent ATP assay via this procedure, and a standard curve relating bacterial ATP to standard plate counts was used to estimate the bacterial concentration in meat samples.
MATERIALS AND METHODS

Preparation of beef and chicken samples

Fresh beef (rump, chuck, steak, and ground) was purchased from local supermarkets or meat processing plants. Whole chicken was purchased from local supermarkets. The fresh beef and chicken meat were separately minced into 1-in. cubes (except ground beef) and divided into 10-g samples. Samples were stored aerobically for up to a week at 5°C to obtain meat of different bacterial spoilage levels. To obtain specific spoilage levels, samples of different storage age were mixed before testing. Samples were assayed in duplicate for bacterial ATP (B-ATP) and for actual colony forming units (A-CFU).

Double filtration

Samples were prepared for double filtration (DF) by macerating a 10-g sample in 90 ml of 3 mM phosphate buffer (pH 7.2) for 30 s using a Colworth 400 stomacher (Seward Laboratory, London, England). To aid in filtration, coarse particles were removed from the homogenate by filtering through glass wool. To allow the filtration of chicken meat, the glass wool-filtered chicken homogenate was treated with 1% trypsin 1:250 (w/v; Difco, Detroit, MI) for 30 min at room temperature (ca. 25°C). Sample homogenates were then diluted further in phosphate buffer (1:5 to 1:10) and 10.0 ml of the diluted sample homogenate was then processed via DF. The DF system consisted of two filters fitted in tandem to a 10.0-ml disposable sterile syringe. A somatic filter (nylon screen type, Packard Instrument Company, Downers Grove, IL) was used to trap food particles that may have contained contaminating somatic ATP while allowing bacteria to pass through. The bacterial filter (0.45 μm, 25 mm Acrodisc, Gelman, Ann Arbor, MI) retained the bacteria. To eliminate somatic ATP that may have been deposited on the bacterial filter, the filter was incubated with 1.0 ml of PICO™ E/C, a Packard enzyme reagent (Packard), for 30 min at 35°C. PICO E/C was washed from the filter with 5.0 ml of Hanks’ balanced salt solution with Tris buffer (HTBSS, Packard). B-ATP was extracted by passing 0.6 ml of PICOEX™ B (Packard) through the bacterial filter into an assay tube for subsequent ATP analysis. A six position pump (PICOPUMP, Packard) was used to process samples through the DF units.

ATP assay

Extracted samples containing B-ATP were assayed (volume 0.3 ml) using the bioluminescent firefly luciferase reaction (9). Light output was measured on a PICOLITE® Model 6200 luminometer (Packard). The luminometer was programmed to automatically inject the assay reagents; PICOZYME® F, 0.2 ml, a purified firefly luciferase plus cofactors enzyme reagent and PICO Internal Standard, 0.1 ml, an ATP internal standard. The PICO Internal Standard was used to correct for sample interference of light output. Sample counts were initiated after a 5-s delay, postinjection, and were counted for 15 s. A standard calibration curve was used to transform light response into ATP values (fg of ATP/g of sample).

Bacterial enumeration

To enumerate the bacterial content of the beef and chicken samples, the glass wool-filtered homogenate was serially diluted in phosphate buffer and surface-spread plated on trypticase soy agar (BBL, Baltimore, MD). Plates were incubated at 25°C for 48 h. These plate counts were used as the reference bacterial count and defined as actual colony forming units (A-CFU)/g of sample.

Predicted CFU standard curve

ATP and CFU data obtained from beef and chicken samples were used to obtain standard curves relating B-ATP to A-CFU. Samples that possessed less than 5 × 10^4 CFU/g were not included in the standard curve. Duplicate samples were averaged to yield individual standard curve points. Predicted CFU (P-CFU) counts, estimates of bacterial spoilage, were obtained by transforming B-ATP values using the standard curve equations. The PICOLITE Model 6200 was programmed with this algorithm so that B-ATP results were automatically transformed into P-CFU counts. A P-CFU standard curve was prepared for both beef and chicken.

Statistical analyses

All data were transformed to log_{10} values before analysis. Least square regression and the student’s t test for independent samples were used (/6) to calculate the standard curve equations and test for differences in means, respectively.

RESULTS

Preliminary studies, performed to assess bacterial recovery through the glass wool and somatic filter indicated recovery of more than 90% when pure cultures and meat samples were tested (results not shown).

The efficacy of the PICO E/C reagent in removing somatic ATP from food samples is demonstrated by data in Table 1. Reductions of up to 99.99% in somatic ATP levels were observed after treatment with the enzyme reagent. When bacterial levels were below 1.0 × 10^6 CFU/g, the B-ATP constituted less than 1.0% of the total ATP present on the bacterial filter. Comparable intracellular ATP levels obtained from samples containing greater than 1.0 × 10^6 CFU/g with and without PICO E/C treatment indicated that the enzyme reagent was specific for non-bacterial ATP. The detection sensitivity of DF was approximately 5.0 × 10^4 CFU/g of fresh meat.

A standard curve relating B-ATP to A-CFU for each product was used to transform the B-ATP results into estimates of bacterial contamination. The equation (linear regression) for fresh beef was log_{10} P-CFU = (log_{10} B-ATP-0.141)/0.936, (n = 9). A similar curve was obtained for chicken, log_{10} P-CFU = (log_{10} B-ATP-0.177)/0.926.

<table>
<thead>
<tr>
<th>Sample range (log_{10})</th>
<th>Mean ATP/CFU</th>
<th>No treatment</th>
<th>PICO E/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 - 4.0</td>
<td>17</td>
<td>10,000</td>
<td>18.2</td>
</tr>
<tr>
<td>5.0 - 6.0</td>
<td>5</td>
<td>100</td>
<td>0.75</td>
</tr>
<tr>
<td>6.1 - 8.0</td>
<td>17</td>
<td>0.98</td>
<td>0.72</td>
</tr>
</tbody>
</table>

- aMinced chicken samples were tested.
- bATP (fg) level per CFU (1 fg = 10^{-15} g).
- cAerobic plate count (25°C).
- dNumber of samples in CFU range.
- eThe bacterial filter was not treated with PICO E/C.

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Both standard curves had a high correlation coefficient of 0.96.

In Fig. 1 and 2, the A-CFU obtained from the fresh beef (n = 74) and chicken (n = 33) samples have been plotted against the P-CFU, respectively. The line of equivalence is defined as that line on which any data point would fall if the same result was obtained from both methods (ATP and plate count). An upper and lower boundary line is drawn on either side of this line. Points that fall between the boundary lines were samples with P-CFU within ± 0.5 log₁₀ of the A-CFU. For both fresh beef and chicken samples above bacterial contamination levels of 1.0 × 10⁵ CFU/g, the percent of samples falling within the boundaries ranged from 88 to 100% (Table 2). Differences (A-CFU - P-CFU) ranged from -0.69 log₁₀ to +0.51 log₁₀, with an average difference of -0.12 log₁₀ and -0.24 log₁₀ for the fresh beef and chicken samples, respectively. The negative average differences indicated that both standard curves were estimating bacterial levels slightly higher than the plate count. Below bacterial levels of 1.0 × 10⁵ CFU/g, the percent agreement, percent of samples in which the difference between the A-CFU and P-CFU was less than ±0.5 log₁₀, decreased to 71% (5 of 7) and 80% (4 of 5) for each product, respectively. The A-CFU/P-CFU relationships were linear (over the range 5.0 × 10⁴ to 5.0 × 10⁸ CFU/g) with correlation coefficients of more than 0.97 for each product.

The ATP content of the bacterial populations isolated from the meat products is summarized in Table 3. As the bacterial contamination level increased in beef samples, the ATP/CFU decreased slightly. At the lowest sample CFU range, the mean ATP/CFU was significantly higher, at a 5% significance level, than that of the higher ranges. The highest sample CFU range possessed the lowest ATP/CFU, which was significantly lower than the lowest CFU range’s mean ATP/CFU. This trend was not apparent in the chicken samples. Overall, the ATP/CFU varied over an approximate ten-fold range from 0.20 to 2.88 for both products.

DISCUSSION

The utility of the bioluminescent ATP assay as a measure of the bacterial content in foods is dependent upon three assumptions: (a) ATP is present in the flora and can be precisely measured, (b) bacteria possess relatively constant intracellular ATP concentrations (ATP/CFU), and (c) nonmicrobial ATP can be separated and/or eliminated from the bacterial population before ATP measurements.

To obtain precise measurements of ATP from microbial cells, an efficient ATP extraction procedure and purified firefly enzyme must be used. Previous work by Sanville (14) demonstrated that PICOEX B is an efficient and rapid extractant. Greater than 80% of the ATP from bacteria tested (up to 1.0 × 10⁷ CFU/ml) was extracted within seconds. PICOZYME F is a highly purified preparation of firefly luciferase plus cofactors, which allows selective measurement of ATP (10).

Figure 1. Scattergram relating predicted CFU log₁₀ and actual CFU log₁₀ for fresh beef, n = 74. The center line (a) is a line of equivalence bounded by an upper (b) and lower (c) ±0.5 log₁₀ boundary line.

Figure 2. Scattergram relating predicted CFU log₁₀ and actual CFU log₁₀ for minced chicken, n = 33. The center line (a) is a line of equivalence bounded by an upper (b) and lower (c) ±0.5 log₁₀ boundary line.
TABLE 2. Comparison of predicted and actual colony forming units for fresh beef and minced chicken.

<table>
<thead>
<tr>
<th>Sample CFU range log&lt;sub&gt;10&lt;/sub&gt;</th>
<th>Fresh beef</th>
<th>Minced chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (A-P)</td>
<td>Percent agreement</td>
</tr>
<tr>
<td>4.00 - 4.99</td>
<td>-0.38</td>
<td>71%</td>
</tr>
<tr>
<td>5.00 - 5.99</td>
<td>-0.88</td>
<td>90%</td>
</tr>
<tr>
<td>6.00 - 6.99</td>
<td>-0.10</td>
<td>100%</td>
</tr>
<tr>
<td>7.00 - 7.99</td>
<td>-0.88</td>
<td>88%</td>
</tr>
<tr>
<td>8.00 - 8.99</td>
<td>+0.01</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Aerobic plate count (25°C).

bNumber of samples in CFU range.

cActual CFU less predicted CFU.

dPercent of samples whose difference (A-CFU - P-CFU) was less than ± 0.5 log<sub>10</sub>.

eNo samples in this range.

TABLE 3. Summary of ATP content of mixed bacterial populations from fresh beef and minced chicken.

<table>
<thead>
<tr>
<th>Sample CFU range log&lt;sub&gt;10&lt;/sub&gt;</th>
<th>Fresh beef</th>
<th>Minced chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP/CFU mean</td>
<td>95% CI</td>
</tr>
<tr>
<td>4.00 - 4.99</td>
<td>1.68</td>
<td>1.22 - 2.14</td>
</tr>
<tr>
<td>5.00 - 5.99</td>
<td>0.89</td>
<td>0.66 - 1.12</td>
</tr>
<tr>
<td>6.00 - 6.99</td>
<td>0.74</td>
<td>0.59 - 0.89</td>
</tr>
<tr>
<td>7.00 - 7.99</td>
<td>0.65</td>
<td>0.44 - 0.86</td>
</tr>
<tr>
<td>8.00 - 8.99</td>
<td>0.42</td>
<td>0.24 - 0.60</td>
</tr>
</tbody>
</table>

*Aerobic plate count (25°C).

bNumber of samples in CFU range.

cMean fg ATP level per CFU for range, (1 fg = 10<sup>-15</sup> g).

d95% confidence interval for mean ATP levels.

eNo samples in this range.

range during exponential growth. These ATP pools turn over several times per second. Evidence indicates that the ATP/CFU level is independent of the growth rate while maximum intracellular levels are dependent on an extent of the growth medium. Two factors that can depress ATP/CFU levels below the steady range are environmental stress and nutrient depletion (19).

In this study, the ATP level of meat bacterial flora was measured. At the beginning of the study, samples contained mixed bacterial populations with cells in various phases of growth. As samples aged, cell densities increased, resulting in nutrient depletion and other changes in the microenvironment. Consequently, the P-CFU standard curve represents the ATP/CFU relationship for each product's bacterial population measured over time. The fresh beef and chicken P-CFU standard curves predicted, on average, P-CFU values 0.12 and 0.23 log<sub>10</sub> higher than the A-CFU values, respectively (Table 3). Beef bacterial flora at the higher contamination levels, greater than 1.0 x 10<sup>8</sup> CFU/g, also possessed lower ATP/CFU than at the lower bacterial levels (Table 3); this did not occur in the chicken samples. This indicated that the ATP/CFU relationship may vary slightly in bacterial populations of the same meat type, intrinsic and extrinsic conditions of meat products (age of sample post slaughter, initial bacterial flora, storage temperature, etc.) will affect the bacterial population found in a sample. Therefore, the standard curve samples should be incubated under product storage conditions to best represent the ATP/CFU relationship for that product. For the bacterial populations studied, the ATP/CFU levels fluctuated over an approximate ten-fold range, 0.20 to 2.88 ATP/CFU. Stannard and Wood (18) and Patel and Wood (11) reported a similar range in ATP levels for fresh meat flora.

Variability in population ATP levels apparently did not affect the overall predictability of the P-CFU standard curves. Greater than 90% of the P-CFU values were within +/− 0.5 log<sub>10</sub> of the corresponding A-CFU (Fig. 1 and 2). Agreement is high even though the CFU is a variable unit of measure. Stannard and Wood (18) reported a +/− 0.21 log<sub>10</sub> 95% confidence interval for fresh meat plate counts. The data presented demonstrate that a weighted average ATP/CFU level (P-CFU standard curve) determined for a mixed bacterial population can be used to predict bacterial levels in meat products. Variability of the ATP/CFU level within these populations does not seem to affect the usefulness of ATP measurements in estimating the bacterial content of fresh meat.

An important factor affecting the sensitivity of any ATP procedure is removal of somatic ATP during sample preparation procedures. Any remaining somatic ATP in the sample will distort the population ATP/CFU levels.
and lead to an inaccurate estimate of bacterial level. This approach combined enzymatic destruction and physical separation of somatic ATP to allow bacterial ATP measurements in food samples. The dual treatment removed approximately 99.99% of the somatic ATP (Table 1). The minimal sensitivity of this method was approximately 5.0 x 10^4 CFU/g, as evidenced by the relatively constant ATP/CFU levels for the fresh meat samples (Table 1 and Table 3). When Cook et al. (Abstr. 44th Annu. IFT Meeting, 1984. No. 237) sampled fresh beef using only selective filtration, a bacterial sensitivity of approximately 1 x 10^6 CFU/g was obtained. Similar sensitivity levels were obtained using other physical separation procedures (11,17,18). Kennedy and Oblinger (8) used a commercially available sampling procedure utilizing an enzymatic degradation procedure and reported high and variable ATP/CFU values below 1 x 10^6 CFU/g. It thus appears that the present physical and enzymatic procedures alone are unable to remove somatic ATP effectively enough to give sampling procedure sensitivities near that of the luminometer. Most manufacturers of luminometers report reagent/instrument sensitivity levels of approximately 1000 bacteria per unit of sample (assuming 1 fg of ATP per bacterial cell). Given the maximum sample dilutions required to filter a sample for this dual procedure, the theoretical lower detectable bacterial level for the PICOLITE is 5 x 10^3 CFU per gram of beef, which is roughly equivalent to the sensitivity of the dual treatment.

The purpose of this study was to demonstrate a sample preparation procedure which allows microbial ATP measurements to be used as estimates of bacterial levels in fresh meat products. Using the dual treatment procedure, the major drawback of ATP analysis in food systems was eliminated. The nonmicrobial ATP was essentially reduced to instrument background levels. This method appears to give good estimates of bacterial population levels when compared with plate count results (Fig. 1 and 2), and indicates that when nonmicrobial ATP is removed by the sample preparation procedure, transformed bacterial ATP (P-CFU) results are essentially identical to A-CFU.

REFERENCES


