Use of Bioluminescence for the Analysis of ATP, ADP and AMP in Fruits

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SUMMARY

The bioluminescence technique has been used for a long time in the measurement of adenosine triphosphate (ATP). Up to the end of the seventies the assay was complicated and the results uncertain because of low purity reagents and poorly controlled reaction conditions. With the introduction of a new type of luciferin-luciferase reagent, the ATP Monitoring Reagent (AMR), these disadvantages have been overcome and as a consequence, the application of the assay has been extended. The new reagent results in the emission of light of an almost constant intensity, proportional to the ATP concentration. This is in contrast to the flash obtained with conventional reagents. This new technique facilitates measurements and improves the sensitivity of the assay. The reagent may also be used to monitor changes in ATP concentration because the ratio between the ATP concentration and light intensity remains constant. This work describes the principle, the methodology for the determination of ATP, ADP and AMP by the luciferin-luciferase reaction in fruits like apples, pears and sweet cherries and some application fields.

RESUMO

A técnica da bioluminescência tem sido usada há muito tempo para medição de adenosina trifosfato (ATP). Até o final da década de 70 a análise era complicada e os resultados não muito precisos em função da baixa pureza dos reagentes e da falta de controle das condições de trabalho. Com a introdução de um novo tipo de reagente envolvendo a reação luciferina-luciferase denominado Reagente de Monitoramento de ATP, essas desvantagens foram superadas e, como consequência, a aplicação deste tipo de análise tem expandido muito. O novo reagente resulta na emissão de luz com intensidade quase constante a qual é proporcional à concentração de ATP das amostras, sendo muito mais eficiente quando comparado com o método convencional que produz somente um “flash” de luz. Essa nova técnica, além de facilitar as medições, aumenta a sensibilidade das análises. O reagente pode ser usado para monitorar mudanças na concentração de ATP, pois a relação entre a concentração de ATP e a intensidade de luz se mantém constante. Este trabalho descreve o princípio da bioluminescência, a metodologia para determinação de ATP, ADP e AMP em maçãs, pêras e cerejas, e apresenta também campos de aplicação da técnica.

PALAVRAS-CHAVE

Bioluminescence, ATP, fruits, methodology, applications.

Bioluminescência, ATP, frutos, metodologia, aplicações.
1. INTRODUCTION

Bioluminescence is a widespread phenomenon characterized by light emission produced in enzyme catalyzed reactions. Most systems consist of an enzyme, luciferase, catalyzing the oxidation of the \( \text{O}_2 \) or \( \text{H}_2\text{O}_2 \) of a substrate, luciferin. The light emission is of high quantum yield and the color varies with the species, from blue to red, depending on the type of luciferin-luciferase system involved (Harvey, 1952).

Among insects bioluminescence is found in a number of species, many of them belonging to the Coleoptera family Lampyridae, the so-called fireflies. These insects emit light from specialized abdominal organs as a mating signal. Much of the present knowledge of the biochemistry of firefly bioluminescence was developed with the North American firefly Photinus pyralis and the work started in 1947 with the important discovery that firefly bioluminescence requires ATP (McElroy et al., 1974). It soon became evident that the firefly system could be used for analytical purposes, and in 1952 the first ATP assay using firefly bioluminescence was demonstrated (Strehler & Totter, 1952).

Together with adenosine 5'-triphosphate (ATP), the nucleoside phosphates adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) form the adenylate system. This system plays an essential role in all living organisms and all reactions which deliver convertible energy for the cell are designed to charge the adenylate pool in an economical way, irrespective of whether it is by respiration, fermentation or photosynthesis (Saquet, 2001).

The various relationships between the adenylates, together with the pool sizes of inorganic phosphate (Pi), are used to describe the energy situation of a system (tissue, single cell, or intracellular compartments such as cytosol, mitochondria and chloroplasts). The terms commonly employed are energy charge, \( \frac{2 \text{[ATP]} + \text{[ADP]}}{2 \text{[ATP]} + \text{[ADP]} + \text{[AMP]}} \); with values between 0 and 1, and ATP/ADP ratio or phosphorylation potential, \( \frac{\text{[ATP]}}{\text{[ADP]}} \times \text{[Pi]} \). The values of these ratios are important measures of the cellular energy status: deviations from standard values indicate disfunctions. Thus the determination of adenylate ratios is of diagnostic value in both animal and plant samples (Praevent & Raymond, 1983; Hampp, 1985).

2. THE FIREFLY LUCIFERASE REACTION

Bioluminescence is an enzyme catalyzed reaction which results in the emission of light. As shown in Figure 1, the firefly luciferase enzyme \( E \) catalyzes the activation of D-luciferin \( \text{LH}_2 \) by ATP and its subsequent oxidation to electronically excited oxyluciferin \( \text{P}^* \). The transition of oxyluciferin to its ground state \( \text{P} \) results in light emission.

A) In an initial activating reaction, similar to the activation of amino acids in protein biosynthesis, luciferin is adenylated by ATP to form luciferyl adenylate bound to the luciferase enzyme and free pyrophosphate.

B) In the next step, the luciferyl adenylate enzyme complex is oxidized by molecular \( \text{O}_2 \) resulting in \( \text{CO}_2 \), AMP, and electronically excited oxyluciferin. AMP and oxyluciferin remain bound to the enzyme.

C) On returning to the ground state the excited complex emits light, the colour of the light depending on the firefly species (i.e. the structure of the luciferase) and the pH: it varies between greenish yellow (560nm) and red (620nm). Under favourable conditions quantum yields close to 100% have been observed.

D) In a final step luciferin and AMP are released from the luciferase enzyme which is then free to enter another reaction cycle.

The simplified reaction can be written as below:

\[
\text{ATP + D-luciferin + O}_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin + AMP + PPI + CO}_2 + 0.9 \text{hv}
\]

In the presence of the luciferase from Photinus pyralis, ATP reacts with Photinus pyralis luciferin to give pyrophosphate and adenylyl luciferin. Adenylyl luciferin is then oxidized by molecular oxygen in the presence of the same enzyme, yielding \( \text{CO}_2 \), AMP and oxyluciferin in the electronically excited singlet state. Upon relaxation, 0.9 photons of wavelength 562 nm, per molecule of ATP are emitted. If optimized conditions are used, the intensity of the emitted light is proportional to the ATP concentration in the sample (Wulff & Döppen, 1985).

Figure 1. Schematic mechanism of the firefly luciferase reaction (Lundin & Thore, 1978).
Optimized conditions for measurement: two parameters govern the luminometric ATP assay: the chemical background due to ATP concentrations originating from the reagents, and the sensitivity of the luminometer used.

In the assay of ATP, the reaction conditions are arranged to provide light emission proportional to the ATP concentration. In this way an extremely sensitive assay for ATP is obtained, which allows for the detection of concentrations as low as 15 nmol of ATP, 12 nmol of ADP and 7 nmol of AMPg\(^{-1}\) DW of pear or apple flesh tissue (SAQUET, 2001).

3. METHODOLOGY FOR FRESH FRUITS (SAQUET, 2001)

3.1. Equipment

The procedure described here is designed for the luminometer 1251 (LKB-Wallac, Turku, Finland). The instrument is fitted with an auto-sampler and a water-through system in order to keep the temperature in the range of 25°C. Other very important factor is the control of the environmental room temperature, which should be about 25°C. The procedure can, of course, be used with any other luminometer of comparable sensitivity, which maintains a constant temperature in the cuvette. However, it should be recognized that other instruments may require a different final volume of the assay mixture for optimal results.

3.2. Reagents and solutions

Purity of the reagents and solution preparation: luciferase and luciferin must be of the highest purity available. All other chemicals must be of reagent grade. For the preparation of solutions, it is very important that they be prepared in re-purified water and the containers must be sterilized to prevent microbial contamination.

3.3. Sources of error

Light emission can be considerably inhibited by the following effects:

a) Physically, by light absorption, if there is a substance present in the sample whose absorption spectrum overlaps the emission spectrum of the luciferase reaction.

b) By inhibition of the luciferase:

- by specific inhibitors of the ATP- or luciferin binding sites of luciferase (DENBURG et al., 1969).

All these interfering factors can be accounted for by carrying out control measurements with an internal ATP standard. The inhibitory effect can be corrected by the difference in signal emitted.

3.4. Extraction and assay procedures

3.4.1. Samples and extraction

Samples of fresh or dried fruits can be used. In the first case, reduced losses are achieved because of the direct use of fresh fruit material without the drying process. In the case of dried samples, they should be obtained from a low temperature vacuum system in order to avoid losses of nucleotides. The advantage of this method is that it can be used for a great number of samples, keeping them at -20°C or below until analysed. Samples can be taken from fruit flesh or the skin, depending of the aim of the research. In this report, results from the flesh of pears, apples and sweet cherries are described.

For the extraction of ATP, ADP and AMP one gram of dried and powdered sample was used. The samples were then placed in centrifuge tubes, on ice, containing a solution of 5% trichloracetic acid (2mM) and homogenized. Samples were taken for 30 min and centrifuged at 18,000g for 15min at 4°C. In order to minimize the inhibition effect of anions of the trichloracetic acid, the samples were diluted 40 times with 0.1M Tris EDTA buffer (2mM, pH 7.75) before assessment.

3.4.2. Assay

For the assessment of ATP the following were mixed in a cuvette: 10µL extract, 50µL AMR (ATP monitoring reagent) and 440µL of 0.1M tris-EDTA buffer (2mM, pH 7.75). The luminescence of this reaction was measured with a 1251 Luminometer at 25°C (LKB Wallac, Turku, Finland). After measurement of each sample, an internal standard was fed and the luminescence recorded again. The ATP concentration was calculated using both values.

To measure ADP, it was first necessary to convert it to ATP. Samples were then incubated with pyruvate kinase (120 units/ml PEP buffer) at 25°C for 30min. The total ATP concentration was assayed as described above and ADP calculated by difference.

Before the AMP assessment, it was converted to ADP and this converted to ATP by incubation of the samples with a mixture of myokinase (180 units/ml PEP buffer) and pyruvate kinase (120 units/ml PEP buffer) for 30 min at 25°C. Myokinase converts AMP to ADP and pyruvate kinase converts ADP to ATP.
4. SOME RESULTS WITH FRUITS

Table 1. Changes in ATP & ADP concentrations (nmol.g\(^{-1}\) DW) and the ATP:ADP ratio of 'Regina' sweet cherries stored under various CA-conditions at 1\(^\circ\)C after four and seven weeks in storage [HARB et al., 2003].

<table>
<thead>
<tr>
<th>%CO(_2) + %O(_2)</th>
<th>ATP</th>
<th>ADP</th>
<th>ATP:ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks</td>
<td>7 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>At harvest</td>
<td>55.3(^g)</td>
<td>-</td>
<td>16.9b</td>
</tr>
<tr>
<td>Air control</td>
<td>196.6a</td>
<td>194.6a</td>
<td>37.4a</td>
</tr>
<tr>
<td>06 + 18</td>
<td>108.2d</td>
<td>-</td>
<td>35.5a</td>
</tr>
<tr>
<td>12 + 18</td>
<td>106.4de</td>
<td>107.2c</td>
<td>27.0a</td>
</tr>
<tr>
<td>18 + 18</td>
<td>119.0c</td>
<td>85.7d</td>
<td>36.4a</td>
</tr>
<tr>
<td>06 + 02</td>
<td>138.3b</td>
<td>137.3b</td>
<td>34.7a</td>
</tr>
<tr>
<td>12 + 02</td>
<td>90.4f</td>
<td>-</td>
<td>30.0a</td>
</tr>
<tr>
<td>18 + 02</td>
<td>101.9e</td>
<td>72.7e</td>
<td>29.4a</td>
</tr>
<tr>
<td>24 + 02</td>
<td>85.6f</td>
<td>68.8f</td>
<td>29.4a</td>
</tr>
</tbody>
</table>

\(^1\)Means within each column followed by different letters indicate significant differences between treatments at P ≤ 0.05, Student-Newman-Keuls range test.

**Figura 2.** Effect of delayed CA on ATP concentrations of 'Conference' pears during six months of storage in 3% CO\(_2\) + 1% O\(_2\) at 0°C. Vertical bars indicate S.D. between treatment means [SAQUET et al., 2003c]. (Delayed CA: fruits were kept 21 days in air at 0°C before storage in CA).

**Figura 3.** ADP concentrations of 'Conference' pears with and without delayed CA stored in 3% CO\(_2\) + 1% O\(_2\) at 0°C. Vertical bars indicate S.D. between treatment means [SAQUET et al., 2003c]. (Delayed CA: fruits were kept 21 days in air at 0°C before storage in CA).
Figure 4. Effect of delayed CA on AMP concentrations of 'Conference' pears stored in 3% CO$_2$ + 1% O$_2$ at 0°C. Vertical bars indicate S.D. between treatment means (SAQUET et al., 2003c). (Delayed CA: fruits were kept 21 days in air at 0°C before storage in CA).

Figure 5. Adenylate energy charge (A.E.C.) of 'Conference' pears with and without delayed CA stored in 3% CO$_2$ + 1% O$_2$. Vertical bars indicate S.D. between treatment means (SAQUET et al., 2003c). (Delayed CA: fruits were kept 21 days in air at 0°C before storage in CA).

Figure 6. ATP concentrations and ATP:ADP ratio of delayed and not delayed 'Braeburn' apples. Vertical bars indicate S.D. between treatment means (SAQUET et al., 2003b).
5. SOME APPLICATION FIELDS OF ATP BIOLUMINESCENCE

Numerous applications based on the firefly bioluminescence assay have been described in the literature. Many of them have great potential interest as routine assays in clinical chemistry, clinical bacteriology, etc. Some of the principal applications of interest are listed in Table 2.

Bioluminescence is a versatile analytical tool for the assay of ATP and other metabolites and enzymes participating in ATP converting reactions. The improvements in the analytical techniques brought about by the introduction of the ATP bioluminescence principle should remove many of the analytical problems which have hindered general acceptance of the firefly assay. The technique should also stimulate the development of new applications in food chemistry and safety, biochemistry, biology and medicine.

6. DISCUSSION

Assays using ATP bioluminescence are as convenient and as rapid as the alternative spectrophotometric or fluorometric procedures but their sensitivity is much higher.

While the spectrophotometric assay is limited to concentrations greater than 1 to 5 mol, the determination of adenylates by bioluminescence offers considerable advantages regarding the sensitivity in the range of nmoles as shown in Fig. 4 with 'Conference' pears with values near to 10 n mole or lower. Furthermore, the technique allows for a large number of samples to be assayed in a reasonable time and the ATP is measured directly rather than after phosphorylation of a substrate participating in a NAD(P)NAD(P)H converting reaction. Thus, the risk of analytical interference by auxiliary reactions is less in the bioluminescence assay. This risk is further reduced by the use of an internal ATP standard.

The firefly assay of ATP has found many applications in the study of cellular systems. The central role of ATP in intracellular metabolism makes ATP an interesting parameter in physiological studies. ATP has been used as an index of biomass in oceanography, limnology, clinical bacteriology, hygiene monitoring, food chemistry and biochemistry, and in connection with sewage treatment plants.

In the field of plant biochemistry, ATP bioluminescence has been extensively applied. The roles of ATP, ADP and AMP in cell metabolism are very important and a lot of studies have been developed. SAQUET et al. (2000, 2001, 2003b, 2003c) used the technique to measure the energy charge related to the incidence of physiological disorders in
Table 2. Some applications of ATP bioluminescence.

<table>
<thead>
<tr>
<th>Metabolite, enzyme, or event assayed</th>
<th>Method (auxiliary reactions, etc…)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>METABOLITES</strong></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Direct reaction with luciferase</td>
</tr>
<tr>
<td>ADP, AMP</td>
<td>Formation of ATP by AK or PK</td>
</tr>
<tr>
<td>cAMP</td>
<td>Formation of ATP by adenylate kinase</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>Consumption of ATP by creatine kinase</td>
</tr>
<tr>
<td>Glucose</td>
<td>Formation of ATP by hexokinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Formation of ATP by nucleotide diphosphate kinase</td>
</tr>
<tr>
<td>Adenosine tetraphosphate</td>
<td>Direct reaction with luciferase</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>Formation of ATP by pyruvate kinase</td>
</tr>
<tr>
<td>1,3 Diphosphoglycerate</td>
<td>Formation of ATP by phosphoglycerokinase</td>
</tr>
<tr>
<td><strong>ENZYMES</strong></td>
<td></td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>Formation of ATP from creatine phosphate</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Consumption of ATP by glucose</td>
</tr>
<tr>
<td>ATP-sulphurylase</td>
<td>Formation of ATP from adenosine sulphate</td>
</tr>
<tr>
<td><strong>METABOLIC REACTIONS</strong></td>
<td></td>
</tr>
<tr>
<td>Oxidative phosphorylation in Mitochondria</td>
<td>Continuous monitoring of ATP</td>
</tr>
<tr>
<td>Photophosphorylation in bacterial chromatophores</td>
<td>Continuous monitoring of ATP</td>
</tr>
<tr>
<td>Photophosphorylation in green plants</td>
<td>Continuous monitoring of ATP</td>
</tr>
<tr>
<td><strong>MICROBIOLOGY</strong></td>
<td></td>
</tr>
<tr>
<td>Determination of bacteriurine</td>
<td>Selective extraction and assay of bacterial ATP</td>
</tr>
<tr>
<td>Determination of biomass in ocean water</td>
<td>Extraction and assay of cellular ATP</td>
</tr>
<tr>
<td>Determination of biomass in soil</td>
<td>Extraction and assay of cellular ATP</td>
</tr>
<tr>
<td>Hygiene monitoring (bacteria, yeasts) in equipments and foods like such as meat, eggs, raw milk, fruit juices, poultry, brewing and beverages.</td>
<td></td>
</tr>
</tbody>
</table>

Apples and pears during controlled atmosphere storage. The role of ATP in the biosynthesis of fatty acids in fruits was also investigated as related to the integrity of the membranes and the production of aroma compounds in apples (SAQUET et al., 2003a) and sweet cherries, related to the aroma production during storage (HARB et al., 2003).

In fruit metabolism ATP plays an essential role because it is the unique source of energy for reactions of synthesis. It is necessary for the maintenance of cell function as well as for repair mechanisms of cell membranes (TAN, 1999). The involvement and importance of ATP in the synthesis and desaturation of fatty acids is well known (MAZLIAK, 1994; OHLROGGE & BROWSE, 1995). However, its possible involvement in the development of physiological disorders in fruits and in reduced aroma production during CA-storage has not been sufficiently studied. In all kinds of fruit investigated, a reduction in the concentrations of ATP during storage under low oxygen and/or combined with high concentrations of CO₂ was observed. Fruits stored under cold storage, showed the highest concentrations of ATP as well as the highest values of energy charge expressed either by the ratio of ATP:ADP or by the adenylyl energy charge (A.E.C.) as proposed by PRADET & RAYMOND (1983). Such results confirm the high dependence of aerobic respiration of the majority of the plant tissues on molecular oxygen. In pears and apples kept under cold storage it was observed that the highest concentrations of ATP coincided with the maximum of the respiratory climacteric (data not shown). BENNETT et al. (1987) observed similar behaviour during the ripening of avocado fruit in normal air using another method for ATP assessment.

The effect of delay in the establishment of CA-conditions in the occurrence of disorders in fruits was early observed (HÖHN et al., 1996) but no physiological or biochemical changes were investigated. Depending on the...
cultivar, the storage of pears and apples in air at 0°C for 21 days before establishment of CA-storage conditions, was shown to be an efficient method to reduce the occurrence of internal browning and/or formation of cavities in the fruit flesh (SAQUET et al., 2001; SAQUET et al., 2003b). The results with ATP measurements (Fig. 2 and Fig. 6) and fatty acids (data not shown) of these fruits showed a very good relationship with the development of browning disorders. During the air storage period before CA-storage, fruit respiration was higher and resulted in higher ATP and fatty acids amounts, which certainly influenced the adaptation of fruits to the remaining storage time under CA-conditions.

The relationship between the energy charge of tissues and fatty acid contents with aroma production during the storage time was also observed. Especially in the apple cultivars studied (ester type), in which lipids are the precursors of the esters, which are the impact aroma compounds, a negative effect of CA-storage on aroma production during controlled atmosphere storage was observed. The lower the O₂ and/or higher the CO₂ concentrations during CA-storage, the lower the ATP and fatty acid contents, and as a consequence a lower production of aroma volatiles was measured. The results of the fatty acids and aroma volatiles can be seen in SAQUET et al. (2003a).

The energy charge of pears was more important in relation to the incidence of physiological disorders, because of its involvement with the membrane fatty acids. Pear aroma production also depends on ester production but they also produce large amounts of alcohols during ripening. A very good relationship between low energy charge and low contents of fatty acids with the occurrence of internal browning was observed, showing that such storage problems are dependent on the energy status of the cells during CA-storage.

In sweet cherries ATP also seems to play an important role in aroma production. As shown in Table 1, the storage of fruits under low oxygen and/or combined with high concentrations of CO₂ resulted in a strong reduction in the ATP levels of fruit tissues and aroma production.

In conclusion, ATP bioluminescence was shown to be a very sensitive and reliable method for investigating fruit biochemistry and metabolism, and in the case of this work, in relation to the incidence of browning disorders and aroma production in fruits.

7. REFERENCES


SAQUET, A.A.; STREIF, J.; BANGERITH, F. Impaired aroma production of CA-stored 'Jonagold' apples as affected by


