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An efficient extraction method for quantitation of adenosine triphosphate in mammalian tissues and cells

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ABSTRACT

Firefly bioluminescence is widely used in the measurement of adenosine 5′-triphosphate (ATP) levels in biological materials. For such assays in tissues and cells, ATP must be extracted away from protein in the initial step and extraction efficacy is the main determinant of the assay accuracy. Extraction reagents recommended in the commercially available ATP assay kits are chaotropic reagents, trichloroacetic acid (TCA), perchloric acid (PCA), and ethylene glycol (EG), which extract nucleotides through protein precipitation and/or nucleotidase inactivation. We found that these reagents are particularly useful for measuring ATP levels in materials with relatively low protein concentrations such as blood cells, cultured cells, and bacteria. However, these methods are not suitable for ATP extraction from tissues with high protein concentrations, because some ATP may be co-precipitated with the insolubilized protein during homogenization and extraction, and it could also be precipitated by neutralization in the acid extracts. Here we found that a phenol-based extraction method markedly increased the ATP and other nucleotides extracted from tissues. In addition, phenol extraction does not require neutralization before the luciferin–luciferase assay step. ATP levels analyzed by luciferase assay in various tissues extracted by Tris–EDTA–saturated phenol (phenol–TE) were over 17.8-fold higher than those extracted by TCA and over 550-fold higher than those in EG extracts. Here we report a simple, rapid, and reliable phenol–TE extraction procedure for ATP measurement in tissues and cells by luciferase assay.

1. Introduction

Measurement of adenylate nucleotides (ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate) levels is widely used to monitor and evaluate energy stasis and metabolic activity in various cells and tissues [1–7]. Particularly, ATP is the "energy currency" of organisms and plays central roles in bioenergetics, whereby the levels are used to evaluate cell viability and proliferation [8–11], cell death [12,13], and energy transmission [14]. Depletion of ATP from tissues and cells is thus a sensitive marker of impaired cellular functions and viability. For example, administration of neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [15], amphetamine [16], methamphetamine [17], 3-nitropropionic acid [18], and kainite [19], induces acute ATP depletion in the brain with significant impairment of psychomotor functions.

Bioluminescence assays using the firefly luciferin–luciferase system have recently become the most popular method for quantitative determination of ATP level due to their high sensitivity and specificity [20]. Such assays for ATP have been used to estimate minute quantities of biomass to indicate living matter in water and sludge [21–23], bacterial contamination in food and drinking water [24–28], and in plant material [29]. The initial step for measuring ATP levels in these cases is extracting the ATP away from the surrounding source material, and it is the solubilized ATP free from proteins in the extracts that is used for the luciferase assay. The efficiency of ATP extraction is therefore the main determinant of accuracy of the bioluminescence assay.

Table 1 lists the main ATP extraction media used for bacteria, plants, and mammalian cells and tissues reported thus far. For determination of ATP levels in bacteria and plants, comparative studies on extraction efficiency have been conducted in detail among boiling water and buffers [26–28], organic solvents [26,29], acids [26,28], and proteinase-K [28]. For measurement of ATP levels in erythrocytes, platelets, cultured cells, and sheep lumen digesta, extraction media such as boiling water and buffers [30,31], organic solvents [31–34], and acids [31,34–39] have also been used. However, these studies mainly assayed ATP levels in microorganisms and cultured cells with relatively low protein concentrations. In addition, these methods were also applied to mammalian tissues without verification of the efficiency of ATP extraction. We recently found that the ATP extraction media reported previously and

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Table 1
Extraction media in literature.

Extraction media	Bacteria and plant	Mammalian cells and tissues
Boiling water or buffer		
Deionized water, Tris–EDTA, Triton X-100, Na_2HAsO_4	Refs. [26–28]	Refs. [30,31]
Organic solvents		
Ethanol, methanol, butanol, octanol, acetonitrile, DMSO	Refs. [26,29]	Refs. [31–34]
Acids		
TCA, PCA, $HClO_4$, H_2SO_4	Refs. [26,28]	Refs. [31,34–39]
Enzymes		
Proteinase-K	Ref. [28]	

recommended in the manufacturer's instructions of commercially available ATP assay kits, such as trichloroacetic acid (TCA), perchloric acid (PCA), and ethylene glycol (EG), are not suitable for ATP extraction from mammalian tissues, such as muscle, liver, and brain, which generally have high protein concentrations. Thus, ATP from such tissues is not fully extracted in the homogenization and deproteinization steps with these extraction media, probably due to co-precipitation with the insolubilized proteins or adsorption of ATP to the acid–salt precipitate during neutralization of the acid extract [37,38].

In attempting to optimize ATP extraction media for mammalian tissues with high protein concentrations, we found that ATP and other nucleotides were most effectively and conveniently extracted from mammalian tissues by phenol-based reagents, such as those generally used for DNA and RNA extraction. The aim of this study was to compare a phenol-based extraction reagent with several commercially used ATP extraction media, such as TCA and EG, based on the efficacy of ATP extraction and the convenience of performance.

2. Experimental

2.1. Animals and tissue sampling

C57 BL female mice, weighing 15.0 ± 2.0 g, were maintained at 12 h light/dark cycle in a temperature-controlled room with free access to food and water. Mouse brain, heart, liver, spleen, and muscle (gastrocnemius muscle) were isolated for the determination of ATP levels. Tissue wet masses were determined in polypropylene tubes (#352059, BD Falcon, Bedford, MA), and 3.0 mL of homogenization buffer was added per 70–150 mg of tissue. Blood samples (200 μL) were immediately transferred to 2.0-mL microtubes containing appropriate volume of pre-cooled extraction media as described below. All studies were approved by the Local Ethics Committee.

2.2. Reagents

All chemicals used were of analytical grade, and all dilutions were made with high purity deionized water (18 MΩ cm resistivity) obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Nucleotides were purchased from Sigma–Aldrich (Tokyo, Japan). Tris–EDTA (10 mmol L^{-1} Tris–HCl, pH 8.0 and 1 mmol L^{-1} EDTA)–saturated phenol (phenol–TE), chloroform, and TCA were obtained from Wako Pure Chemical Industries (Osaka, Japan). Luciferase and D-luciferin potassium salt were from ABD (Sunnyvale, CA).

2.3. Phenol–TE extraction

For extraction of ATP and other nucleotides, freshly prepared tissues were homogenized with 3.0 mL of ice-cold phenol–TE using an Ultra-Turrax® (Ika Japan, Nara, Japan) operated in pulsed mode for three cycles of 30-s homogenization and 30-s cooling. One mL of homogenate was transferred into 2.0-mL microtubes containing 200 μL of chloroform and 150 μL of de-ionized water. The homogenate was thoroughly shaken for 20 s and centrifuged at 10,000 × *g* for 5 min at 4 °C. The aliquot from the supernatant was diluted 1000-fold with deionized water, and 10 μL of the diluted extract was used for luciferin–luciferase assay and high-performance liquid chromatography (HPLC) analysis. For measurement of blood ATP levels, isolated blood samples (200 μL) were immediately transferred to 2.0-mL microtubes containing pre-cooled extraction media (600 μL of phenol–TE, 200 μL of chloroform, and 200 μL of de-ionized water). The sample was thoroughly shaken for 20 s and centrifuged (10,000 × *g*, 5 min at 4 °C) to achieve phase separation; 50 μL of the upper aqueous phase was diluted 10,000-fold with deionized water, and 10 μL of this diluted extract was used for luciferin–luciferase assay and HPLC analysis.

2.4. TCA extraction

ATP extraction from tissues using TCA was performed according to the protocol supplied by the manufacturer (Enliten® ATP Assay System, Promega, Madison, WI). Freshly prepared tissues were immediately homogenized with 3.0 mL of ice-cold homogenization buffer (0.25 mol L^{-1} sucrose and 10 mmol L^{-1} HEPES–NaOH, pH 7.4) by Ultra-Turrax® using three cycles of 30-s homogenization and 30-s cooling. After homogenization, the homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. Three hundred microliters of the supernatant was quickly added to an equal volume of ice-cold 10% TCA and shaken for 20 s. The supernatant was then transferred into a 2.0-mL microtube for centrifugation (10,000 × *g* for 10 min at 4 °C), and 400 μL of supernatant was collected and added to 200 μL of 1 mol L^{-1} Tris–acetate buffer (pH 7.75) for neutralization. The aliquot from the supernatant was diluted 30-fold with deionized water, and 10 μL of the diluted extract was used for luciferin–luciferase assay and HPLC analysis. Blood samples (200 μL) were immediately transferred to 2.0-mL microtubes containing 200 μL of ice-cold 10% TCA and homogenized or mixed well by vortex mixer. The samples were then added to 200 μL of 1 mol L^{-1} Tris–acetate buffer, pH 7.75, and centrifuged at 10,000 × *g* for 5 min at 4 °C. The aliquot from the supernatant was diluted 10,000-fold with deionized water, and 10 μL of this diluted extract was used for the following analyses.

2.5. EG extraction

ATP extraction from tissues using EG was performed according to the manufacturer's protocol (Tissue ATP measurement kit®, Toyo Ink, Tokyo). Freshly prepared tissues were immediately homogenized with 3.0 mL of ice-cold homogenization buffer (0.25 mol L^{-1} sucrose and 10 mmol L^{-1} HEPES–NaOH buffer, pH 7.4) by Ultra-Turrax® three cycles of 30 s homogenization and 30 s cooling. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The