Brief communication

Bioluminometric assay of ATP in mouse brain: Determinant factors for enhanced test sensitivity

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Firefly luciferase bioluminescence (FLB) is a highly sensitive and specific method for the analysis of adenosine-5-triphosphate (ATP) in biological samples. Earlier attempts to modify the FLB test for enhanced sensitivity have been typically based on *in vitro* cell systems. This study reports an optimized FLB procedure for the analysis of ATP in small tissue samples. The results showed that the sensitivity of the FLB test can be enhanced several fold by using ultraturax homogenizer, perchloric acid extraction, neutralization of acid extract and its optimal dilution, before performing the assay reaction.

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1. Introduction

Recent studies have suggested a pivotal role of energy metabolism in the pathogenesis of various neurological disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, tardive dyskinesia and epilepsy (Calabrese *et al* 2001; Andreassen and Jorgensen 2000). Studies on experimental animals have shown significant depletion of brain ATP levels following administration of neurotoxins like MPTP (Chan *et al* 1993), amphetamine (Wan *et al* 1999), methamphetamine (Chan *et al* 1994), 3-nitropropionic acid (Matthews *et al* 1998) and kainate (Farooqui *et al* 2001). Tissue ATP content is the measure of energy status and provides valuable information on understanding the role of energy impairment in neurodegeneration. Although sophisticated noninvasive procedures are used to determine energy status (ATP) in human brain, biochemical assay of ATP continues to be the method of choice in experimental animal studies.

Firefly luciferase bioluminescence (FLB) is a sensitive and accurate technique for the estimation of ATP in biological samples (Leach 1981). The enzyme luciferase catalyses the oxidative decarboxylation of luciferin in the presence of ATP and produces a bioluminescence signal, which is linearly proportional to the concentration of ATP. In the past, several modifications have been made to improve the sensitivity of bioluminescence procedure for ATP estimation (Kamidate 1997; Ford *et al* 1994; Nyren 1994), however, those studies were mainly aimed for *in vitro* applications. Considering the fact that the assay sensitivity would have significant analytical impact while dealing with minute samples like tissue biopsies or discrete brain regions of small animals, it was rationalized to optimize the test conditions for FLB assay of ATP in small tissue samples.

2. Materials and methods

2.1 *Animals and tissue sampling*

C57 BL female mice, weighing 27 ± 2 g, were maintained at 12 h light/dark cycle in a temperature-controlled room with free access to food and water. For the determination of ATP, the striata were isolated from the brain and immediately frozen in liquid nitrogen and stored at – 80° until analysed (within 3 days).

Keywords. ATP assay; bioluminescence; mouse brain

Abbreviations used: ATP, Adenosine-5-triphosphate; FLB, firefly luciferase bioluminescence; SW, sterile water.

2.2 *Reagents*

ATP assay mix (luciferase+luciferin) and ATP standard were purchased from Sigma Chemical Company, USA. The sterile water (SW) was obtained from McGaw Inc., USA. All other reagents used were of analytical grade. The extraction media used were SW; 0·1 M Tris-acetate buffer containing 2 mM EDTA, pH 7·75 (Tris-EDTA) and perchloric acid ($HClO_4$ 10% v/v). Prior to assay, ATP assay mix (100 mg) was dissolved in 5 ml of ice-cold SW. The solution of assay mix was kept on ice, while protecting from light.

2.3 *Instruments*

The luminescence signal was measured by a luminometer (Luminoscan, Labsystems, Finland). Different homogenizing systems used for tissue disruption were Sonic probe (Fisher Scientific, USA), Teflon homogenizer and Ultraturax T-25 homogenizer (both from Janke and Kunkel, Germany). For centrifugation purpose a refrigerated centrifuge (Hermle Z382K, Germany) was used.

2.4 *Procedure of ATP assay*

Striatum (approx. 3–4 mg) from one side of the cerebrum was weighed and homogenized with pre-cooled extractant. The homogenate was centrifuged at 4500 rpm for 10 min in a refrigerated centrifuge at 4°C. One hundred microlitres of supernatant were used for ATP analysis, when the extraction medium was SW or Tris-EDTA. However, in case of $HClO_4$, 500 µl of supernatant were first neutralized with 200 µl of 2·5 M KOH, and the precipitate was removed by centrifugation at 4500 rpm for 5 min. The aliquot from the supernatant was diluted 5–40-folds with Tris-EDTA, and 100 µl of this diluted extract were used for ATP assay. For the analysis of ATP, 100 µl of sample extract or standard solution were placed in the well of microplate (White Cliniplate, Labsystems, Finland), followed by the addition of 50 µl of ATP assay mix. The luminescence signal was measured in a luminometer set at a lag time of 1 s and integration time of 5 s. In FLB reaction, light production begins after a lag of about 40 ms and within 500 ms the maximal luminescence is observed which is stable for several seconds (Lemasters and Hackenbrock 1979). Therefore a lag time of 1 s was considered to be optimum, whereas, the integration time may be increased to amplify the luminescence signal (total RLUs), if the sample has very low concentration of ATP.

3. Results

Ultraturax was found to be the most efficient homogenizing technique for producing high signal intensity from the homogenate, followed by teflon homogenizer, whereas the sonic probe was the least efficient method of homogenization (figure 1). Amongst the various homogenizing media used for the extraction of ATP, HClO4 showed about 14 times higher sensitivity in the assay as compared to SW or Tris-EDTA (figure 2). The effect of dilution of $HClO_4$ extract on the sensitivity of

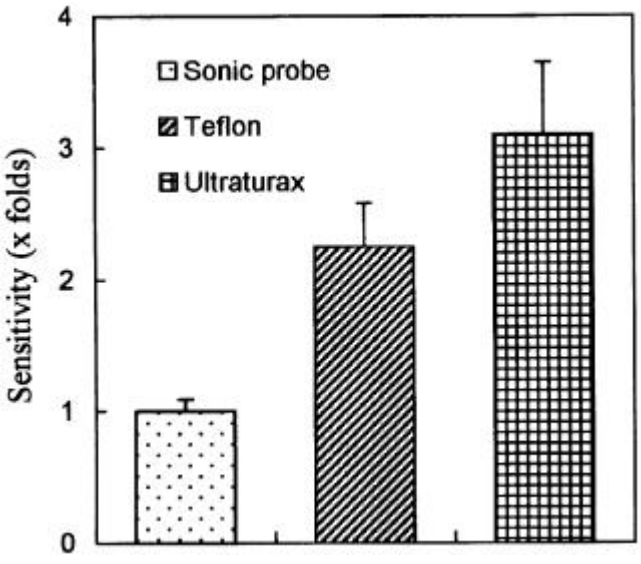

Figure 1. Efficiency of different homogenizing techniques for the analysis of ATP in mouse striatum. The sensitivity was compared against sonic probe to be unity. Values are mean of four observations; the error bars show standard error of means.

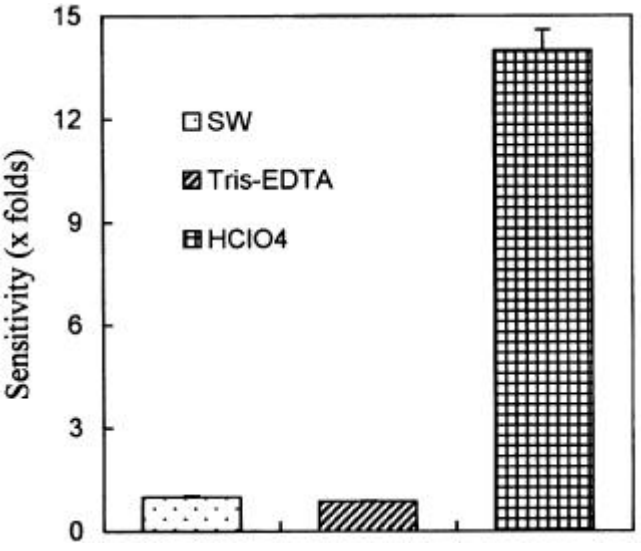

Figure 2. Efficiency of different extraction media for the analysis of ATP in mouse striatum. $HClO_4$ was highly effective extractant which showed about 14-folds higher sensitivity in the test as compared to SW.

test is shown in figure 3. There was steep rise in test sensitivity up to 10-fold dilution of extract; further dilution produced minimal effect on the test sensitivity (figure 3). The luminescence signal was found to be quite stable when the test was performed in $HClO_4$, whereas a rapid decline in signal intensity was observed when SW was used as the extractant (figure 4). Tris-EDTA showed a medium level of signal stability, falling in between $HClO_4$ and SW (figure 4).

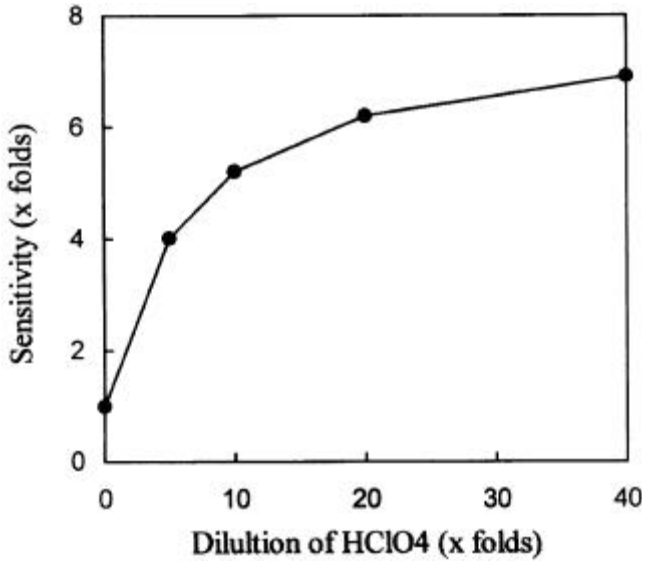

Figure 3. Effect of dilution of $HClO_4$ extract on sensitivity of the test. The extract was neutralized with KOH before dilution.

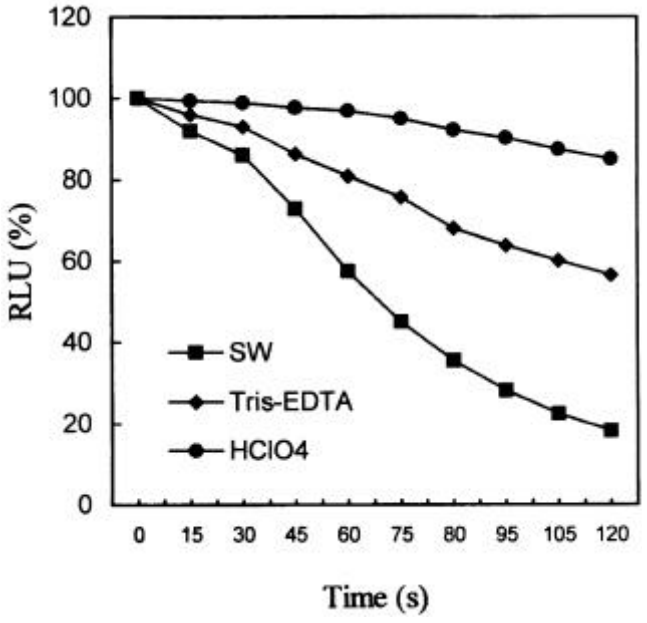

Figure 4. Effect of different extractants on the stability of luminescence signal. RLU, Relative light unit.

4. Discussion

The results of this study clearly demonstrate that the sensitivity of ATP assay in tissue can be enhanced several folds by using ultraturax homogenizer, and perchloric acid extraction followed by the removal of acid and appropriate dilution of the extract. The use of sonication for homogenizing striatal tissue for ATP assay has been reported earlier (Chan *et al* 1993), however, the present study shows that the efficiency of ATP extraction can be enhanced about 3-folds by using ultraturax homogenizer (figure 1). The use of $HClO_4$ extraction resulted in higher yield of ATP as compared to the other two media (figure 2). Since $HClO_4$ has a tendency to inhibit the enzyme luciferase, an efficient removal of $HClO_4$ and/or a sufficiently high dilution of extract are needed to minimize the inhibitory effect of $HClO_4$, before the assay is performed. This study showed that a combined approach including removal of $HClO_4$ from the extract, followed by an appropriate dilution significantly enhanced the sensitivity of the test (figure 3). Furthermore, the resulting luminescence signal was highly stable (figure 4), indicating an efficient removal of interfering components from the extract. The stability of luminescence signal with Tris-EDTA medium was comparatively higher than with SW medium, probably due to the chelatory effect of EDTA on ionic cofactors involved in ATP converting reaction. In conclusion, the sensitivity of the FLB test can be enhanced several folds by using ultraturax homogenizer, perchloric acid extraction, neutralization of acid extract followed by an optimum dilution, prior to bioluminescence reaction.

Acknowledgements

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