Bull. Environm. Contam. Toxicol. 22,715-716 (1979)

A Note on the Determination of ATP by the Luciferin-Luciferase Bioluminescence Reaction in Samples Containing Heavy Metals

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Iron- and sulfur-oxidizing bacteria have been intensely studied for their role in the microbiological leaching of metals from ores and for contributing to acid pollution problems of mine waters through the formation of sulfuric acid and ferric-iron. In our studies on characterizing the growth of Thiobacillus ferrooxidans on soluble ferrous-iron and inorganic sulfur compounds, and on metal sulfides, we endeavoured to measure ATP as one of the growth parameters reflecting the metabolic state and biomass of the cultures. Heavy metals including iron were observed to remain at low levels in extracts of ATP samples inhibiting the luciferin-luciferase bioluminescence reaction used for measuring ATP. The inhibition could be partially accounted for by using an internal ATP standard for each sample measured. However, better reproducible results and an increased recovery of ATP from acidic samples were obtained by modifying the method for extracting ATP as described in the present note.

In the first series of experiments using samples of chemostat cultures of T.ferrooxidans growing on 120 mM $FeSO_4$ at pH 1.8, ATP was extracted by mixing 1 ml sample with 0.2 ml 35 %(w/v) $HClO_4$ containing 67 mM EDTA, neutralizing with 0.78 ml 2.5 N KOH and diluting to 4 ml with 200 mM phosphate buffer (pH 7.3). Samples were centrifuged and the supernatants were assayed for ATP using 0.1 ml aliquots. A Lumac Celltester and partially purified luciferin-luciferase extracts of the firefly were used to monitor the ATP-dependent light emission which could be related to the ATP content of the sample by using a standard curve or an internal standard. Samples extracted in this way had a negligible ATP content probably owing to the low density of the bacteria in the samples. In checking the probable cause for the low amount of ATP, Baker's yeast was added to the samples (5 mg/ml) to increase the ATP content before extraction. Comparative tests consistently showed a 16 % improvement in the recovery of ATP if iron was removed from the extracts by adding, prior to centrifugation, 0.1 ml 4 mM Na_2S to precipitate soluble iron followed by 0.1 ml 4 mM $K_2S_4O_6$ to remove the excess sulfide by precipitation as elemental (colloidal) sulfur. The extraction with 10 mM Tris-H_2SO_4 containing 10 mM EDTA was also tested but deleted since an intense blue color developed

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0007-4861/79/0022-0715 $01.00

in Tris-extracts interfering with the light emission measurements. The perchloric acid extraction showed, however, that soluble ferrous/ferric iron remained in the extracts but could be further removed by the sulfide-tetrathionate treatment to alleviate the inhibition.

In another series of experiments, tests showed that copper, uranyl and zinc ions could be readily removed from the extracts by sulfide precipitation before centrifugation. Soluble nickel could also be precipitated but its deposition during centrifugation was not complete and some NiS remained as a surface film in the supernatants. The sulfide treatment was tested for practical application by mixing partially dissolved suspensions of NiS and CuS (synthetic sulfides obtained from K&K Laboratories) with washed cells of T.ferrooxidans. 1 ml samples (pH 1.5) containing about 50 mg metal sulfide were extracted with 0.2 ml perchloric acid, neutralized with 0.54 ml 2.5 N KOH and diluted to 2.1 ml with 200 mM phosphate buffer. By adding a known amount of ATP to parallel samples it was estimated that 86 and 34 % of the added ATP could be recovered in the NiS and CuS samples respectively by the routine measurement which included an internal standard for each sample. (The light emission was now measured in a Packard Tricarb liquid scintillation spectrometer by diluting 0.01 ml aliquots of samples with 3 ml phosphate-arsenate-$MgCl_2$ buffer before adding the enzyme.) 0.2 ml aliquots of the extracted samples were mixed with 125 nmoles $S^=$ to precipitate nickel and copper from the solution and after centrifugation the ATP was assayed. The sulfide treatment increased the recovery of the added ATP to the range of 93-106 % normally attained in control measurements. The tests also indicated that, owing to the relatively low level of sulfide in the final diluted sample in the vial, it was not necessary to remove the excess sulfide to further increase the efficiency of measurement. In fact, if sulfide was removed by boiling until the spot test indicated a $S^=$-free sample, the recovery of ATP decreased by almost 50 % for the NiS-samples. However, acidification of the sulfide-treated extracts with H_2SO_4 for 10 min at 80-90°C volatilized most of the excess sulfide and after neutralization with NaOH no inhibitory effects nor degradation of ATP were observed in the extracts.

The luciferin-luciferase enzyme complex is very sensitive to heavy metals. Although the extent of adsorption and coprecipitation of ATP with metals is not known for samples described in the present communication, it seems that modifications may be designed for various ATP extraction procedures to partially or completely abolish the inhibitory effects of metal ions in the bioluminescence assay. These observations may be useful for developing methods for determining ATP in studies of acid mine water pollution and related problems which arise from microbiological activities of sulfuric acid generation and metal dissolution.

Acknowledgments. O.H.T. was sponsored by the Academy of Finland. The work was partially funded by the Australian Research Grants Committee (at the Waite Agricultural Research Institute, South Australia) and the Foundation for Research of Natural Resources in Finland, and related to Research Agreement No. 1949/CF with the International Atomic Energy Agency.