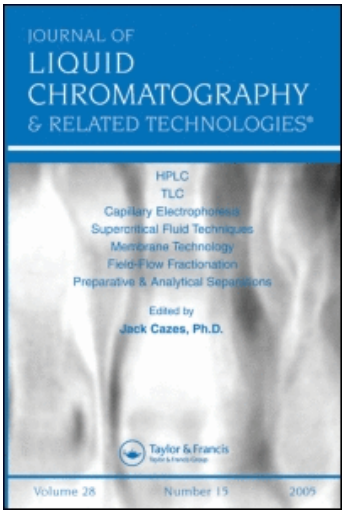

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ABSTRACT

A quick and sensitive method for the determination of AMP, ADP, and ATP levels in mantle tissue of the sea mussel *Mytilus galloprovincialis Lmk.* is described. The method uses ion-pair high-performance liquid chromatography with diode array detection, allowing obtaining of the UV absorption spectrum for each peak. Method precision was good. The detection limit for all three nucleotides was 20 pmol (much lower than the minimum concentrations in this tissue).

INTRODUCTION

The various species of *Mytilus* are sessile organisms that occupy intertidal habitats, and they are, therefore, useful experimental models for studies of mechanisms of adaptation to different types of stress, particularly in view of their ability to withstand very low oxygen levels for long periods. A number of

authors[1-5] have reported that *Mytilus* species adapt to hypoxia by depressing metabolic rate, and particularly glycolysis (i.e. the principal ATP-production pathway under anaerobic conditions). The same authors suggest that one of the mechanisms by which glycolysis is depressed is regulation of the activity of 6-phosphofructo 1-kinase (PFK-1; EC 2.7.1.11) by its allosteric modulators. PFK-1 is the principal regulator of glycolysis in various tissues of *Mytilus galloprovincialis Lmk.*[6,7] Its allosteric modulators include ATP (inhibitor at high concentrations) and AMP (activator).[8,9] It is, thus, of interest to be able to monitor the concentrations of these nucleotides in *Mytilus* tissues during hypoxia.

Accurate evaluation of the tissue concentration of any biological molecule requires 1) adequate conservation of the tissue prior to extraction, 2) the use of an appropriate extraction procedure, and 3) the use of an appropriate analytical procedure. A number of good enzymatic methods are available for the determination of nucleotides;[10,11] however, all such methods are time-consuming. High-performance liquid chromatography (HPLC) techniques are faster, and have proved useful for the isolation and quantification of nucleotides in both biological fluids and tissue extracts. Both anion-exchange HPLC[12-14] and reversed-phase ion-pair HPLC[15-17] are rapid and sensitive techniques that permit simultaneous measurement of the concentrations of different nucleotides.[18,19] Nevertheless, nucleotide elution profiles vary widely from one tissue to another, and it is thus necessary to develop assays specifically for each extract of interest.

In the present study, we developed a simple ion-pair HPLC assay, with diode array detection, for determination of AMP, ADP, and ATP levels in *Mytilus galloprovincialis Lmk.* Ion-pair chromatography was used because this technique has been reported to give better results than anion-exchange chromatography.[20] Tetrabutylammonium dihydrogen phosphate (TBA) was used as pairing ion, in view of a report indicating that it is the most effective reagent for retaining most nucleotides.[16]

EXPERIMENTAL

Reagents

Adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), and inosine 5'-monophosphate (IMP) were from Boehringer (Mannheim, Germany). Hexokinase (EC 2.7.1.1), pyruvate kinase (EC 2.7.1.40) and AMP deaminase (EC 3.5.4.6) were from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH) and perchloric acid (PCA) were from

Merck (Darmstadt, Germany). Tetrabutylammonium dihydrogen phosphate (TBA) was from Aldrich (Steinheim, Germany). Acetonitrile was from Panreac (Barcelona, Spain). All water used was previously purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA). Before use, all solutions were filtered through 0.45 μm membranes (Millipore) and degassed in an ultrasonic bath.

Stock Solutions of Nucleotides

Stock aqueous solutions of AMP, ADP, and ATP (3 mM) were made up previously, then divided into 1 mL aliquots and stored at -80°C. Working solutions (as required for peak identification, construction of calibration curves, and assessment of extraction efficiency) were made up daily from these stocks.

Tissue Samples

Mussels (*Mytilus galloprovincialis Lmk.*) were collected from the Ría de Betanzos (La Coruña Province, northwest Spain). On arrival at the laboratory, the mussels were maintained for 24 h in tanks with continuously aerated seawater at 18°C. The water was then drained off, leaving the mussels exposed. Samples of 20 mussels were taken immediately before draining, and at regular intervals between 3 h and 9 days after draining. In all cases, the mantle was immediately dissected out and weighed, then frozen by immersion in liquid nitrogen (-196°C) and stored at -80°C until extraction.

The extraction procedure was as follows. The still-frozen samples were rapidly homogenized in a Potter homogenizer in an ice bath, after addition of cooled 0.6 M PCA (3 mL per g of tissue). The homogenate was left to stand for 10 min, then centrifuged at 4°C for 15 min at 12000 rpm. The supernatant was collected, adjusted to pH 4 with 2 M KOH, left to stand for 30 min in an ice bath, then centrifuged for 2 min at 12000 rpm to sediment out the $KClO_4$ formed. The supernatant was filtered through a 0.45 μm Millipore membrane, divided into 500 μL aliquots, frozen by immersion in liquid nitrogen, then stored at -80°C until analysis.

For estimation of recovery, three standard solutions of each nucleotide (1, 2, and 3 mM) were subjected to the above extraction procedure, in each case in triplicate. To this end, each sample of standard solution was divided into three aliquots of 1 mL, to each of which was added 3 mL of 0.6 M PCA. The subsequent procedure was identical to that used for tissue samples.

Table 1

Elution Schedule Used*

t (Min.)	% Buffer A	% Buffer B	Curve
0	100	0	---
2	100	0	---
15	30	70	0
25	30	70	---
27	100	0	0
37	100	0	---

* See Figure 1.

Chromatography

AMP, ADP, and ATP contents in the tissue extracts were determined using a Beckman HPLC system (Arlington Heights, IL, USA). This system comprises an injection valve (module 210A), two pumps (module 126), and a diode array detector (module 168) programmed to simultaneously monitor absorbance at 254 and 280 nm with a 4 nm bandwidth, and record the UV absorption spectra of the peaks between 220 and 320 nm.

Separation was done on a 5-μm Ultrasphere ODS reversed-phase analytical column (250 mm x 4.6 mm i.d.), as follows: flow rate 1 mL/min; injection volume 20 μL; mobile-phase, 25 mM KH_2PO_4 containing 2 g/L of TBA as buffer A, and a 1:3 (v/v) mixture of acetonitrile and 125 mM KH_2PO_4 containing 2 g/L of TBA as buffer B (both at pH 5.5 adjusted with 2 M KOH). The elution schedule used is summarized in Table 1.

The peaks corresponding to each nucleotide were identified a) by comparison of retention times with those obtained in analysis of standard solutions, b) by spiking of samples with known amounts of adenine nucleotides, c) by comparison of UV absorption spectra of the sample peaks recorded during the run using the diode array detector with those obtained in analysis of standard solutions, and d) by the enzymatic peak-shift method of Brown.[12]

Nucleotide concentrations were estimated from peak areas. Solvent programming, data collection and data analysis were done with the aid of the System Gold program.

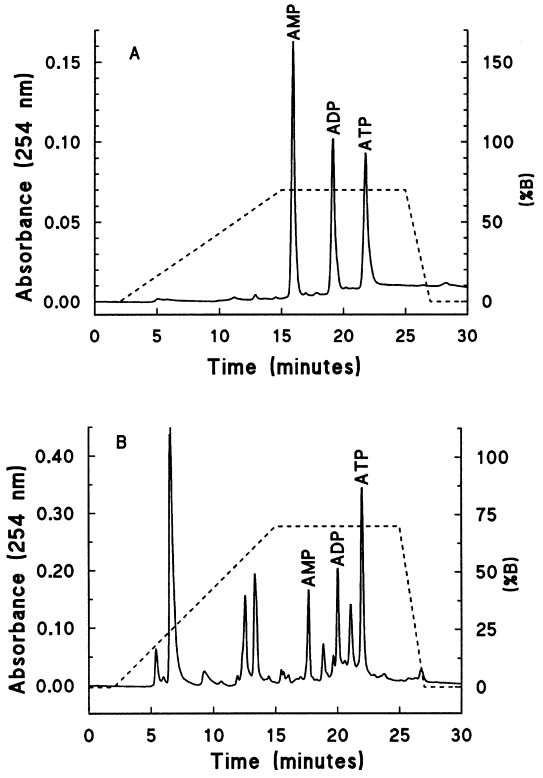

Figure 1. Elution profiles of a standard mixture of adenine nucleotides (each 250 μM) (A) and an extract from mussel mantle (B). Chromatographic conditions are detailed in Material and Methods. Dotted lines show percentage of Buffer B (right vertical axis).

RESULTS AND DISCUSSION

Chromatographic Conditions

The aim of the present study was to develop a rapid and precise method for the routine determination of adenine nucleotides in the mussel *Mytilus galloprovincialis Lmk.* To this end, we used reversed-phase ion-pair chromatography with TBA as the pairing ion. Elution profiles obtained after injection of a standard solution of adenine nucleotides and an extract from *M. galloprovincialis* mantle are shown in Figs. 1A and 1B, respectively. The three nucleotides were well separated with a 30 minutes run. Gradient elution was essential, in view of the complex composition of the tissue samples.

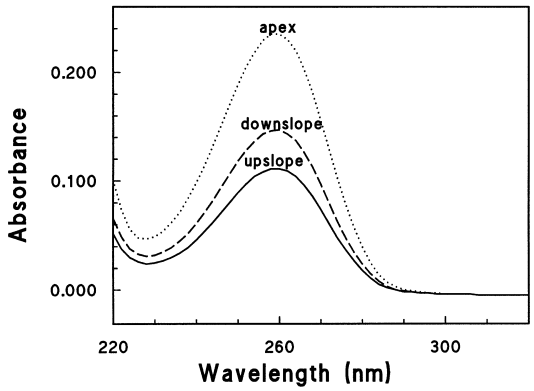

Figure 2. UV absorption spectra (as recorded with the diode array detector) of the upslope, apex and downslope parts of the ATP peak obtained in chromatography of an extract from mussel mantle (see Fig. 1B). The similarity of these spectra confirms the purity of the peak.

Table 2

Intra-Assay Variation (i.e., Reproducibility) of Determinations of Adenine Nucleotides on the Basis of Peak Areas (PA) or Retention Times (RT)*

	RT_{SS}	PA_{SS}	RT_{TE}	PA_{TE}
AMP	16.1 ± 0.15 (0.9%)	52.5 ± 4.5 (8.6%)	16.0 ± 0.07 (0.47%)	23.4 ± 2.6 (11.4%)
ADP	19.3 ± 0.12 0.62%)	33.5 ± 2.05 (6.1%)	19.17 ± 0.1 (0.57%)	55.4 ± 1.17 (2.1%)
ATP	22.0 ± 0.05 (0.22%)	33.9 ± 2.48 (7.3%)	21.7 ± 0.08 (0.4%)	139.4 ± 3.4 (2.45%)

* Values shown are means ± standard deviations, with coefficients of variation in brackets, in each case for five replicate assays of the same 100 μM standard solution (SS) or tissue extracts (TE).

Identification of Peaks and Confirmation of Peak Purity

In each sample, peaks were identified by comparing retention times to those of standard solutions (Table 2). The identifications were confirmed by spiking (addition of 1 nmol of AMP, ADP, and ATP to samples gave the

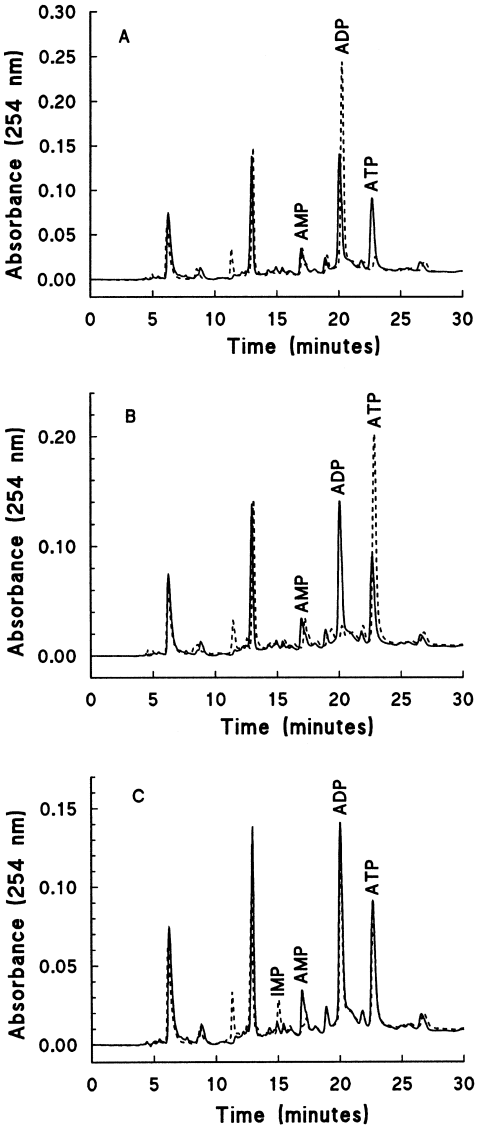

Figure 3. Results of enzymatic peak-shift assays to investigate the purity of the adenine nucleotide peaks obtained in chromatography of extracts from mussel mantle (see Fig. 1B). Solid lines (—) show elution profiles of untreated extracts, and dotted lines (---) elution profiles of extracts treated with hexokinase (ATP → ADP) (A), pyruvate kinase (ADP → ATP) (B) or AMP deaminase (AMP → IMP) (C). Chromatographic conditions were the same as for Figure 1, and are detailed in Experimental.

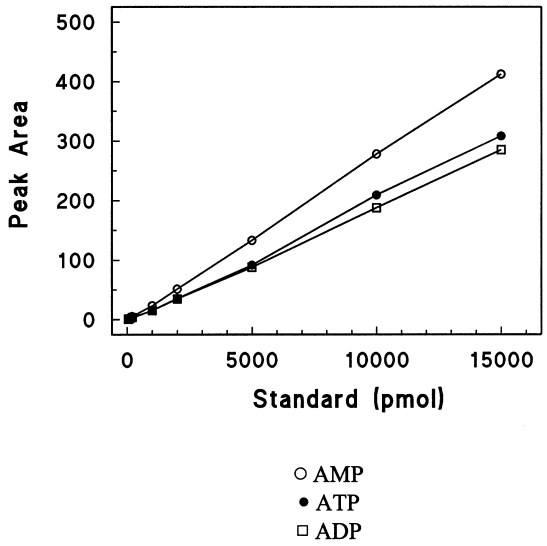

Figure 4. Representative calibration curves for AMP, ADP and ATP, obtained by linear regression of peak area (PA) on amount of nucleotide injected (ANI) (four replicates at each concentration). PA_{AMP} = -1.95 + 0.027*ANI, r = 0.999; PA_{ADP} = -2.19 + 0.019*ANI, r = 0.999; PA_{ATP} = -3.62 + 0.02*ANI, r = 0.999. Chromatographic conditions were the same as for Figure 1, and are detailed in Experimental.

expected increases in the absorbance of each peak) and by comparison of UV absorption spectra with those of standard solutions. Comparison of the UV absorption spectra of the upslope, apex, and downslope parts of the different peaks (Fig. 2, showing the results for ATP) indicated that the peaks were of high purity. Note that the three adenine nucleotides have identical UV absorption spectra (since the purine base is common to all three), so that the other identification methods are necessary.

Finally, incubation of sample aliquots with a) hexokinase,[10] b) pyruvate kinase,[11] or c) AMP deaminase[21] led to the complete disappearance of the peaks corresponding to ATP, ADP, and AMP respectively, and increases in the corresponding reaction products (ADP, ATP, and IMP respectively) (Fig. 3), thus confirming peak purity.

Method Quality

Typical calibration curves for AMP, ADP, and ATP are shown in Figure 4. A close linear relationship was observed between injected nucleotide

Table 3

Adenine Nucleotide Concentrations in Mantle Extracts from Mussels Sampled Immediately Before Draining the Tank*

Nucleotide	nmol/g of Fresh Weight
AMP	451.51 ± 51.28
ADP	2034.11 ± 300.00
ATP	3576.23 ± 204.20

* Values shown are means ± SD for 20 mussels.

concentration and detector response over a wide nucleotide concentration range (50-15000 pmol). For all three nucleotides, the detection limit was 20 pmol. Signal-to-noise ratio at the detection limit was 2. Both retention-time and peak-area measurements gave good reproducibility, as indicated by low intra-assay variation in assays of both standard solutions and tissue extracts (Table 2).

The nucleotide extraction method used in the present study has been extensively characterized.[22,23] Nevertheless, we investigated its efficiency using three standard solutions of adenine nucleotides (1, 2, and 3 mM), each of which was subjected to the complete analytical procedure in triplicate. Recoveries were excellent: 105.8 ± 8.5% for AMP, 102.0 ± 5.8% for ADP, and 108.1 ± 7.8% for ATP.

Adenine Nucleotide Levels in *Mytilus galloprovincialis* Mantle

Table 3 lists the concentrations of AMP, ADP, and ATP in *M. galloprovincialis* mantle, in tissues frozen immediately before draining the tank (i.e. from animals that had been maintained under aerobic conditions). The values obtained are similar to those obtained in this tissue by conventional enzymatic methods.[24]

Figure 5 shows levels of the three nucleotides in tissues frozen after different periods of hypoxia. As can be seen, AMP concentration increased steadily throughout the 9-day hypoxia period, with a very rapid increase during the first 24 h. In contrast, ATP concentration declined steadily throughout the hypoxia period. ADP concentration did not vary significantly ($p > 0.05$) over the hypoxia period. Since PFK-1 activity in this tissue is stimulated by AMP and inhibited by ATP,[8,9] these findings are consistent with increased glycolysis during the first 24 h of hypoxia.

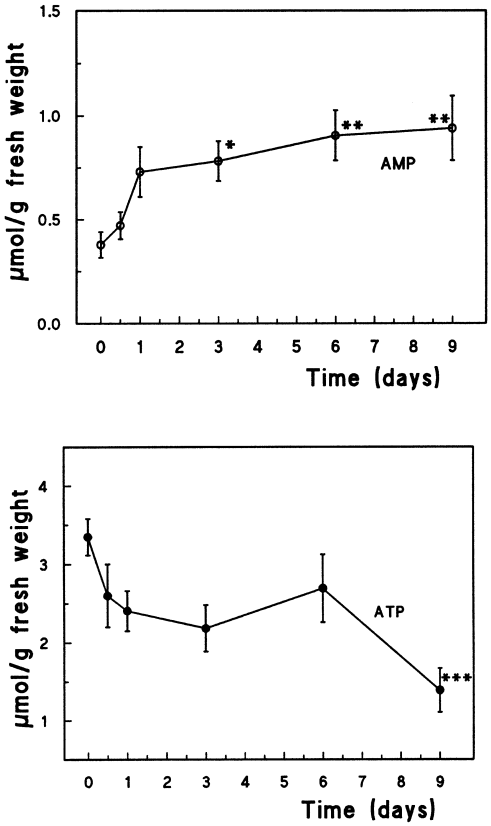

Figure 5. Time-courses of AMP and ATP concentrations in mussel mantle over the 9-day period following draining of the tank. Values shown are means and standard deviations for 20 samples. In both cases one-way analysis of variance (ANOVA) indicated that "period of hypoxia" had a significant effect on adenine nucleotide concentration; Tukey-Kramer test was then performed to identify means that differed significantly from the corresponding day-0 value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In contrast, a previous study has found that hypoxia leads immediately to reduced glycolysis.[25] This apparent discrepancy is probably attributable to the fact that we reduced oxygen levels simply by draining the tank (so that the mussels maintained some oxygenated water within their shells, as occurs naturally at low tide), whereas Isani induced immediate anoxia by bubbling nitrogen into the water. After the first 24 h, we found that AMP levels continued to increase, and ATP levels continued to decline, though much less rapidly than during the first 24 h.

However, the levels of fructose 2,6-bisphosphate (a PFK-1 activator) declined rapidly after the first 24 h (results not shown; manuscript in preparation), which accords with previous findings that hypoxia leads to reduced glycolytic activity.

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