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Short communication

Optimized method for the determination of phosphoarginine in abalone tissue by high-performance liquid chromatography

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Abstract

A rapid high-performance liquid chromatography method for the determination of phosphoarginine (PArg) in invertebrate tissue has been redeveloped and validated. The method employs a reversed-phase amino column and a KH_2PO_4–acetonitrile mobile phase. PArg peak identity was confirmed by comparison with a known standard and via enzymatic conversion. Additionally linear calibration data, low intra-assay variability (<4%), and a detection limit of 5 pmol were determined. The method was demonstrated using PArg extracted from red abalone (*Haliotis rufescens*) adductor muscle. Validation of the extraction procedure was also completed, including the measurement of a 100.2±0.9% extraction efficiency.

Keywords: Phosphoarginine

1. Introduction

Phospho-L-arginine (PArg; Fig. 1) is the primary phosphagen in invertebrates, and serves as both a spatial and temporal energy buffer in tissues with high-energy demands. This process is catalyzed by arginine kinase, which exchanges a phosphate from PArg to ADP, thus maintaining a stable ATP concentration:

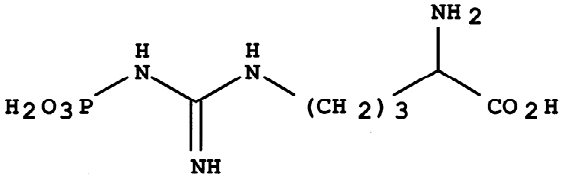

Fig. 1. Structure of phospho-L-arginine.

$$\text{PArg} + \text{ADP} \rightleftarrows \text{arginine} + \text{ATP} \quad (1)$$

An increase in organismal energy demand due to either an elevated metabolism or exposure to toxicants that impede ATP formation will be reflected by changes in the PArg concentration ([PArg]). In fact, the ratio of [PArg] to the concentration of inorganic phosphate can serve as a more sensitive indicator of energetic status than the adenylate energy charge [1]. Using in vivo ^{31}P nuclear magnetic resonance spectroscopy we have successfully employed this indicator to characterize the actions of copper [2], pentachlorophenol [3], and 3-trifluoromethyl-4-nitrophenol [4] in red abalone. However, determining [PArg] by high-performance liquid chromatography (HPLC) would be desirable, since this would enable

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a more quantitative analysis of a greater number of samples.

To date, very few HPLC methods for the determination of PArg have been reported. In a study of adenylate nucleotides in muscle extracts using ion-pair HPLC and a reversed-phase C_{18} column, Shofer and Tjeerdema reported that PArg and arginine were not reliably separated [5]. Although Teague Jr. and Dobson successfully employed a strong anion-exchange column to isolate PArg from a simple in vitro reaction mixture [6], this method was not recommended for the analysis of tissue extracts [7]. Most recently, Shimada et al. briefly described the determination of PArg in lobster muscle extracts using a reversed-phase NH_2P-50 column [8]. Unfortunately no method validation, calibration data, or chromatograms were reported.

Due to this lack of characterization, and because our attempt to reproduce their method failed, a comprehensive redevelopment and validation of the procedure was necessary. Specifically, the chromatographic conditions, including buffer concentration, solvent ratio, and temperature were optimized. Separation of PArg in tissue extracts was validated by comparison with a known standard and via enzymatic conversion. Furthermore, calibration data, intra-assay precision, and the detection limit were determined. This revised method is demonstrated using PArg extracted from the adductor muscle of red abalone. The validation of the extraction procedure is also discussed, including extraction efficiency and the storage stability of PArg.

2. Experimental

2.1. Chemicals

Phospho-L-arginine (90% minimum purity), magnesium chloride, hexokinase (EC 2.7.1.1) and arginine kinase (EC 2.7.3.3) were purchased from Sigma (St. Louis, MO, USA), while potassium phosphate (monobasic; ultrapure bioreagent for LC), acetonitrile (HPLC grade) and water (HPLC grade) were obtained from VWR Scientific Products (Brisbane, CA, USA). Perchloric acid (70%) and potassium carbonate were acquired from Fisher Scientific (Pittsburgh, PA, USA), and glucose was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Chromatographic equipment and conditions

Equipment comprised of a Hewlett-Packard Model 1090 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Rheodyne manual injection valve and a UV–Vis diode-array detector set at 205 nm. Separation of PArg was achieved using a reversed-phase SphereClone NH_2 column (250×4.6 mm I.D., 5 μm particle diameter; Phenomenex, Torrance, CA, USA) fitted with an NH_2 SecurityGuard cartridge (4×3.0 mm I.D.; Phenomenex), at room temperature. The isocratic mobile phase was composed of KH_2PO_4 buffer (20 m*M*, pH 2.6)–acetonitrile (72:28). Solvents were filtered through a 0.44-μm membrane filter, helium degassed, and used at a flow-rate of 1 ml/min. The PArg peak was identified by (i) comparison to a known standard, and (ii) enzymatic conversion (see Section 2.5); total analysis time was 20 min.

2.3. Preparation of standard solutions and calibration curve

A stock solution of 10 m*M* PArg in KH_2PO_4 buffer (20 m*M*, pH 7.0) was prepared, and then diluted to concentrations of 5000, 2000, 1000, 500, 250, 100, 25 μ*M* PArg. These standards were aliquoted and stored at −80°C to minimize degradation. A calibration curve was generated by injecting 10 μl of each of these standards (in triplicate), and then subjected to linear regression analysis.

2.4. Extraction procedure

Red abalone (*Haliotis rufescens*) were purchased from The Cultured Abalone (Goleta, CA, USA) and maintained in a seawater aquarium. The adductor muscle was rapidly dissected and freeze clamped in liquid nitrogen. Samples were pulverized to a fine powder in a pre-cooled mortar and pestle, transferred to pre-weighed and pre-cooled microcentrifuge tubes, and then lyophilized for 48 h. Next, samples were weighed, extracted using 40 ml/g (dry mass) of ice-cold 6% perchloric acid (PCA), vortexed (30 s), incubated on ice (10 min), and then centrifuged (12 000 *g*, 10 min, 4°C). The supernatants were neutralized (pH 7.0) with 2 *M* K_2CO_3, incubated on ice (30 min), and then centrifuged again (12 000 *g*, 10 min, 4°C). Finally, the samples were passed

through 0.45-μm nylon syringe filters before being analyzed by HPLC (20 μl injected) or stored at −80°C.

2.5. Enzymatic confirmation of phosphoarginine

Verification of metabolite identity was achieved via enzymatic degradation of PArg according to Eq. (1). An aliquot of muscle extract was added to a reaction mixture containing 50 m*M* phosphate buffer (pH 7.6), 50 m*M* glucose, 6.7 m*M* magnesium chloride, 10 U hexokinase and 33 U arginine kinase. The hexokinase converts ATP to ADP [9], thereby shifting the equilibrium in Eq. (1) in the forward direction, towards the degradation of PArg. The reaction was incubated at 25°C for 20 min, filtered, and then analyzed by HPLC. An identical reaction mixture, except without enzymes, was used as control.

2.6. Tests of method robustness

Intra-assay precision was determined by injecting 2.5 and 20.0 nmol PArg standards and a muscle extract, five times each. Extraction efficiency was calculated by subjecting a 1000 μ*M* PArg standard (in triplicate) to the extraction procedure described above, followed by HPLC analysis. PArg peak areas were compared to those from an equivalent 1000 μ*M* PArg standard (in triplicate) maintained at −80°C.

Two storage stability experiments were conducted, the first to determine the thermal stability of a 1000 μ*M* PArg standard at −80°C, and the second to check for the decay of PArg by residual enzymatic activity in a PCA-extracted muscle sample. In both cases, samples (in triplicate) were analyzed by HPLC, stored at −80°C for 15 days, and then re-analyzed by HPLC.

3. Results and discussion

3.1. Extraction procedure

The rate of PArg depletion in fresh tissue was determined by freeze clamping at increasing time periods after the initial dissection. A 20% decrease in PArg was observed in tissue that sat for only 3 min before freezing. Thus, to obtain an accurate measure of PArg, both dissection and freeze clamping must be extremely rapid. Although PArg is susceptible to hydrolysis during the PCA extraction, we observed <3% loss following a 30-min incubation in 6% PCA. Consequently, the 10-min incubation employed in the optimized extraction procedure causes minimal loss of analyte. Also, no additional degradation of PArg was caused during lyophilization.

3.2. Chromatographic separation

Representative chromatograms of a PArg standard solution and of adductor muscle extract are presented in Fig. 2. The PArg peak in the tissue extract was identified by comparing its retention time (t_R = 6.27 min) to that of the standard (t_R = 6.13 min), and confirmed via enzymatic conversion, as illustrated in

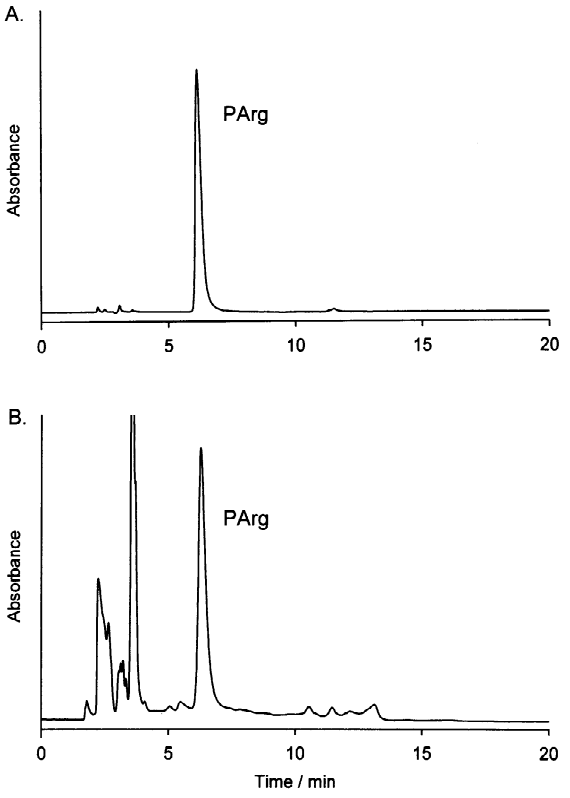

Fig. 2. Representative chromatograms of (A) phosphoarginine (PArg) standard (2000 μ*M*), and (B) abalone adductor muscle extract, analyzed on a reversed-phase SphereClone NH_2 column (5 μm, 250×4.6 mm I.D.) in a KH_2PO_4 (20 m*M*, pH 2.6)–acetonitrile (72:28) mobile phase, with detection at 205 nm.

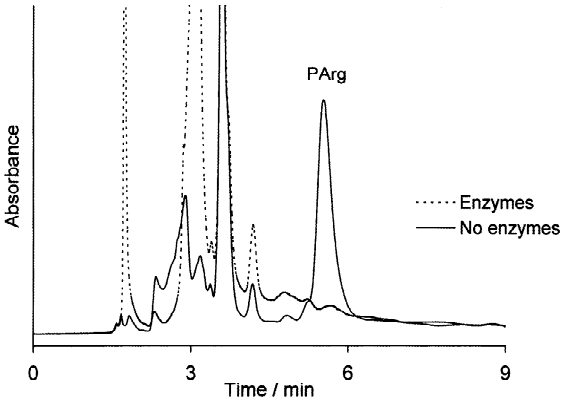

Fig. 3. Results of the enzymatic conversion assay to confirm the identification and purity of the phosphoarginine (PArg) peak in abalone adductor muscle extract. A neutralized aliquot of extract was incubated with hexokinase (ATP→ADP) and arginine kinase (PArg + ADP→arginine + ATP) at 25°C for 20 min, thereby depleting the PArg signal. Chromatographic conditions as in Fig. 2.

Fig. 3. The results of the enzymatic conversion also establish that PArg does not co-elute.

3.3. Calibration curve

The calibration data ($n = 7$ concentrations, each in triplicate) was subjected to linear regression analysis, yielding: PArg peak area = 119.9 × nmol PArg ($R^2 = 0.9999$). Excellent linearity was observed between peak area and analyte injected over the range 0.25–50 nmol. A detection limit of 5 pmol was achieved (equivalent to 138 ng/ml for a 10 μl injection), at which the signal-to-noise ratio was two.

This calibration data yields a [PArg] in abalone adductor muscle extract of 3.142±0.012 (mean±SD) nmol/μl, which corresponds to a [PArg] of 15.96±0.06 μmol/g (wet mass) in the muscle itself; this calculation assumes that the PArg standard is 100% pure. However, the standard has a 90% minimum purity, as declared by the manufacturer. Thus the determination of [PArg] has an inherent error of ≤10%, which leads to 14.36≤[PArg]≤15.96 (±0.06) μmol/g (wet mass) in the muscle. This is similar to the 11.77±2.92 μmol/g (wet mass) reported for [PArg] in the adductor muscle of another abalone species, *H. lamellosa*, which was determined enzymatically [10].

3.4. Tests of method robustness

PArg peak area measurements gave good reproducibility as indicated by the following low intra-assay variations determined by injecting each sample five times: peak area = 3856.8±14.3 (mean±SD in units of detector absorbance × peak width; 0.37% variability) for muscle extract, 2471.6±9.63 (0.39% variability) for 20.0 nmol PArg standard, and 308.4±9.42 (3.05% variability) for 2.5 nmol PArg standard. The extraction efficiency for PArg, determined using a 1000 μ*M* PArg standard (in triplicate), was calculated to be 100.2±0.9%, confirming excellent analyte recovery. Finally, the storage stability of PArg at −80°C for 15 days was determined to be 99.9±0.4% for 1000 μ*M* PArg standard, and 99.7±4.9% for muscle extract. The increased variability in the muscle extract potentially resulted from residual enzymatic activity.

4. Conclusions

We have reported an optimized method for the determination of phosphoarginine in tissue extracts. The HPLC procedure has been validated, including: (i) the confirmation of PArg identity by comparison to a known standard and via enzymatic conversion; (ii) the confirmation of linearity between peak area and detector response over a wide range of analyte concentrations ($R^2 = 0.9999$); (iii) the observation of low intra-assay variations (<4%); (iv) the determination of a 5 pmol PArg detection limit; (v) the determination of a 100.2±0.9% extraction efficiency based upon results from the complete analytical method; and (vi) the proof of applicability of the method following the analysis of phospho-L-arginine in abalone adductor muscle.

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