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A Colorimetric Microplate Assay Method for High Throughput Analysis of Arginase Activity *in Vitro*

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Abstract

Several analytical methods have been developed for the determination of arginase activity in physiological samples. These methods are limited by the considerable effort and time required to obtain reliable and reproducible measurements. Here we describe a simple high throughput colorimetric assay for the determination of arginase activity based on the ornithine-ninhydrin reaction. This method is an improvement over the original single cuvette assay developed by Chinard in that no boiling step is required. The turn-around time has been reduced, with improved precision and reproducibility. The method was extended to the determination of arginase activity in human leukemic (K562) cells and sickle erythrocytes. We believe that the method will find applications for routine analysis as well as for characterizing the action of novel and potent inhibitors on arginase activity.

Introduction

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is found predominantly in the liver and kidneys, but is also present in human red blood cells [1]. Since arginine and ornithine compete for the same transport system for cellular uptake, a decrease in the ratio of arginine to ornithine resulting from increased arginase activity could limit arginine bioavailability for nitric oxide (NO) synthesis. Arginase has important roles in human pathogenesis, and its activity modulators have been suggested as potent pharmaceuticals for the treatment of inflammation-mediated diseases including certain neoplastic conditions [2-5].

Different assays for arginase activity, including the utilization of ¹⁴C-labeled L-arginine as substrate to produce ¹⁴C-labeled urea, have been developed [6]. A spectroscopic method involving the coupling of urea product with urease and glutamate or the utilization of an alternate substrate for arginase has also been developed [7-10]. These methods are limited by the considerable effort and time required to obtain reliable and reproducible measurements. Moreover, some of these assays require the use of a radio labeled substrate. The colorimetric arginase assay, which utilizes the reaction of ninhydrin reagent with ornithine, was first

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described by Chinard [11]. One of the chief benefits of this method is that it appears to accurately measure the activity of arginase isozymes (types I and II) in several mammalian tissues, including intact erythrocytes [5,12]. Also, the physiological enzyme substrate utilized in this procedure is readily available.

Despite its clear utility, widespread application of the ninhydrin-ornithine single cuvette assay for arginase activity is limited due to the considerably wide variability/low reproducibility and increased turn-around time required from sample to sample analysis. A typical arginase activity assay experiment demands triplicate analysis of the test mixture as well as similar analyses of three control samples. As a single cuvette reaction may take more than 2 h to prepare and analyze, several days may be required to complete about 96 samples. The development of a high-throughput method of assaying arginase activity would thus simultaneously reduce the required effort, enhance the sensitivity of the method, and improve the reproducibility of the data. The current work describes a high-throughput method for the determination of arginase activity, based on modification of the ninhydrin-ornithine enzyme assay, permitting up to 96 reaction mixtures to be evaluated within the space of a few hours.

Methods

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Preparation of erythrocyte arginase hemolysate

Whole blood samples were collected in heparinized tubes from pediatric patients with homozygous sickle cell (Hb SS) disease. To be eligible for blood donation for this study, sickle cell disease (SCD) patients must have had no blood transfusions for at least six months before the initiation of the study. All procedures involving the use of human subjects were approved by the University of Missouri Pediatric Institutional Review Board, and informed consent was obtained from all subjects prior to blood collection. Whole blood samples were processed within 24 hours of collection and the red blood cell (RBC) portion was separated from whole blood in several steps as described earlier [5]. Erythroleukemic (K562) cells (ATCC, Manassas, VA) were cultured in RPMI medium for 72 hours followed by cell lysis and the supernatant was utilized for the arginase assay. Purified bovine arginase 1 was purchased from AXXORA, LLC, San Diego, CA. One unit of enzyme activity is defined as the amount of the enzyme that produces 1 μmol of ornithine/min at 37°C. Specific activity is expressed in enzyme units per mg of protein. Total protein was measured by the Bradford assay.

Microplate method

Reactions mixtures were assembled at 4°C containing 16.6 μL of known units of purified arginase 1 in the reaction buffer (100 mM Tris—HCl) at pH 7.4, and 1.0 mM MnCl_2 supplemented with 100 μM arginine (adjusted to pH 7.4 with HCl) to initiate the reaction. The mixtures were incubated at 37°C in a water bath with gentle shaking for 30 minutes. The reaction was stopped by the addition of 166 μL of 0.72M HCl, followed by centrifugation at 5000 RPM for 5 minute at ambient temperature. Aliquots (30 μL) of the supernatant were transferred to a Costar polystyrene 96-well microplate (Corning, NY), followed by the addition of 66 μL of 6% ninhydrin, and overlaid with 50 μL of optically inert TW oil (Inland Vacuum, Churchville, NY). Thereafter, the samples were preheated to 100 °C (no boiling) in a temperature-controlled LC Oven (Barnstedt LabLine, Melrose Park, IL) for 25 minutes and were allowed to cool at room temperature before reading the absorbance at 505 nm using the SpectraMax® spectrophotometer (Molecular Device, Sunnyvale, CA). The level of arginase activity was expressed as μmoles of ornithine produced per min (U) per milligram protein.

Conventional single cuvette method

Single reactions were prepared as above, but contained 0.25 ml enzyme hemolysate and 0.5 ml 100 μ M arginine to initiate the reaction. The samples were incubated for 60 minutes (not 30 minutes), followed by the addition of 1 ml of 0.72 M HCl to stop the reaction. After centrifugation, 2 ml of 6% ninhydrin was added to 1 ml of the filtered sample in a spectronic cuvette and the samples were boiled (without oil overlaid) for 25 minutes, with a marble placed on top of each cuvette. Cooled samples were read using a Coleman 124 spectrophotometer (Perkin—Elmer, Shelton, CT) at an absorbance of 505 nm.

Results and Discussion

A high-throughput arginase assay and reproducibility

Methods for determination of arginase activity have important applications in understanding the structure-activity relationship of this important enzyme involved in cell proliferation and in elucidating cell signaling and testing mechanisms of action of potent inhibitors of arginase [2,3]. In general, the ninhydrin-ornithine method has been underutilized, for these purposes as it is time consuming and subject to considerable experimental variability, as well as low reproducibility. A high-throughput method offering high reproducibility would provide the opportunity to reliably evaluate arginase activity *in vitro* for these purposes. In response to these needs, we devised a method for the simultaneous analysis of up to 96 samples using supplies and equipment common to many research venues. Each buffered reaction contains defined quantities of one or more units of arginase to which a small volume of ninhydrin was added to react with ornithine produced. Individual samples were overlaid with an optically inert oil to prevent evaporation of the sample during the preheating step (without boiling). With this microplate assay, about 96 samples could be read in less than 5 minutes. As replicate samples and controls are assayed simultaneously, time-dependent variations in technique or reagents do not occur, eliminating their potential impact on data quality.

To test the precision of the method, five replicate samples of the time-course hydrolysis of arginine were measured (Fig. 1). As shown, the ornithine produced per unit time (as a measure of arginase activity) of the replicate mixtures were practically indistinguishable, with standard deviations ranging from 1 to 5% of the mean for mixtures containing high and low units of arginase, respectively. Figure 1b shows the linearity of measured arginase activity by the microplate method. The inter-day assay reproducibility of the five replicate samples (samples prepared fresh daily) was also assessed on six consecutive days. The coefficient of variation (CV, %) for inter-day assay precision was 1.5 to 5.6%.

Microplate method is fast and highly reproducible

In order to demonstrate the advantage of the microplate method, we assessed arginase activity in erythrocyte hemolysate and cultured erythroleukemic cells (K562 cells) using the single-cuvette and the microplate methods. The reactions were initiated in triplicate at three different substrate concentrations. As shown, there was a large discrepancy between the data obtained by the single cuvette in comparison with the microplate method (Fig. 2a and 2b respectively). The time-dependent variations in operator technique and the larger volume of the reaction mixture likely contributed to the significant variability between samples observed in the single cuvette method. Further, our microplate method was found to be 6 times more sensitive than the single cuvette method. The increased sensitivity was largely due to the reduced volume of the reaction mixture. However, after correction for dilution, the difference in sensitivity between the two methods was not significant, as shown in Fig 2. We believe that the reproducibility of the assay could be further increased, if desired, by increasing the number of replicates. In theory, enormous numbers of replicates could be assayed simultaneously using

a method adapted to analysis in a 384-well microplate. Further, since boiling of samples is omitted in this modification, accidental contamination of samples from boiling water is also averted.

For the interference studies, we determined whether proline, citrulline and hydroxyurea (HU) (the only drug currently approved by the FDA for the treatment of sickle cell disease) interfere with ornithine. Our results indicate that only proline significantly interfered with ornithine determination at pH 7.4. Based on this observation, this microplate procedure may be supplemented with a chromatographic separation technique in certain myeloid tumors where proline synthesis is increased as was also suggested in the original procedure. However we have shown that our microplate method is fast, simple and highly reproducible. Further, we showed that HU, which has structural similarity with urea [13], did not interfere with ornithine determination. This lack of interference of HU lends further credence to the belief that our microplate method will find increased utility not only in routine analysis but also in patients on HU therapy.

In summary, the modifications described herein eliminate practical and scientific limitations of the arginase assay, by the single cuvette method, thereby increasing its utility for routine analysis and for characterizing the action of novel and potent inhibitors of arginase activity.

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1 Abbreviations used

NO, nitric oxide; Hb SS disease, homozygous sickle disease; SCD, sickle cell disease; RBC, red blood cell.

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FIG. 1.

Arginase activity determined using the microplate method is highly reproducible. Using the microplate method, standard reaction mixtures were prepared with 0.5, 1.0, 3.0 and 5.0 Units of 100 μ M arginase and incubated at 37 °C. The absorbances were recorded at different time points, (A). The linearity was evaluated by preparing different amounts of arginase (0.5-20 U), incubated at 37°C for 30 min, (B). Each data point represents the mean from triplicate determinations, and the error bar represents the standard deviation.

FIG. 2.

The microplate method generates reliable and highly reproducible arginase activity from various cell extracts. The arginase activities of reactions containing defined amounts of hemolysate from sickle erythrocytes or erythroleukemic (K562) cells were determined using the single-cuvette method (A) and the microplate method (B) at various substrate concentrations. The amounts of ornithine produced per unit time at different enzyme substrate concentrations were established as described in Methods section. Each data point represents the mean from triplicate determinations, after correction for dilution, and the error bar represents standard deviation.