

**FIG. 1.** Detection of *bcr/abl* fusion transcript in archival bone marrow biopsies Patient 34 is positive for the b2a2 transcript (96-bp product), whereas patient 33 is positive for both fusion transcript variants (b2a2, 96-bp product; and b3a2, 85-bp product). M, molecular size marker pBR322/BsuRI (MBI Fermentas), RT, reverse transcriptase, 1  $\mu$ g of total RNA was transcribed using 200 U of SuperScript II RNase<sup>-</sup> reverse transcriptase (Gibco BRL) following the manufacturer's protocol. PCR primers and reaction conditions are according to Branford *et al.* (7).

bone marrow trephines also provides reproducible quantitative data using real-time PCR technology. The efficiency of RNA extraction measured as successful amplification of several different cDNAs is more than 98% for bone marrow trephines processed in our institute. Up to now more than 200 routinely processed bone marrow specimens have been analyzed successfully. In addition RNA has been extracted from several dozens of other biopsies following the described protocol.

In a small series we could also show that from plastic-embedded bone marrow biopsies (6) RNA suitable for PCR analysis can be extracted following the described protocol (data not shown). This will now enable expression analysis in large archival collections of plastic-embedded biopsies for which immunohistochemical analysis is possible only for a few antigens.

In conclusion, we have developed a simplified cost-effective and robust protocol for the extraction of RNA from formalin-fixed paraffin-embedded tissue specimens, which is also suitable for decalcified and even for plastic-embedded biopsies. Our results clearly show that in addition to qualitative data reproducible quantitative results using real-time PCR technology can be achieved following the extraction protocol described herein. Thus quantitative mRNA analysis of large archival tissue collections for which extensive clinical data are available becomes possible.

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## A Spectrophotometric Assay of Arginase<sup>1</sup>

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The amino acid arginine is found at a critical junction between two major metabolic pathways. Arginine is hydrolyzed to ornithine and urea by the enzyme arginase as the final step in the excretion of excess nitrogen through the urea cycle. Arginine is also oxidized by the enzyme nitric oxide synthase, leading to citrulline and nitric oxide (NO).<sup>3</sup> NO is an important cell signaling molecule that regulates a number of functions in tissues and cells including vasodilation and neurotransmission (1, 2). The partitioning of arginine between these two fates will have an influence on nitrogen flux, multiple cell signaling pathways, and also its availability for protein and polyamine biosynthesis. Reciprocal regulatory mechanisms between

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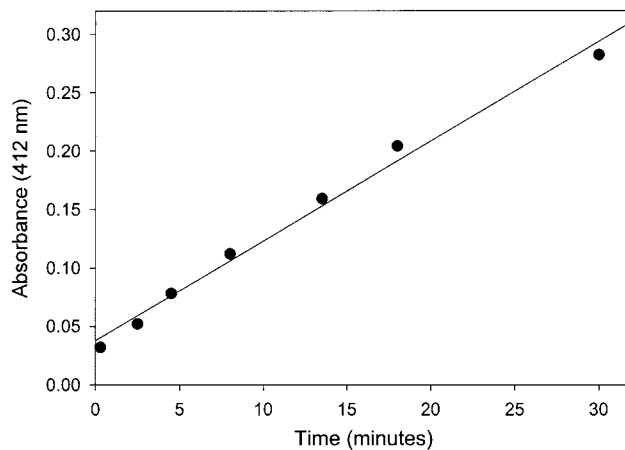
<sup>3</sup> Abbreviations used: DTNB, 5,5'-dithiobis(2-nitrobenzoate); NGB, 1-nitro-3-guanidinobenzene; NO, nitric oxide; thioarginine, L-2-amino-5-isothioureidovaleric acid.

these pathways have been proposed in which enhanced arginase activity leads to substrate depletion and therefore inhibition of NO synthase (3), while arginase inhibition by an NO pathway intermediate leads to enhanced NO production (4).

The catalytic activity of arginase is currently assayed either by a fixed-point assay or by a continuous spectrophotometric assay. The fixed-point assay uses  $^{14}\text{C}$ -labeled L-arginine as the substrate to produce  $^{14}\text{C}$ -labeled urea (5). The unreacted labeled substrate is separated from the product by adsorption on an ion-exchange resin and the eluted urea is then quantitated by scintillation counting (6). The spectrophotometric assays involve either the coupling of the urea product with urease and glutamate dehydrogenase (7) or the use of an alternative substrate of arginase, 1-nitro-3-guanidinobenzene (NGB), that yields a chromophoric product (8). The fixed-point assay is quite time consuming and requires the use of radiolabeled materials, and the continuous assays that have been developed to date are not extremely sensitive.

*Synthesis of a new alternative substrate.* Reagents were purchased from Aldrich. NMR spectra were run on a Varian Unity 400 and mass spectra on a Bruker Esquire LC/MS. Elemental analysis was conducted by Midwest Microlab.

Glutamic acid, protected at the  $\alpha$ -carboxyl and  $\alpha$ -amino groups with *t*-butyl and *t*-butoxycarbonyl groups (2*S*-*N*-(*t*-butyloxycarbonyl)glutamic acid *t*-butyl ester), was converted to an anhydride by the addition of 1 eq of ethyl chloroformate in anhydrous THF with stirring at  $-5^\circ\text{C}$  for 20 min. The filtered solution of the anhydride was reduced to the alcohol by the addition of excess sodium borohydride in water with overnight stirring at room temperature. The alcohol product, purified by silica gel chromatography, was dissolved at room temperature in dry  $\text{CH}_2\text{Cl}_2$  and reacted with methanesulfonyl chloride in the presence of 1.2 eq of triethylamine overnight. After acidic (5% HCl) and basic (50% saturated  $\text{NaHCO}_3$ ) washes the tosylated product was purified on silica gel to yield a pale yellow liquid. The product was dissolved in acetone and refluxed with thiourea overnight. The resulting residue was dissolved in  $\text{CH}_2\text{Cl}_2$ , filtered to remove the excess thiourea, and then purified by silica gel chromatography. The protected thioarginine product was dissolved in  $\text{CH}_2\text{Cl}_2$  at  $-76^\circ\text{C}$  and deprotected by the dropwise addition of a 1.0 M  $\text{BCl}_3$  solution in  $\text{CH}_2\text{Cl}_2$ . A pale yellow solid was obtained after drying in vacuum over  $\text{P}_2\text{O}_5$ . Further purification over silica gel yielded a white solid that was confirmed to be L-2-amino-5-isothioureidovaleric acid (thioarginine) by NMR [ $^1\text{H}$  (DMSO):  $\delta$  9.33 (s, 4H), 8.56 (s, 3H), 3.90 (t, 1H), 3.23 (t, 2H), 1.60–2.0 (broad, 4H);  $^{13}\text{C}$  (DMSO): 170.61, 169.92, 51.31, 29.33, 28.64, 24.49], mass spectroscopy (parent



**FIG. 1.** Time course of the hydrolysis of L-2-amino-5-isothioureidovalerate by arginase. Reaction was conducted in 50 mM HEPES buffer, pH 8.0, aliquots were removed at the indicated times, and the level of thiol product was quantitated as described in the fixed-point assay.

ion  $\text{C}_6\text{H}_{14}\text{N}_3\text{O}_2\text{S}^+$ , calculated: 192.26, found: 192.10), and elemental analysis (calculated ratios: C:H:N:S = 2.247:0.440:1.311:1, found C:H:N:S = 2.249:0.434:1.299:1).

*Fixed-point assay.* An assay was developed to quantitate the amount of thiol product produced by the arginase-catalyzed hydrolysis of the alternative substrate thioarginine. A reaction mixture was prepared in 50 mM HEPES buffer (pH 8.0) with varying concentrations of thioarginine in 1 mL total volume. The reaction was initiated by the addition of 20  $\mu\text{L}$  of arginase (0.14  $\mu\text{g}$ ). Aliquots were removed at fixed times and quenched by addition to a borate solution (9). Ten microliters of a 10 mM solution of 5,5'-dithiobis(2-nitrobenzoate) (DTNB) was added and the absorbance was read immediately after mixing. A molar extinction coefficient of  $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  at 412 nm was used to quantitate the product of the reaction (10). An increase is observed in the level of thiol product that is linear over at least 30 min (Fig. 1). Thioarginine is stable under these reaction conditions in the absence of enzyme, and only a small background absorbance from DTNB is observed in controls examined in the absence of either thioarginine or arginase.

*Continuous assay.* To develop a continuous spectrophotometric assay a buffered solution was prepared with thioarginine and DTNB present, with arginase added to initiate the reaction. A linear time course was observed after addition of enzyme. The  $k_{\text{cat}}$  for arginase-catalyzed hydrolysis of thioarginine at pH 9 is  $18,000 \pm 1700 \text{ min}^{-1}$  and the  $K_m$  is  $0.5 \pm 0.1 \text{ mM}$ . These values are quite similar to those observed for the physiological substrate arginine under these assay conditions (6). In contrast, the spectrophotometric assay with the alternative substrate NGB yields *m*-nitroaniline as the chromophoric product ( $E_{372} = 1.28 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ) with a  $k_{\text{cat}}$  of only  $0.09 \text{ min}^{-1}$  (8).

TABLE 1

Comparison of the Uncatalyzed and Arginase-Catalyzed Hydrolysis of Thioarginine<sup>a</sup>

pH	DTNB hydrolysis <sup>b</sup>	Thioarginine hydrolysis <sup>c</sup>	Arginase-catalyzed rate <sup>d</sup>
7.0	n.m. <sup>e</sup>	n.m.	$2.2 \times 10^{-2}$
7.5	n.m.	n.m.	$4.5 \times 10^{-2}$
8.0	n.m.	$2.3 \times 10^{-4}$	$1.0 \times 10^{-1}$
8.5	n.m.	$1.6 \times 10^{-3}$	$1.5 \times 10^{-1}$
9.0	n.m.	$6.3 \times 10^{-3}$	$2.5 \times 10^{-1}$
9.5	$1.0 \times 10^{-3}$	$1.8 \times 10^{-2}$	$6.0 \times 10^{-1}$
10.1	$4.0 \times 10^{-3}$	$5.3 \times 10^{-2}$	$4.8 \times 10^{-1}$

<sup>a</sup> Rates are reported in min<sup>-1</sup>.

<sup>b</sup> Nonenzymatic hydrolysis with 0.1 mM DTNB incubated at each pH and the increase in absorbance monitored at 412 nm.

<sup>c</sup> Nonenzymatic hydrolysis with 0.9 mM thioarginine incubated at each pH in the presence of 0.1 mM DTNB.

<sup>d</sup> For the arginase-catalyzed reaction 0.9 mM thioarginine and 0.1 mM DTNB were incubated at each pH and the reaction was initiated by the addition of 20 μL of arginase (0.14 μg).

<sup>e</sup> n.m., no measurable rate observed.

To determine the utility of this assay the reaction was examined across a range of pH values. The rate of hydrolysis of DTNB, even at high pH, is negligible compared to the enzyme-catalyzed rates. At high pH thioarginine was observed to slowly hydrolyze, releasing the mercaptovalerate product that will react with DTNB. However, even at pH 10 where the nonenzymatic hydrolysis of thioarginine is the fastest, this rate is still only about 10% of the rate observed in the presence of arginase (Table 1). This difference can be further enhanced simply by increasing the levels of arginase in the assay.

**Summary.** An alternative substrate of arginase has been synthesized in which the bridging guanidium nitrogen has been replaced with a sulfur. This compound is a good substrate for arginase, leading to urea and 2-amino-5-mercaptovaleric acid, with a  $k_{\text{cat}}$  that is comparable to and a  $K_m$  that is less than half that of arginine. The thiol product reacts with DTNB to produce a disulfide adduct and release 2-nitro-5-thiobenzoate as a chromophoric product. A continuous spectrophotometric assay of arginase has been designed and optimized based on this reaction. This new assay is significantly more sensitive than existing continuous assays, and is much less time consuming than the fixed-point assays of arginase that are currently being used. The dramatically higher  $k_{\text{cat}}$  with thioarginine for arginase, coupled with a 10-fold greater extinction coefficient for 2-nitro-5-thiobenzoate as compared with *m*-nitroanaline, results in greater than a million-fold enhanced sensitivity for this improved spectrophotometric arginase assay.

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## Enhanced Protein Recovery and Reproducibility from Pull-Down Assays and Immunoprecipitations Using Spin Columns

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“Pull-down” assays are a commonly used affinity purification method for determining protein–protein interactions.

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