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Determination of dimethylarginine dimethylaminohydrolase activity in the kidney

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Abstract

Dimethylarginine dimethylaminohydrolase (DDAH) metabolizes asymmetric dimethylarginine to generate L-citrulline and is present in large quantities in the kidney. We present a new study that optimizes the Prescott–Jones colorimetric assay to measure DDAH-dependent L-citrulline generation in kidney homogenates. We found that the removal of urea with urease is necessary since urea also produces a positive reaction. Deproteinization with sulfosalicylic acid was found to be optimal and that protease inhibitors were not necessary. All assays were conducted in phosphate buffer, since other common additives can create false positive and false negative reactions. Arginase or nitric oxide synthase isoenzymes were not found to influence L-citrulline production. Our optimized L-citrulline production assay to measure DDAH activity correlated closely with the direct measure of the rate of asymmetric dimethylarginine consumption. Using this assay, we found that both superoxide and nitric oxide inhibit renal cortical DDAH activity *in vitro*.

Keywords

asymmetric dimethylarginine; L-citrulline; dimethylarginine dimethylaminohydrolase; nitric oxide; urea

Dimethylarginine dimethylaminohydrolase (DDAH) metabolizes the methylarginines asymmetric dimethylarginine (ADMA) and N^{W}-monomethyl-L-arginine, to generate L-citrulline, and is highly expressed in the kidney.[1] ADMA is elevated in many systemic diseases, including renal failure, possibly due to impaired renal DDAH activity.

DDAH activity can be measured by rate of substrate (e.g. ADMA) consumption, but these assays are time consuming and costly.[2,3] A colorimetric method that detects L-citrulline production can also be used providing that (1) other pathways that generate or remove L-citrulline are inactivated and (2) interfering compounds have been removed.

Here, we have optimized the Prescott–Jones method[4] using diacetyl monoxime derivatization of the ureido group in L-citrulline to form color[5,4] that has been adapted to a 96-well format.[6] Particular attention was paid to nonspecific color generation by urea.[7] Furthermore, we

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compared this modified L-citrulline assay to the direct high-pressure liquid chromatography method measuring rate of ADMA consumption.

Results

Optimization of L-citrulline assay

The enzyme is saturated at between 100 μM and 1 mM substrate (ADMA) and we therefore use 1 mM ADMA in all studies. We compared four different homogenization buffers, and found sodium phosphate buffer, pH = 6.5, was without effect on color formation (Table 1). Without deproteinization, both bovine serum albumin and the kidney homogenate caused turbidity. Sulfosalicylic acid (4%) gave the lowest blank absorbance and was used for deproteinization. With deproteinization, there was no background color with bovine serum albumin although the kidney homogenate still had high color, suggesting the presence of interfering factors.

Effect of urea on L-citrulline assay

As shown in Figure 1, the high background color seen in the deproteinized kidney homogenate was reduced by >95% at t = 15 min after incubation with urease. Addition of 1, 5, 10, 50, and 100 mM urea gave color equivalent to ~21, 49, 106, 195, and 221 μM L-citrulline, respectively, but gave no background color at t = 0 when preincubated with urease for 15 min.

After preincubation with urease, citrulline production in kidney and other tissue homogenates incubated with 1 mM ADMA was linear from 0 to 120 min (Figure 2). We used a 45-min incubation in subsequent studies.

Comparison of DDAH activity measured by L-citrulline accumulation with ADMA consumption

In kidney cortex, the rate of production of L-citrulline = 0.3976 μM/g protein/min and the corresponding rate of consumption of ADMA = 0.4378 μM/g protein/min are similar (Figure 3), suggesting that this assay gives a faithful measurement of DDAH activity. Other tests (Figure S2) confirmed that there was no citrulline to arginine conversion and no citrulline generation by activity of arginase or nitric oxide synthase.

The renal cortex and medulla DDAH activity was 0.39±0.01 (n = 15) and 0.30±0.01 (n = 3) μM/g protein/min, respectively. Inter- and intra-assay coefficient of variation are 5.61±0.28% (n = 12) and 4.82±0.19% (n = 9). The NO donors and superoxide donor inhibited DDAH activity (Figure 4).

Discussion

We have used the Prescott–Jones method to measure kidney DDAH activity from rate of L–citrulline production and found that urea markedly raises background and must be removed; deproteinization is essential and the choice of deproteinization method influences background color; the modified method correlates well with rate of ADMA consumption; and both superoxide and NO, known to inhibit DDAH activity, produce declines in rate of L-citrulline formation in kidney homogenates.

Knipp and Vasak[6] adapted the Prescott–Jones assay to a 96-well plate method for measurement of activity of the purified DDAH enzyme. However, in complex tissues, there may be agents that reduce or increase color development and alter L-citrulline metabolism. Of particular note, the development of color is not specific for L-citrulline but also occurs with urea,[7] and there is a urea concentration gradient in kidney (~4 mM in cortex and ~20 mM in inner medulla).[8] Even in renal cortex, urea accounts for more than 90% of baseline absorbance, and therefore obscures

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DDAH-induced changes in color due to citrulline formation. In the presence of urease, the effect of urea (up to 100 mM) can be completely removed from kidney cortex and medulla. Kulhanek *et al.*[9] reported that urease treatment was not required for citrulline assay in liver and brain. Our results, however, demonstrate that where urea concentration is measurable, urease should be used. Although the urea effect can be prevented by initial ion-exchange chromatography,[10] this is more costly and time consuming compared to urease.

Another difficulty is that the diacetyl monoxime reagent can detect protein-bound L-citrulline as well as free L-citrulline. Although no separate protein-removing step was required in the purified enzyme system,[6] in tissues, protein precipitation is mandatory. We found that 4% sulfosalicylic acid gave lowest background.

Buffer/additives also influence color development, for example, 2-mercaptoethanol (in HB2) reduces color formation,[5] which might explain our observation of a higher renal DDAH activity than a previous study using HB2.[10] Without urease treatment, the high background color due to urea obscures DDAH-dependent L-citrulline formation until ~ $t = 45$ min (Figure S1), which might also explain the longer incubation time used previously.[11–13]

In addition to DDAH, ornithine carbamoyltransferase and nitric oxide synthase generate L-citrulline. In this assay, activity of both enzymes can be ignored since ornithine carbamoyltransferase is not detectable in kidney and 1 mM ADMA used as substrate is a potent nitric oxide synthase inhibitor. On the other hand, L-citrulline can be converted to L-arginine by argininosuccinate synthase and lyase. However, in tissue homogenate, citrulline consumption by argininosuccinate synthase and lyase requires added aspartate[15] and ATP, and we found no citrulline consumption under the conditions of our assay (Supplementary Material). Furthermore, arginases, which might indirectly increase L-citrulline consumption by increasing rate of L-arginine utilization,[16] are not active since arginase inhibition did not affect L-citrulline formation and there was no l-arginine consumption.

Both oxidative and nitrosative stress have been reported to inhibit DDAH activity,[17,18] and in this study we show that both superoxide and NO donors have an acute inhibitory action on renal cortex DDAH activity, measured from L-citrulline production.

In conclusion, this colorimetric assay of L-citrulline accumulation is a simple and inexpensive method optimized for detection of renal tissue DDAH activity *in vitro*, which agrees well with the more costly and time-consuming method of measuring ADMA consumption. This can also be adapted for other tissues, even with low activity such as cerebellum, but should be optimized before use.

Materials and Methods

Preparation of tissue and reagents

Male Sprague–Dawley rats from Harlan (Indianapolis, IN, USA) were used. Tissues were collected after perfusion with cold phosphate-buffered saline and stored at –80°C. Protein concentration was determined by Bradford assay. Tissue homogenate was adjusted to the concentration of 20 mg/ml.

Assay procedures

Several pilot studies were conducted to optimize the assay including evaluation of homogenization buffer, deproteinization reagents, and other pathways of citrulline metabolism (Supplementary Material & Table S1). Recommended assay procedures are summarized in Table 2. A time-course study was conducted with preincubation of urease with homogenates of rat kidney cortex, liver, cerebellum, and aorta, then incubation with 1 mM ADMA from 0 to

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120 min. We also investigated the impact of NO and superoxide (diethylamine NONOate, sodium nitrate, and 2,3-dimethoxy-1,4-naphthoquinone) on DDAH activity.

Comparison of L-citrulline assay and ADMA degradation by High-pressure liquid chromatography

We compared the rate of L-citrulline production by DDAH with the rate of ADMA degradation at $t = 0$, 30, 45, 90, and 120 min. In this study, 400 μl of 1 mM ADMA was mixed with the 100 μl of kidney homogenate (20 mg/ml), and 100 μl of the mixture was collected for high-pressure liquid chromatography analysis of ADMA at the various times, as shown above. ADMA (and L-arginine) levels were measured in tissue homogenate using reverse-phase high-pressure liquid chromatography with the Waters AccQ-Fluor fluorescent reagent kit as published previously.[19]

Statistical analysis

Data are presented as mean±s.e.m. The effects of arginase inhibitor, NO, and superoxide were compared by unpaired *t*-test. The correlation between L-citrulline formation and ADMA consumption was analyzed by Pearson's correlation coefficient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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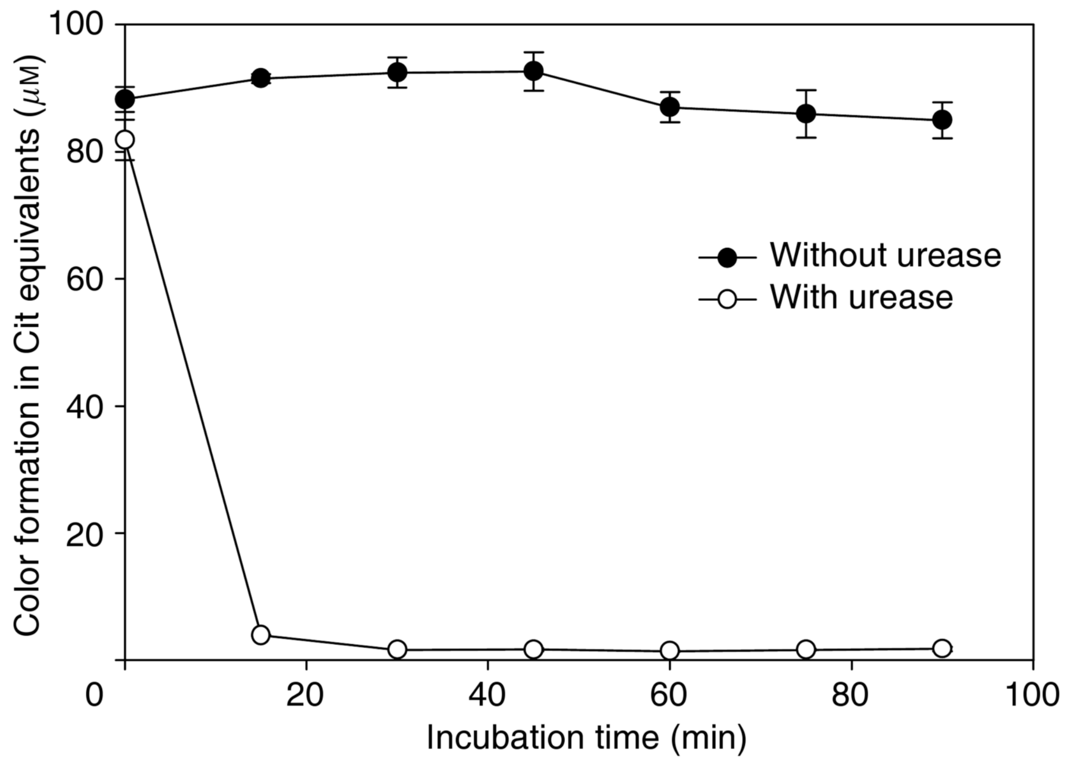

Figure 1. Time course of the urea effect on color formation without substrate (ADMA) in the absence (solid circle) and presence of urease (open circle)

Each time point determined in triplicate.

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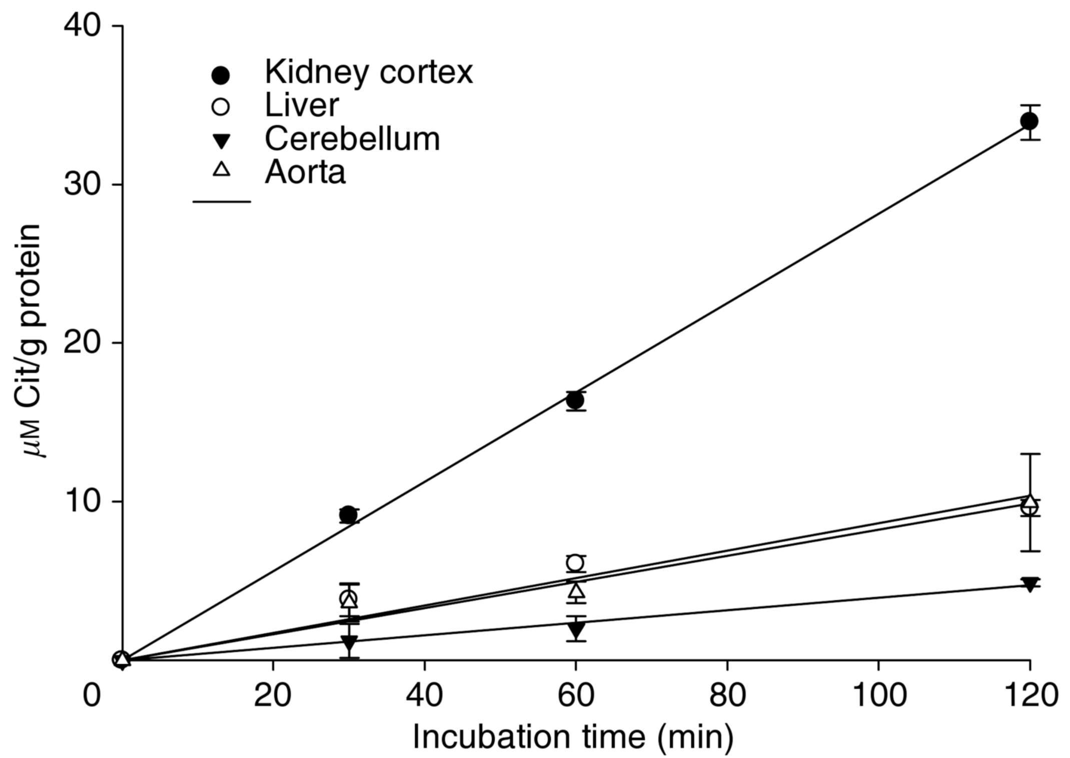

Figure 2. Time course of DDAH activity in different rat tissues: kidney cortex (solid circle), liver (open circle), cerebellum (inverted solid triangle), and aorta (open triangle)

Each time point determined in triplicate.

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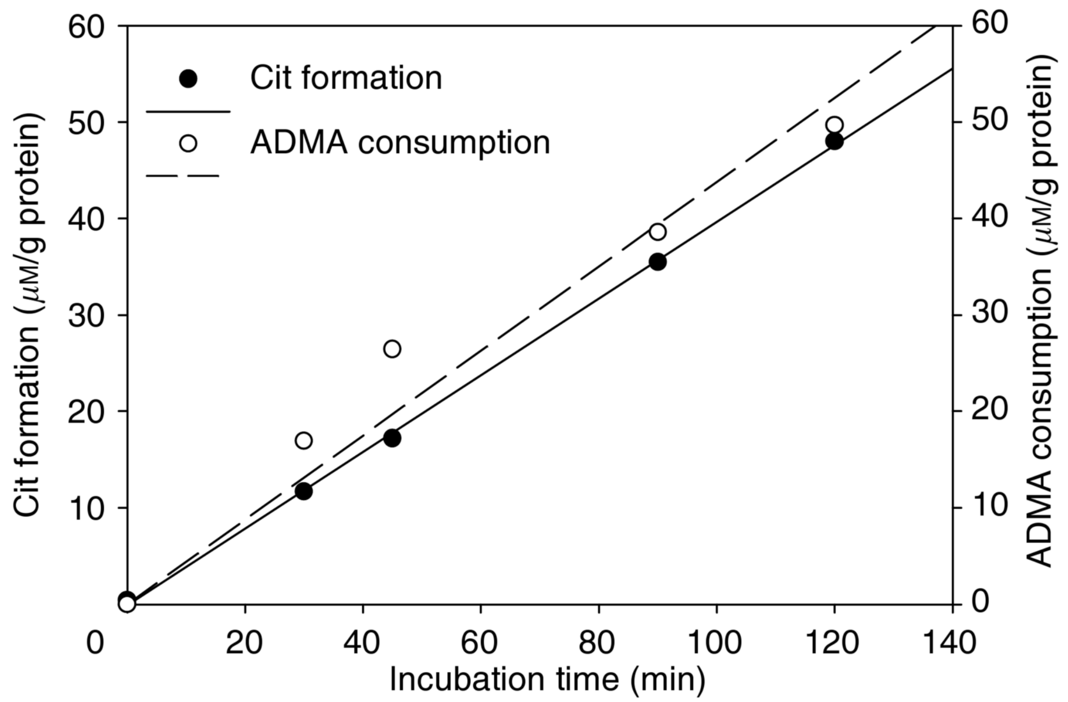

Figure 3. Correlation of L-citrulline formation as a measure of DDAH activity (solid circle, solid line) with the rate of ADMA consumption (open circle, dashed line)

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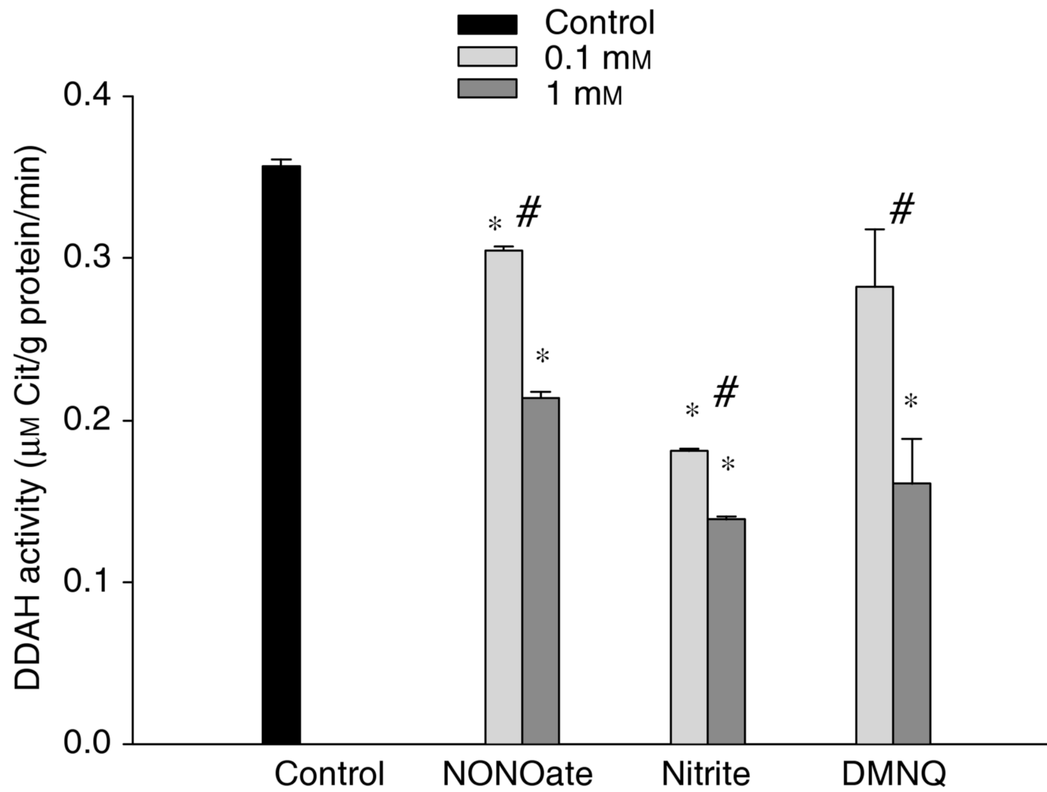

Figure 4. The effect of NO and superoxide on the L-citrulline assay to detect renal DDAH activity
DEA NONOate and nitrite were used as NO donors; and 2,3-dimethoxy-1,4-naphthoquinone was used as superoxide donor. All measurements were in triplicate. **P*<0.05 vs control; #*P*<0.05 0.1 mM vs 1 mM.

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Table 1

Effect of buffers and additives on the L-citrulline assay in the presence of 25 μM L-citrulline

	L-citrulline μM	*Color as % of control*
Homogenization buffer[a]		
HB1	17.0±0.8	68
HB2	<Minimal	<1
HB3	24.9±0.6	100
HB4	>100[b]	464
Buffer base		
1% Triton	13.0±0.9	52
1 M HEPES	27.5±0.4	110
0.3 M sucrose	>100	500
0.9% normal saline	24.6±0.6	98
0.1 M sodium phosphate	24.9±0.6	100
100 mM urea	>100	1419
Additives		
0.1 M DTT	>100	384
1% 2-mercaptoethanol	<Minimal	<1
0.5% Tween	22.2±1.0	89
1% SDS	22.3±3.7	89
0.5 M EDTA	25.6±3.0	102
0.2 M EGTA	33.6±0.6	134
1 mM ADMA	22.2±0.8	89
Protease inhibitors[c]	23.2±0.3	95
4% sulfosalicylic acid	28.2±1.0	113
Protein		
1 mg/ml BSA	33.6±2.8[b]	134
2 mg/ml BSA	43.3±7.8[b]	173
1 mg/ml kidney homogenate	76.3±12.1[b]	305
2 mg/ml kidney homogenate	>100[b]	415
Protein with deproteinization		
1 mg/ml BSA	26.1±0.8	107
2 mg/ml BSA	25.7±0.8	103
1 mg/ml kidney homogenate	63.4±1.1	253
2 mg/ml kidney homogenate	82.6±1.2	330

ADMA, asymmetric dimethylarginine; BSA, bovine serum albumin; EDTA, ethylene-diaminetetraacetic acid; EGTA, ethylene glycol-bis(*â*-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid; HEPES, hydroxyethylpiperazine-*N*′-2-ethanesulfonic; SDS, sodium dodecyl sulfate; DTT, DL-dithiothreitol.

The values reflect effect of an additive on the color generated by a 25 μM L-citrulline standard (taken as 100%).

[a]HB1, pH=6.8 contained 20 mM Tris, 1% Triton X-100, 5 mM EDTA, 10 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and aprotinin; HB2 contained 0.1 M sodium phosphate, pH=6.5 containing 2 mM 2-mercaptoethanol;[10] HB3 contained 0.1 M sodium phosphate, pH=6.5;[12] and HB4 was RIPA buffer (Santa Cruz, CA, USA), containing 20 mM Tris, pH=7.6, 137 mM sodium chloride, 0.2% Nonidet P-40, 0.1% sodium deoxycholate, 0.02% SDS, 0.0008% sodium azide, and protease inhibitors.

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[b]The supernatant was opalescent.

[c]Protease inhibitors: 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and aprotinin.

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Table 2

Recommend assay procedures/conditions for the measurement of renal cortical DDAH activity

1	Homogenize tissue with 5 × sodium phosphate buffer, pH=6.5
2	Adjust protein concentration to 20 mg/ml
3	Preincubate urease (100 U/ml homogenate) with tissue homogenate in 37°C water bath for 15 min
4	Add 100 μl sample to 400 μl 1 mM ADMA in sodium phosphate buffer (respective blank is sample omitting ADMA)
5	Incubate mixture in 37°C water bath for 45 min
6	Stop reaction by addition of 0.5 ml of 4% sulfosalicylic acid
7	Vortex and centrifuge at 3000 g for 10 min
8	Add 100 μl supernatant into a 96-well plate in triplicate
9	Serially dilute 100 μm L-citrulline standard to 0, 3.125, 6.25, 12.5, 25, 50, and 100 μm
10	Add 100 μl of standard into the 96-well plate in triplicate
11	Mix one part of oxime reagent with two parts of antipyrine/H_2SO_4 reagent to make the 'color mixture'
12	Add 100 μl of color mixture into the wells
13	Cover the plate with a sealing tape
14	Shake on a plate shaker for 1 min
15	Incubate the plate in 60°C water bath for 110 min in the dark
16	Cool the plate in an ice bath for 10 min
17	Measure the absorbance by spectrophotometric analysis at 466 nm
18	Subtract the value of respective blank
19	The DDAH activity is represented as μm L-citrulline formation/g protein/min at 37°C

ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase.