Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 170 (2017) 184–190

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Fluorometric enzymatic assay of L-arginine

Nataliya Stasyuk [a], Galina Gayda [a,*], Hasmik Yepremyan [b], Agnieszka Stepien [c,d], Mykhailo Gonchar [a]

[a] Institute of Cell Biology, National Academy of Science of Ukraine, Drahomanov Str. 14/16, 79005 Lviv, Ukraine
[b] Scientific and Production Center "Armbiotechnology", National Academy of Sciences of Armenia, Armenia
[c] Department of Dietetics, Institute of Nursing and Health Sciences, Medical Faculty, University of Rzeszow, Al. mjr.W.Kopisto 2 a, 35-310 Rzeszów, Poland
[d] Centre for Innovative Research in Medical and Natural Sciences, Medical Faculty of the University of Rzeszow, Warzywna 1A, 35-310 Rzeszów, Poland

ARTICLE INFO

Article history:
Received 19 February 2016
Received in revised form 7 July 2016
Accepted 9 July 2016
Available online 11 July 2016

Keywords:
L-arginine
Enzymatic assay
Recombinant arginase I
Urease
o-Phthalaldehyde
Sulfite

ABSTRACT

The enzymes of L-arginine (further — Arg) metabolism are promising tools for elaboration of selective methods for quantitative Arg analysis. In our study we propose an enzymatic method for Arg assay based on fluorometric monitoring of ammonia, a final product of Arg splitting by human liver arginase I (further – arginase), isolated from the recombinant yeast strain, and commercial urease. The selective analysis of ammonia (at 415 nm under excitation at 360 nm) is based on reaction with *o*-phthalaldehyde (OPA) in the presence of sulfite in alkali medium: these conditions permit to avoid the reaction of OPA with any amino acid. A linearity range of the fluorometric arginase-urease-OPA method is from 100 nM to 6 μM with a limit of detection of 34 nM Arg. The method was used for the quantitative determination of Arg in the pooled sample of blood serum. The obtained results proved to be in a good correlation with the reference enzymatic method and literature data. The proposed arginase-urease-OPA method being sensitive, economical, selective and suitable for both routine and micro-volume formats, can be used in clinical diagnostics for the simultaneous determination of Arg as well as urea and ammonia in serum samples.

1. Introduction

L-Arginine (further — Arg), the most basic amino acid, is found in particularly large amounts in protamines and histones. Arg is glucogenic and half-essential for humans and an important metabolite of the urea cycle [1–3]. Arg can be utilized as therapeutics for the number of diseases: angina pectoris, congestive heart failure, hypertension, coronary heart disease, preeclampsia, intermittent claudication, erectile dysfunction, diabetes mellitus and others [2]. Arg is involved in numerous areas of human biochemistry, including ammonia detoxification, hormone secretion, and immune modulation. Arg is a precursor to nitric oxide (NO), a key component of endothelial-derived relaxing factor, an endogenous messenger molecule involved in a variety of endothelium-dependent physiological effects in the cardiovascular system. In the same time, Arg is a precursor of symmetrically and asymmetrically guanidine (N^G)-dimethylated derivatives (SDMA and ADMA), as well as of L-homoarginine (hArg), the methylene homologue of Arg [4–7].

As substrates for NO synthase, Arg and its derivatives have lately become the important biomarkers in clinical diagnostics. The study of relationships between the level of these biomarkers and the details of illness' process was carried out for kidney and liver pathology as well as for cardiovascular disorders [3–7], neuroinflammatory and autoimmune diseases (endocrine diseases, rheumatoid arthritis and systemic lupus erythematosus) [7–9], pulmonary disorders [10–12], sepsis [13], argininemia and ammonemia [14,15].

Despite the close but opposite associations of hArg and ADMA with cardiovascular disease of adults, children and adolescents, the underlying biochemical processes are largely unknown, presumably not restricted to NO, and require further investigation [3,4]. The accumulation of Arg and the resulting abnormalities in the metabolism of guanidine compounds and NO seem to play a major pathophysiological role, but the influence of the Arg/NO pathway in the pathophysiology of the listed diseases remains unclear [3,6].

Hyperargininemia (HA) or argininemia is caused by deficiency of arginase I, which catalyzes the hydrolysis of Arg to urea as the final enzyme in the urea cycle [1,14–20]. In contrast to other urea cycle defects, arginase I deficiency, caused by mutation in the corresponding gene, usually does not cause catastrophic neonatal hyperammonemia but rather leads to progressive neurological symptoms including seizures and spastic paraplegia in the first years of life and hepatic pathology, such as neonatal cholestasis, acute liver failure, or liver fibrosis [15]. To detect HA among the newborns, in some economically developed countries government-funded programs of blood screening for Arg take place: timely diagnostics of HA will allow to start medical treatment of infants and to stop the development of this dangerous genetic disease [14,15]. The pathogenesis of arginase I deficiency is not fully understood so far.

* Corresponding author.
E-mail address: galina.gayda@gmail.com (G. Gayda).

http://dx.doi.org/10.1016/j.saa.2016.07.019
1386-1425/

Arginase I (L-arginine amidinohydrolase, EC 3.5.3.1) plays the main role in the urea cycle, catalyzing the cytosolic hydrolysis of Arg to ornithine and urea. Recent studies have shown that human liver arginase I can be an effective drug in enzymatic cancer therapy for certain types of cancer, causing the Arg starvation of malignant tumors cells (*e.g.*, melanoma, hepatocellular and pancreatic carcinoma) [21–26]. To control such therapy the monitoring of Arg level in blood and urine is required.

A variety of chemical and physico-chemical approaches for Arg monitoring was developed, the advantages and problems of these standard and modern analytical methods were described in detail in our previous papers [27,28]. These methods are based on ion exchange chromatography, fluorometry, spectrophotometry, capillary electrophoresis, biosensors. Usually the adapted extractive techniques and pre-concentration steps (for this procedure some hours is needed) are followed by liquid chromatography (LC) coupled to mass spectrometry (LC-MS; LC-MS/MS). High-performance liquid chromatography (HPLC) and semi-quantitative spectroscopic methods (infra-red) are also proposed as valuable alternatives to the classical ones.

A scientific collaboration of the specialists in high-tech analytical methods with professionals from medical skills is usually fruitful. As an example of such successful work in the field of studied problem are the joint investigations of the scientific groups from Hannover Medical School (Germany). D. Tsikas and his colleagues are the specialists in analytical chemistry with using high-tech devices (HPLC, GS-MS, LC-MS-MS et al.). J. Frölich and R. Böger, being professionals in medicine, had put the actual healthcare problems - to develop valid methods of quantitative determination of Arg and its derivatives in blood. As a result, the novel analytical approaches for assay of these prospective biomarkers have been proposed, a lot of blood samples of patients with different diagnosis have been tested and analyzed as well as the new knowledge about level of biomarkers as risk factors of various malignances were obtained [3,4,7,8,29–31].

All high-tech methods require skillful labor techniques, are time-consuming and expensive, so the further development of novel highly selective and sensitive methods for fast Arg determination is actual. Enzymes of Arg metabolism - arginase I, arginine deiminase and arginine decarboxylase - seem to be promising tools for elaboration of such methods [26–28].

Recently we have described the development of a novel enzymatic method for Arg determination, based on using recombinant ADI and *o*-phthalaldehyde (in the presence of sulphite) with the fluorometric and spectrophotometric detection of the product (ammonia) and evaluation of the proposed method on real samples of wines and juices [27]. Although there is a lot of papers devoted to determination of ammonium in the presence of primary amines (including amino acids), the exact mechanism of an essential increase in the selectivity to ammonium in the presence of sulfite (instead of mercaptoethanol) is not still elucidated.

Due to high cost of arginase I, the search for an alternative source of this enzyme, namely, microbiological one mas well as the development of an effective technology for enzyme isolation is a relevant issue. To solve it, the recombinant yeast strains, producing human liver arginase I (further - arginase), were gene-engineered in the Institute of Cell Biology, NAS of Ukraine [26]. The optimal schemes for isolation of highly purified enzyme preparations were developed and the enzymatic methods for Arg assay, including biosensors, were proposed [27,28,32–37]. A number of food products (juices, wines, brandies, teas) and Arg-containing commercial pharmaceuticals were analyzed using the proposed arginase-based approaches in comparison with the reference methods [27,28,32–35]. The estimated Arg contents demonstrated high correlation with those declared by producers (R near 1.0) and obtained by reference methods.

In this paper, we describe the development of a novel enzymatic method for Arg determination, based on using recombinant arginase, urease and *o*-phthalaldehyde (in the presence of sulfite) with the fluorometric detection of the product (ammonia) and evaluation of the proposed method on real samples of blood serum.

2. Materials and methods

2.1. Chemicals and devices

o-Phthalaldehyde (OPA), 2,3-butanedione monoxime (DMO), sulfuric acid (95–98%), trichloroacetic acid (TCA), ethanol were purchased from Sigma-Aldrich. Amino acids and inorganic salts were obtained from Merck (Darmstadt, Germany). Diagnostic kit for Urea analysis was manufactured by LTD SIMKO (Lviv, Ukraine). All buffers and standard solutions were prepared with Milli-Q system (Millipore) purified water.

Fluorescence emission values of resulted product were registered with 2 devices: spectrofluorometer Infinite M-200 (TECAN, Switzerland) and Quantech digital filter fluorometer (Thermo Scientific, United States).

2.2. Enzymes

Human liver arginase I was isolated from a cell-free extract of the recombinant yeast *Hansenula polymorpha* NCYC 495 pGAP1–HsARG1 (*leu2car1 Sc:LEU2*), carrying the *HsARG1* target gene under the control of a constitutive promoter of the gene coding for glyceraldehyde-3-phosphate dehydrogenase [26]. Highly-purified arginase preparation with a specific activity of 13 U/mL (600 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein) was used in current work. Arginase activity was determined as the rate of urea formation under arginase-catalyzed hydrolysis of Arg. Urea content was estimated on the second step of the reaction with the usage of a commercial kit for Urea analysis based on colorimetric method [38].

Urease (EC 3.5.1.5, type IX from Jack Beans, 26,100 $U \cdot g^{-1}$) were purchased from Sigma-Aldrich. To prepare urease solution (210 U/mL), 3 mg of powder was dissolved in 1 mL of 50 mM phosphate buffer, pH 7.0 (PB) and stored at 4 °C for up to 1 month.

2.3. The development of arginase-urease/OPA-based method

2.3.1. Preparation of OPA reagent

0.2 g OPA was dissolved in 5 mL 95% ethanol solution and mixed with 100 mL 0.1 M borate buffer, pH 10. The final reagent (14.2 mM OPA) was supplemented by sodium sulfite to the final concentration of 0.16 mM [39] and stored in darkness at room temperature until usage. OPA reagent in the presence of sulfite in alkali medium was demonstrated to react only with ammonium ion (not with any amino acid). The resulted OPA-ammonium product may be detected by spectrophotometry and fluorometry [39].

2.3.2. Study of analytical parameters of OPA-based fluorometric methods

The dependencies of fluorescent intensities for the resulted OPA-ammonium products on ammonia concentration, urea (after urease digestion) and Arg (after arginase/urease hydrolyses) contents were studied, the corresponding calibration graphs for these analytes were built. Each experiment was repeated for 3 times.

2.3.2.1. Ammonium ions calibration. An aliquot of 0.15 mL ammonium chloride solution with concentration from 0.005 mM to 1.25 mM in 50 mM phosphate buffer, pH 7.0 (PB) was mixed in plastic tube with 3 mL OPA reagent, closed carefully and heated at 60 °C for 15 min. As control, OPA reagent without ammonia (with PB) was used. Fluorescence emission value of the tested sample was registered at 415 nm (under excitation at 360 nm) using a Quantech digital filter fluorometer (Thermo Scientific, United States). The analytical results were statistically processed using the OriginPro 8.5 software.

2.3.2.2. Urea calibration. Calibration for urea was performed after enzymatic hydrolysis of urea to ammonia. An aliquot of 0.125 mL urea solution in PB with concentration from 0.005 mM to 0.625 mM was mixed in