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Rapid enzymatic assays for L-citrulline and L-arginine based on the platform of pyrophosphate detection

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ABSTRACT

Rapid determination of L-citrulline and L-arginine, physiologically important amino acids, is a beneficial technique from the scientific and medical viewpoints. In this study, enzymatic assays for L-citrulline and L-arginine were established and evaluated. L-Citrulline assay was constructed by coupling argininosuccinate synthetase to a pyrophosphate detection system, in which pyruvate phosphate dikinase was employed, so that the citrulline-dependent production of pyrophosphate could be determined. Furthermore, the L-arginine assay was developed by coupling arginine deiminase to the L-citrulline assay. Both assays exhibited high selectivity to L-citrulline and L-arginine without any significant reactivity to other proteinaceous amino acids. These assays were also resistant to various contaminants that interfered with the conventional L-citrulline and L-arginine assays. The high accuracy of these assays was demonstrated by measurements in the presence of human plasma. Because these assays can be conducted under the neutral pH without terminating the reaction progress, they allow not only measurements in static analyte solutions, but also real-time monitoring of L-citrulline and L-arginine synthesis in the reaction mixture. The features of these assays also demonstrated that the pyrophosphate detection system served as a useful platform to develop selective and robust enzymatic assays by being coupled to a pyrophosphate-producing enzyme.

1. Introduction

L-Citrulline and L-arginine are amino acids constituting the urea cycle, a central metabolic pathway distributed among almost all organisms (Fig. 1). This cycle contributes to the biosynthesis of these amino acids as well as the excretion of excess nitrogen in the form of urea in mammals. The conversion of L-arginine to L-citrulline also serves in the synthesis of NO catalyzed by nitric oxide synthase (NOS; EC 1.14.13.39), which has a wide range of functions in cellular physiology including cell signaling and the host defense system [1]. Since L-citrulline and L-arginine have attracted scientific interest for revealing the function of NO [2], methods to detect these amino acids have been intensely developed to assay the enzymatic activities of NOS and other enzymes involved in NO synthesis [3]. Furthermore, some deficiencies in the urea cycle cause fluctuations in the levels of these amino acids in patients accompanying serious diseases known as urea cycle disorders, such as citrullinemia, argininemia, and argininosuccinic aciduria [4]. In addition to these diseases, plasma citrulline levels have been proposed as an effective biomarker of bowel activity and intestinal function in various pathologies [5,6]. These findings indicate that the rapid determination of L-citrulline and L-arginine is a beneficial technique not only for scientific research, but also for medical applications.

Instrumental analysis of L-citrulline has been developed by using various kinds of analyzer, such as HPLC and LC–MS/MS [7], as well as those for proteinaceous L-amino acids [8]. Although these methods are widely used in laboratories because of their high accuracy and precision, they need bulky and expensive instruments and often require pretreatment and derivatization with skilled operation. In addition, instrumental analysis is time-consuming when the number of analytes is large, because this method cannot simultaneously analyze multiple samples.

Colorimetric assays for citrulline have been developed on the basis of the chemical modification of citrulline by deacetyl monoxime [9]. Some of these assays allow high-throughput simultaneous analysis of multiple samples [3]. However, these methods cannot be used for the rapid and real-time monitoring of citrulline

Abbreviations: ADI, arginine deiminase; ASS, argininosuccinate synthetase; BSA, bovine serum albumin; CV, coefficient of variation; GdmCl, guanidinium chloride; HRP, horseradish peroxidase; NOS, nitric oxide synthase; PEP, phosphoenolpyruvate; POX, pyruvate oxidase; PPDK, pyruvate phosphate dikinase; PPi, pyrophosphate; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine.

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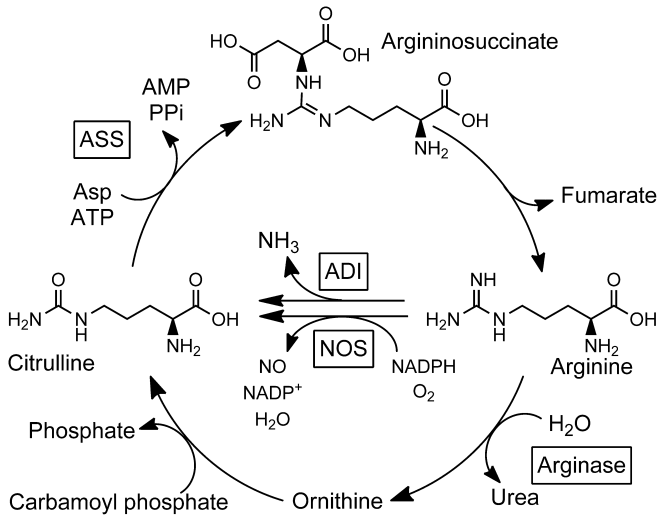

Fig. 1. Enzymes and metabolites constituting the urea cycle. The names of enzymes are shown in boxes.

synthesis because they require a heat treatment at 95 °C on a 96-well plate equipped with a sealing lid system to prevent evaporation of the reaction mixtures. Another problem associated with these methods is the severe disturbance caused by contamination with urea and proteins [3,9]. The Sakaguchi reaction and its modifications [10–12] are well-known as a colorimetric method for the determination of arginine. However, these methods are time-consuming and are disturbed by substances including Tris, glycine, proteins, and guanidyl compounds [12,13]. An enzymatic assay for L-arginine was developed coupling arginase (EC 3.5.3.1), urease (EC 3.5.1.5), and glutamate dehydrogenase (EC 1.4.1.3) [14]. Although its selectivity is higher than that of the colorimetric assays, this enzymatic assay has the drawback of severe interference by urea and ammonia, which often contaminate biological samples and analytical instruments.

Enzymatic assays for L-amino acids have been developed using various kinds of enzymes, and many of the assays use L-amino acid oxidases and L-amino acid dehydrogenases because of their easy detection [15–18]. Another strategy was recently adopted to develop an enzymatic assay for L-methionine by coupling a pyrophosphate (PPi) detection system to adenosylmethionine synthetase [19]. This assay allowed more selective and robust measurements of L-methionine than known enzymatic assays. In this study, the PPi detection system was coupled with argininosuccinate synthetase (ASS; EC 6.3.4.5) and arginine deiminase (ADI; EC 3.5.3.6) for the development of rapid and selective L-citrulline and L-arginine enzymatic assays (Fig. 2).

2. Materials and methods

2.1. Bacterial strains

Escherichia coli JM109 and *E. coli* BL21 (DE3) were used as the hosts for cloning and overexpression, respectively. *E. coli* W3110 and *Pseudomonas aeruginosa* PAO1 were used as the source of the ASS and ADI genes, respectively.

2.2. Plasmid construction

argG, which encodes ASS (YP_491358), was amplified from the *E. coli* W3110 genome using the two primers, 5′-aaggatccatatgacgacgattctcaagcatc-3′ and 5′-aaaaagcttactggccttttgttttccag-3′. *arcA*, which encodes ADI (NP_253858), was amplified from the *P. aeruginosa* PAO1 genome using the two primers, 5′-aaactgcagcatatgagcacggaaaaaaccaaac-3′ and 5′-aagaattcagtagtcgatcgggtc-3′. The amplified fragments were inserted into pET-28a adjacent to the N-terminal His-tag to construct the expression plasmids for each enzyme.

2.3. Expression and purification of enzymes

Pyruvate phosphate dikinase (PPDK; EC 2.7.9.1) from *Propionibacterium freudenreichii* NBRC 12426 was heterologously expressed in *E. coli* and purified as described previously [19].

E. coli BL21 (DE3), harboring the expression plasmid of ASS or ADI, was cultivated in LB medium until the optical density reached 0.6–0.8, followed by the addition of IPTG at a final concentration of 0.5 mM. Cells were harvested four hours after the induction by IPTG. Cells were disrupted by sonication and centrifuged to remove cell debris. The supernatant was applied to an open column packed with 5 ml of Ni-Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK). After eluting unbound proteins with 20 mM Tris–HCl, 300 mM NaCl, and 50 mM imidazole-HCl (pH 8.0), the His-tagged protein was eluted with 20 mM Tris–HCl, 300 mM NaCl, and 500 mM imidazole-HCl (pH 8.0). Enzyme solutions were kept at 4*circ*C throughout ASS and ADI purification. The purified fractions of ASS and ADI were mixed with glycerol at a final concentration of 10% (v/v) and stored at −80◦C until use.

2.4. ASS assay

A previous study proposed three forms of PPi-detecting assays, namely, colorimetric, ultraviolet, and fluorescent assays [19]. To develop a citrulline assay, the colorimetric assay for PPi was coupled with ASS in the present study. The reaction mixture contained 50 mM Mes-KOH (pH 6.5), 10 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM sodium L-aspartate, 0.5 mM phosphoenolpyruvate (PEP), 0.1 mM AMP, 0.5 mM Na_2HPO_4, 1 mM 4-aminoantipyrine, 1 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) [20], 1.5 U/ml pyruvate oxidase (POX; Toyobo, Osaka, Japan), 7.5 U/ml horseradish peroxidase (HRP; Wako, Osaka, Japan), 0–100 μM L-citrulline, PPDK, and ASS. The mixtures were incubated at 30 °C, and the increase in absorbance at 555 nm was monitored using a microplate reader. After the reaction was complete, absorbance was measured to construct L-citrulline standard curves. The detection limit of the assay was defined as three times the standard deviation of citrulline-free blank samples ($n = 20$).

2.5. ADI assay

The reaction mixture contained 50 mM Mes-KOH (pH 6.5), 10 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM sodium L-aspartate, 0.5 mM PEP, 0.1 mM AMP, 0.5 mM Na_2HPO_4, 1 mM 4-aminoantipyrine, 1 mM TOOS, 1.5 U/ml POX, 7.5 U/ml HRP, 0–100 μM L-arginine, PPDK, ASS, and ADI. The mixtures were incubated at 30◦C, and the increase in absorbance at 555 nm was monitored using a microplate reader. After the reaction was complete, absorbance was measured to construct L-arginine standard curves. The detection limit of the assay was defined as three times the standard deviation of arginine-free blank samples ($n = 20$).

2.6. Selectivity of the assays

To investigate the selectivity of the assays, L-citrulline and L-arginine in the reaction mixtures of ASS and ADI assays, respectively, were replaced by one of the following amino acids: 0.5 mM L-alanine, L-cysteine, L-aspartic acid, L-glutamic acid, L-phenylalanine, glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-methionine, L-asparagine, L-proline, L-glutamine, L-arginine, L-serine, L-threonine, L-valine, L-tryptophan, L-tyrosine, and L-citrulline.

2.7. Effects of additives that interfere with conventional assays

To verify the robustness of the assay, 10 mM NH_4Cl, 50 mM urea, 50 mM sucrose, 75 μM bovine serum albumin (BSA), 50 mM guanidinium chloride (GdmCl), 1 mM Gly, 10 mM Tris, or 10 mM creatine was added to the assay mixture. Each assay mixture contained 50 μM L-citrulline or L-arginine as the substrate.

2.8. Spike and recovery tests with human plasma

Human plasma from a single anonymous donor was purchased from Kohjin Bio (Saitama, Japan). Frozen plasma was thawed and centrifuged to remove precipitates just before use. Plasma was added at a final concentration of 20% (v/v) in the assay mixtures. The previous study demonstrated that the PPi detection system can accurately function in the presence of 20% plasma. L-Citrulline or L-arginine was added to each mixture at a final concentration of 0, 10, 20, and 50 μM ($n = 3$ and 12 for inter-assay and intra-assay tests, respectively). The recoveries of the extrinsic amino acids were calculated by subtracting the absorbance of the negative control containing no additive amino acids. Standard curves were constructed by assay mixtures containing no plasma in order to calculate the recovery rates of the plasma-containing samples.

3. Results

3.1. L-Citrulline determination

ASS catalyzes the conversion of L-citrulline, L-aspartate, and ATP into argininosuccinate, AMP, and PPi as a member of the urea cycle