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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.

Key words

L-Citrulline; L-arginine; argininosuccinate synthetase; arginine deiminase; pyrophosphate detection

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 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

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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; PPK, pyruvate phosphate dikinase; PPI, pyrophosphate; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfoethyl)-*m*-toluidine.

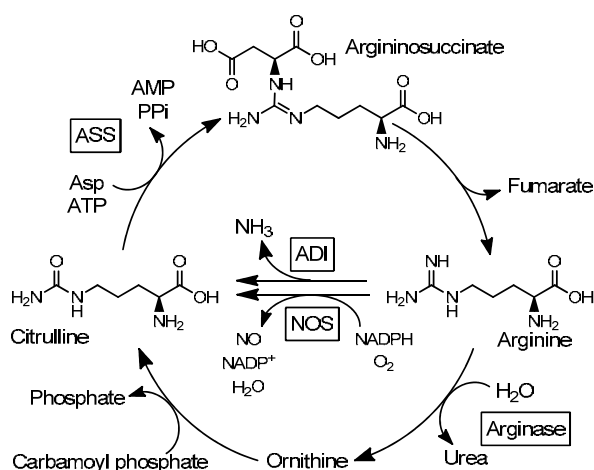


Fig. 1. Enzymes and metabolites constituting the urea cycle. The names of enzymes are shown in boxes.

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'-aaggatccatgatgacgacgattctcaagcatc-3' and 5'-aaaaagcttactggcctttgtttccag-3'. *arcA*, which encodes ADI (NP_253858), was amplified from the *P. aeruginosa* PAO1 genome using the two primers, 5'-aaactgcagcatatgagcacggaaaaacaaac-3' and 5'-aagaattcagtagtcgatcggttc-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°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,

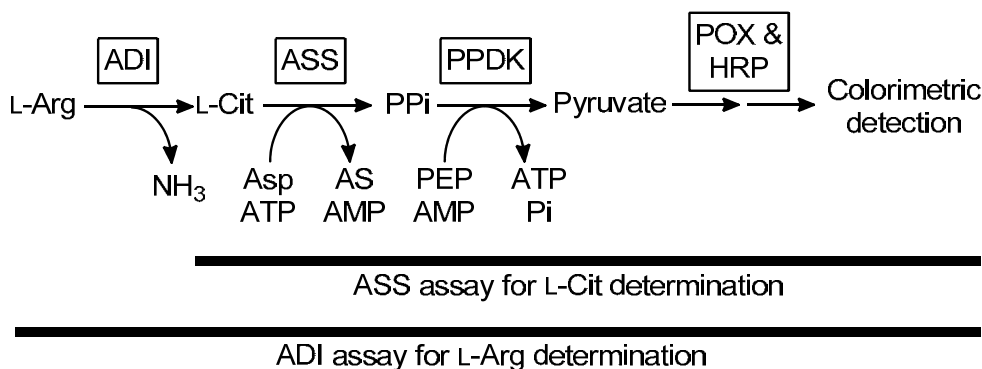


Fig. 2. Overall scheme of the L-citrulline and L-arginine assays. The coupling reaction gives a visible absorbance change depending on the amount of L-citrulline or L-arginine. The names of the enzymes used are shown in boxes. AS, argininosuccinate; Cit, citrulline.

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 con-

centration 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 (Fig. 1) [21-23]. This enzyme recognizes the ureide group located in the side chain of L-citrulline and transfers L-aspartate to this group, which is absent in other amino acids. Because of this reaction mechanism, ASS was expected to endow the assay system with high selectivity to L-citrulline.

The ASS gene was cloned from *E. coli* and overexpressed. The gene product was completely purified by Ni-Sepharose chromatography, yielding a single band on SDS-PAGE analysis of the purified fraction. The citrulline assay system was constructed by coupling purified ASS with the PPI detection system [19]. The colorimetric detection system for PPI consisted of PPDk, POX, and HRP, resulting in PPI-dependent coloring (Fig. 2). When L-citrulline was added to the ASS assay mixtures, the absorbance of each reaction mixture immediately started to increase and reached the endpoints within 10 min. The endpoints of absorbance were correlated to the L-citrulline concentrations added. The obtained standard curve for L-citrulline showed high linearity with a coefficient of correlation of 0.9998 (Fig. 3), which indicates that the ASS assay allows the accurate determination of L-citrulline. When reaction mixtures in which L-citrulline was replaced by the same concentrations of PPI were prepared as positive controls, no significant difference was observed between absorbance changes in the citrulline- and PPI-added groups. This result indicated that the citrulline added was efficiently converted to equimolar PPI by ASS. The detection limit of L-citrulline by this assay was estimated to be 0.4 μM .

3.2. L-Arginine determination

ADI catalyzes the deamination of L-arginine and produces L-citrulline and ammonia [24]. This enzyme functions in some bacteria as the first step of the arginine deiminase pathway, in which ATP is anaerobically synthesized using L-arginine as a substrate. ADI also attracts attention as a promising anticancer drug that regresses tumors through arginine deprivation [25].

The ADI gene was cloned from *P. aeruginosa* and heterologously overexpressed in *E. coli*. ADI was puri-

fied by Ni-Sepharose chromatography as well as ASS. When ADI was coupled to the above ASS assay to construct the arginine assay system, the coupling assay showed absorbance increases that were dependent on the amount of L-arginine added (Fig. 4). The coefficient of correlation of the standard curve was estimated to be 0.9994. The high linearity of the standard curve indicates that L-arginine can be accurately measured by this assay. The slope of the L-arginine standard curve was similar to that of L-citrulline (Fig. 4), which demonstrated that ADI efficiently yielded L-citrulline from L-arginine, followed by its conversion into PPi in the ASS reaction. The detection limit of L-arginine by this assay was estimated to be 0.4 μ M.

Because this assay detected not only L-arginine but also L-citrulline, the total amount of these two amino acids could be determined. Standard reaction mixtures in which these amino acids were mixed at various ratios were prepared and analyzed by the ASS and ADI assays (Table 1). While the ASS assay provided L-citrulline concentrations, the ADI assay accurately estimated the total concentrations of L-arginine and L-citrulline. Thus, the L-arginine concentration

can be calculated by subtracting the former from the latter when L-citrulline coexisted in the analytes.

3.3. Selectivity and robustness of the assays

The selectivities of the ASS and ADI assays were examined by replacing their substrates with another amino acid in the assay mixtures. No significant reactivity toward amino acids other than L-citrulline and L-arginine was observed (Table 2), which clearly demonstrated the high selectivity of these assays toward L-citrulline and L-arginine.

Several molecules are known to interfere with the conventional colorimetric and enzymatic assays for L-citrulline and L-arginine (Table 3). The effects of these molecules on the ASS and ADI assays were evaluated by adding them to the assay mixtures. Urea, sucrose, BSA, GdmCl, and glycine, which were reported to disturb the colorimetric assay for citrulline [3, 9], caused no significant disturbance in the ASS and ADI assays. In addition to these molecules, ammonium, Tris, and creatine, which interfere with the assays for arginine [12-14], did not affect the ASS and ADI assays. These results show that the ASS and ADI assays are more robust than the conventional assays for citrulline and arginine. It is noteworthy that the ASS and ADI assays require no pretreatment for deproteinization because of their tolerance to proteins, while the conventional colorimetric assays for L-citrulline and L-arginine are known to be sensitive to proteins [3, 12].

3.4. Spike and recovery test in human plasma

Urea cycle disorders are known to often cause fluctuations in L-citrulline and L-arginine in patients' blood samples; therefore, the concentrations of these amino acids in plasma are useful for the diagnoses of these diseases [4]. To test whether the ASS and ADI assays could detect increases in L-citrulline and L-arginine in plasma, assay mixtures containing 20% human plasma and an additional 0-50 μ M L-citrulline or L-arginine were prepared, and their recovery rates were estimated.

In each assay mixture, L-citrulline and L-arginine concentrations were estimated to be similar to the added concentrations (Table 4), which demonstrates that the ASS and ADI assays can accurately determine these amino acids in plasma. The reproducibility of this quantification was also indicated by the low CV values. However, CV values relatively increased when the concentration of L-citrulline or L-arginine was low (10 μ M), probably because of the absorbance derived from plasma. Intra-assay CVs for these samples (13% and 11%) suggested that 10 μ M was similar to the limits of quantification when the assay mixtures contained 20% plasma. The standard deviation of the negative control samples, which contain plasma but no additive amino acids, was estimated to be 1 μ M. The limit of quantification should vary in accordance with the intensity of the absorbance derived from plasma or analytes.

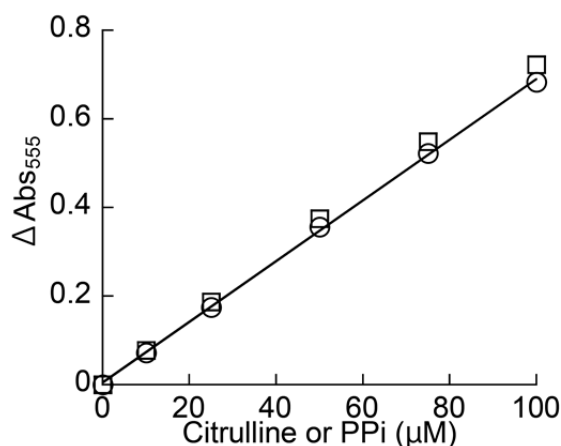


Fig. 3. Standard curve for L-citrulline. Open circles and a solid line indicate the L-citrulline standard curve. Open squares indicate the results from assay mixtures in which PPi was substituted for L-citrulline.

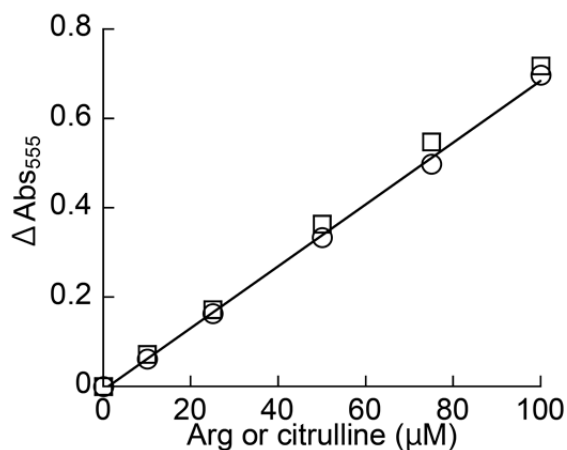


Fig. 4. Standard curve for L-arginine. Open circles and a solid line indicate the L-arginine standard curve. Open squares indicate the results from assay mixtures in which L-citrulline was substituted for L-arginine.

Table 1. L-Citrulline and L-arginine determination under the coexistence of these amino acids.

Added concentration (μM) ^a		Estimated concentration (μM)	
L-Citrulline	L-Arginine	L-Citrulline ^b	L-Citrulline + L-Arginine ^c
100	0	99.8 ± 0.7	104 ± 1.9
70	30	71.0 ± 0.2	101 ± 0.7
30	70	30.0 ± 0.2	99.8 ± 2.4
0	100	0.1 ± 0.1	98.2 ± 2.3

^a Final concentrations in the assay mixtures.

^b Estimated by the ASS assay.

^c Estimated by the ADI assay.

Table 2. Selectivity of the L-citrulline and L-arginine assays.

Amino acid	ASS assay	ADI assay ^a
L-Citrulline	[100]	100
L-Arg	ND	[100]
L-Ala, L-Cys, L-Asp, L-Glu, L-Phe, Gly, L-His, L-Ile, L-Lys, L-Leu, L-Asn, L-Pro, L-Gln, L-Ser, L-Thr, L-Val, L-Trp, and L-Tyr	ND	ND

The reactivities toward L-citrulline and L-arginine were taken as 100% for the ASS and ADI assays, respectively. ND, not detected.

^a An excess amount of ADI was added to the assay mixtures relative to that of ASS.

Table 3. Tolerance of citrulline (Cit) and arginine assays to contaminants in the assay mixture.

	ASS assay for L-Cit ^a , ADI assay for L-Arg ^a	Colorimetric assay for Cit ^{b, c}	Colorimetric assay for Arg ^{d, e}	Arginase assay for L-Arg ^f
Ammonia	+ (10 mM)	+ ^b	– (0.7 mM) ^d	– ^f
Urea	+ (50 mM)	– (50 mM) ^b	– (3.3 mM) ^d	– ^f
Sucrose	+ (50 mM)	– (50 mM) ^b	NR	NR
Proteins	+ (75 μM BSA)	– (50 μM BSA) ^b	– ^d	NR
GdmCl	+ (50 mM)	– (50 mM) ^b	NR	NR
Gly	+ (1 mM)	– ^c	– (0.1 mM) ^e	NR
Tris	+ (10 mM)	+ ^c	– ^e	NR
Creatine	+ (10 mM)	NR	– (1.5 mM) ^d	NR

+, Tolerant; –, sensitive; NR, not reported. Final concentrations of contaminants added to the assay mixtures are shown in the parentheses.

^a This study.

^b Ref. [3].

^c Ref. [9].

^d Ref. [12].

^e Ref. [13].

^f Ref. [14].

Table 4. Spike and recovery tests by adding L-citrulline or L-arginine to human plasma samples.

Amino acid	Addition (μM) ^a	Intra-assay ^b		Inter-assay ^c	
		Recovery (μM)	CV (%)	Recovery (μM)	CV (%)
L-Citrulline	10	11	13	11	6.8
	25	25	5.1	23	5.2
	50	47	3.1	46	3.2
L-Arginine	10	10	11	10	4.1
	25	24	5.6	24	3.7
	50	48	3.6	47	4.0

^a Final concentrations of extrinsic L-citrulline or L-arginine added to the assay mixtures.

^b Determined by assaying plasma samples in a single run (n=12).

^c Determined by assaying plasma samples in six independent runs (n=3 for each run).

4. Discussion

In this study, selective, robust, and rapid enzymatic assays for L-citrulline and L-arginine were developed by coupling ASS, ADI, and the PPi detection system. The rapid and robust nature of the assays is expected to widen the range of analytes, including food samples, biological samples, and reaction mixtures for biochemical research. The results of the spike tests demonstrated that L-citrulline and L-arginine could be accurately determined by this assay in biological samples. In addition, no pretreatment, which is required for some enzymatic assays to remove proteins or other biomolecules from biological samples [18], was required for the ASS and ADI assays. These assays can be conducted under neutral pH without acidic or alkaline reagents, and allow real-time monitoring of L-citrulline and L-arginine. These features are also advantageous in detecting enzyme activities that produce L-citrulline or L-arginine as the product because their reaction progress can be monitored by these assays without terminating the reaction. All enzymes constituting the ASS and ADI assays can be readily prepared by overexpression in *E. coli* (ASS, ADI, and PPDK) or by commercial purchase (POX and HRP), which facilitates the use of these assays. In a previous study, the PPi assay system was embodied as three kinds of detection methods, namely, ultraviolet, colorimetric, and fluorescent detection [19]. Although only colorimetric detection was employed in this study, ultraviolet and fluorescent detection can be also adopted by the ASS and ADI coupling assays to allow a wider use according to the experimental situations.

The high substrate specificity of ASS, as well as the robustness of the PPi detection system shown previously [19], is supposed to contribute to the high selectivity and robustness of the ASS coupling assay. A number of enzymatic assays for amino acids have been

developed using L-amino acid oxidases and L-amino acid dehydrogenases [15-17, 26], which react with amino acids at the amino group. Because the amino group is a common structure among all amino acids, these enzymes often exhibit reactivity to multiple amino acids with low selectivity. In contrast with L-amino acid oxidases and dehydrogenases, ASS reacts with L-citrulline at the ureide group in its side chain, a structure unshared among other amino acids. This catalytic feature intrinsically ensures the high substrate specificity of the ASS assay, as demonstrated in the present study (Table 2). While the transfer of L-aspartate onto the ureide group is energetically unfavorable in itself, ATP hydrolysis serves as a driving force for efficient and irreversible reaction progression in the direction of PPi synthesis. Thus, the ATP-dependent ASS reaction at the side chain of the amino acid may be one of the primary factors for the selectivity and accuracy of the ASS and ADI assays. Moreover, continuous ATP regeneration from PPi and AMP by PPDK may add to the reaction efficiency of these coupling assays.

A previous study showed that a selective and robust assay for L-methionine can be developed by coupling the PPi detection system with adenosylmethionine synthetase, an enzyme producing PPi from ATP and L-methionine [19]. Thus, the results obtained in the present study demonstrated not only the usefulness of the citrulline and arginine assays, but also the potential of enzymatic assays coupled to the PPi detection system (Fig. 5). In this scheme, enzymatic assays for another biomolecule may be developed by replacing ASS with another PPi-producing enzyme that reacts with the target biomolecule as the substrate. The high efficiency and selectivity are not limited only in ASS and adenosylmethionine synthetase, but are shared by other PPi-producing enzymes when they are coupled to the PPi detection system. Selective and robust enzymatic assays for various biomolecules are expected to be developed based on the PPi detection system as a platform.

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References

- [1] Kone BC. Nitric oxide synthesis in the kidney: isoforms, biosynthesis, and functions in health. *Semin Nephrol.* 2004;24:299-315.
- [2] Morris SM. Arginine metabolism: boundaries of our knowledge. *The Journal of Nutrition.* 2007;137:1602S-9S.
- [3] Knipp M, Vasak M. A colorimetric 96-well microtiter plate assay for the determination of enzymatically formed citrulline. *Anal Biochem.* 2000;286:257-64.
- [4] Summar M, Tuchman M. Proceedings of a consensus

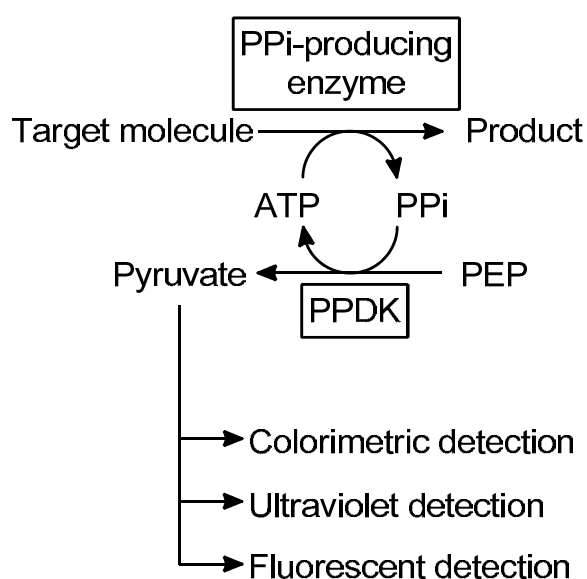


Fig. 5. Construction of a coupling assay based on PPi detection as a platform. Any of the colorimetric, UV, or fluorescent detection assays are available for determination, giving a signal proportional to the amount of the target molecule.

- conference for the management of patients with urea cycle disorders. The Journal of Pediatrics. 2001;138:S6-10.
- [5] Curis E, Nicolis I, Moinard C, Osowska S, Zerrouk N, Benazeth S, et al. Almost all about citrulline in mammals. Amino acids. 2005;29:177-205.
- [6] Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. Clin Nutr. 2008;27:328-39.
- [7] Demacker PN, Beijers AM, van Daal H, Donnelly JP, Blijlevens NM, van den Ouweland JM. Plasma citrulline measurement using UPLC tandem mass-spectrometry to determine small intestinal enterocyte pathology. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:387-92.
- [8] Kaspar H, Dettmer K, Gronwald W, Oefner PJ. Advances in amino acid analysis. Anal Bioanal Chem. 2009;393:445-52.
- [9] Boyde TR, Rahmatullah M. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. Anal Biochem. 1980;107:424-31.
- [10] Sakaguchi S. A new method for the colorimetric determination of arginine. J Biochem (Tokyo). 1950;37:231-6.
- [11] Notenboom CD, Veerdonk FCG, Kamer JC. A fluorescent modification of the Sakaguchi reaction on arginine. Histochemie. 1967;9:117-21.
- [12] Weber CJ. A modification of Sakaguchi's reaction for the quantitative determination of arginine. J Biol Chem. 1930;86:217-22.
- [13] Gilboe DD, Williams JN, Jr. Evaluation of the Sakaguchi reaction for quantitative determination of arginine. Proc Soc Exp Biol Med. 1956;91:535-6.
- [14] Mira de Orduna R. Quantitative determination of L-arginine by enzymatic end-point analysis. J Agric Food Chem. 2001;49:549-52.
- [15] Kameya M, Onaka H, Asano Y. Selective tryptophan determination using tryptophan oxidases involved in bis-indole antibiotic biosynthesis. Anal Biochem. 2013;438:124-32.
- [16] Yamasaki-Yashiki S, Tachibana S, Asano Y. Determination of L-methionine using methionine-specific dehydrogenase for diagnosis of homocystinuria due to cystathionine beta-synthase deficiency. Anal Biochem. 2012;428:143-9.
- [17] Ueatrongchit T, Asano Y. Highly selective L-threonine 3-dehydrogenase from *Cupriavidus necator* and its use in determination of L-threonine. Anal Biochem. 2011;410:44-56.
- [18] Matsuda M, Asano Y. Determination of plasma and serum L-lysine using L-lysine epsilon-oxidase from *Marinomonas mediterranea* NBRC 103028^T. Anal Biochem. 2010;406:19-23.
- [19] Kameya M, Himi M, Asano Y. Rapid and selective enzymatic assay for L-methionine based on a pyrophosphate detection system. Anal Biochem. 2014;447:33-8.
- [20] Tamaoku K, Ueno K, Akiura K, Ohkura Y. New water-soluble hydrogen donors for the enzymatic photometric determination of hydrogen peroxide. II. N-Ethyl-N-(2-hydroxy-3-sulfopropyl) aniline derivatives. Chem Pharm Bull (Tokyo). 1982;30:2492-7.
- [21] Lemke CT, Howell PL. Substrate induced conformational changes in argininosuccinate synthetase. J Biol Chem. 2002;277:13074-81.
- [22] Hilger F, Simon JP, Stalon V. Yeast argininosuccinate synthetase. Purification; structural and kinetic properties. Eur J Biochem. 1979;94:153-63.
- [23] Lemke C, Yeung M, Howell PL. Expression, purification, crystallization and preliminary X-ray analysis of *Escherichia coli* argininosuccinate synthetase. Acta Crystallogr D Biol Crystallogr. 1999;55:2028-30.
- [24] Baur H, Luethi E, Stalon V, Mercenier A, Haas D. Sequence analysis and expression of the arginine-deiminase and carbamate-kinase genes of *Pseudomonas aeruginosa*. Eur J Biochem. 1989;179:53-60.
- [25] Fayura LR, Boretzky YR, Pynyaha YV, Wheatley DN, Sibirny AA. Improved method for expression and isolation of the *Mycoplasma hominis* arginine deiminase from the recombinant strain of *Escherichia coli*. J Biotechnol. 2013;167:420-6.
- [26] Tachibana S, Suzuki M, Asano Y. Application of an enzyme chip to the microquantification of L-phenylalanine. Anal Biochem. 2006;359:72-8.