Journal of Bioscience and Bioengineering
VOL. 119 No. 3, 369–374, 2015

www.elsevier.com/locate/jbiosc

New enzymatic methods for selective assay of L-lysine using an L-lysine specific decarboxylase/oxidase from *Burkholderia* sp. AIU 395

Asami Sugawara,[1] Daisuke Matsui,[2,3] Miwa Yamada,[1] Yasuhisa Asano,[2,3] and Kimiyasu Isobe[1,*]

Department of Biological Chemistry and Food Science, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020-8550, Japan,[1] Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan,[2] and Asano Active Enzyme Molecule Project, ERATO, JST, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan[3]

Received 28 July 2014; accepted 21 August 2014
Available online 2 October 2014

We developed new enzymatic methods for the selective assay of L-lysine by utilizing an oxidase reaction and a decarboxylation reaction by the L-lysine-specific decarboxylase/oxidase (L-Lys-DC/OD) from *Burkholderia* sp. AIU 395. The method utilizing the oxidase reaction of this enzyme was useful for determination of high concentrations of L-lysine. The method utilizing the decarboxylase reaction, which proceeded via the combination of the L-Lys-DC/OD and putrescine oxidase (PUO) from *Micrococcus rubens*, was effective for determination of low concentrations of L-lysine. Both methods showed good linearity, and neither was affected by other amino acids or amines. In addition, the within-assay and between-assay precisions of both methods were within the allowable range. The coupling of L-Lys-DC/OD with PUO was also useful for the differential assay of L-lysine and cadaverine. These newly developed methods were applied to the assay of L-lysine in biological samples and found to be effective.

[**Key words:** L-Lysine; Cadaverine; L-Lysine oxidase; L-Lysine decarboxylase; Putrescine oxidase; Burkholderia]

L-Lysine is an essential amino acid for humans and plays important roles in calcium absorption and recovery from surgery or sports injuries. L-Lysine is also known to be present in high concentrations in the blood of individuals with hyperlysinemia. Since L-lysine is an important amino acid in the nutrition and diagnostic fields, several assay methods for this amino acid have been developed using an amino acid analyzer (1), high performance liquid chromatography (HPLC) (2), and enzymes (3–5). Among these methods, the enzymatic approaches have the advantages of being fast and relatively simple. In addition, the two enzymatic methods developed using L-lysine α-oxidase (EC 1.4.3.14) from *Trichoderma viride* (3,4) and L-lysine ε-oxidase (EC 1.4.3.20) from *Marinomonas mediterranea* (5) also have advantages in terms of sensitivity, because the hydrogen peroxide generated by the oxidative deamination of L-lysine can be assayed by color development methods with high sensitivity. However, these enzymatic methods also have a disadvantage in terms of the substrate specificity for selective assay of L-lysine, since the enzymes employed oxidize several other amino acids in addition to L-lysine (6,7). Recently, we identified a new enzyme, L-amino acid oxidase/oxygenase (L-AAO/MOG) from *Pseudomonas* sp. AIU 813 (8), and used it to develop an enzymatic method for the selective assay of L-lysine. The method utilizing L-AAO/MOG exhibited good selectivity in the L-lysine assay, but the sensitivity was lower than that of the other L-lysine assay methods, because hydrogen peroxide was formed by the oxidative deamination reaction, but not by the oxygenation reaction. The sensitivity of the L-AAO/MOG method was then improved by coupling with a new amine oxidase from *Aspergillus carbonarius* AIU 205, but the improved method still had low sensitivity (9). Thus, there is need of a more selective assay method with high sensitivity for L-lysine.

More recently, we found a novel enzyme, L-lysine decarboxylase/oxidase (L-Lys-DC/OD), which exhibited L-lysine oxidase activity as well as L-lysine decarboxylase activity, from *Burkholderia* sp. AIU 395 (10). The enzyme rapidly oxidized L-lysine and slowly oxidized cadaverine and 5-hydroxy-L-lysine, but did not oxidize other L-amino acids. In addition, the K_m values of this enzyme for cadaverine and 5-hydroxy-L-lysine were much higher than that for L-lysine, and the V_{max} values for both substrates were lower than that for L-lysine. Thus, the substrate specificity and kinetic characteristics of the L-Lys-DC/OD had merits for the selective assay of L-lysine compared to the other enzymes, although the enzyme catalyzed both decarboxylation and oxidation reactions and the speed of the former reaction was much greater than that of the latter one. In this study, therefore, we developed new enzymatic methods for the selective assay of L-lysine using the L-Lys-DC/OD. In addition, we describe a differential assay method for L-lysine and cadaverine using the L-Lys-DC/OD and putrescine oxidase (PUO) from *Micrococcus rubens* IFO 3768.

MATERIALS AND METHODS

Chemicals and enzymes L-Lysine, L-ornithine, 5-hydroxy-DL-lysine, other L-amino acids, and cadaverine were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of analytical grade and commercially available.

* Corresponding author at: Present address, Asano Active Enzyme Molecule Project, ERATO, JST, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan. Tel.: +81 766 88 2280; fax: +81 766 88 2422.
E-mail address: kiso@iwate-u.ac.jp (K. Isobe).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.08.013

Peroxidase (POD) was a gift from Amano Enzyme (Nagoya, Japan). The L-Lys-DC/OD from *Burkholderia* sp. AIU 395 was purified according to our previous report (10). The PUO from *M. rubens* IFO 3768 was purified according to the procedure of Adachi et al. (11) with slight modification.

Assay of enzyme activity The L-lysine oxidase (L-Lys-OD) activity of L-Lys-DC/OD was assayed by measuring the initial rate of hydrogen peroxide formation at pH 6.0 as follows. The standard reaction mixture contained 5 μmol of L-lysine, 0.12 μmol of 4-aminoantipyrine (4-AA), 0.38 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate (TOOS), 1.8 units of POD, 0.1 mmol of potassium phosphate, pH 6.0, and an appropriate amount of enzyme in a final volume of 1.0 ml. The assay of enzyme activity was started by addition of enzyme solution, and hydrogen peroxide formation was spectrophotometrically followed at 30°C for 5 min by measuring the absorbance at 555 nm.

The PUO activity was assayed by measuring the hydrogen peroxide formation rate under the same assay conditions as in the above L-Lys-OD activity measurement, except that 5 μmol of cadaverine and 0.1 mmol of potassium phosphate, pH 7.0, were used.

One unit of enzyme activity for the above two enzymes was defined as the amount of enzyme catalyzing the formation of 1 μmol of hydrogen peroxide per min. The molar absorptivity value of 16.5×10^3 M^{-1} cm^{-1} was used to calculate the enzyme activity.

Principle of the selective assay of L-lysine and differential assay of L-lysine and cadaverine The L-Lys-DC/OD from *Burkholderia* sp. AIU 395 is specific to L-lysine and catalyzes different reactions, i.e., decarboxylation and oxidation, for L-lysine. In the former reaction, L-lysine is converted into cadaverine without the formation of hydrogen peroxide, while the latter reaction catalyzes conversion of L-lysine into 6-oxo-2-aminohexanoic acid and hydrogen peroxide is formed (10). In addition, the former reaction speed is much greater than that of the latter reaction. The PUO from *M. rubens* IFO 3768 oxidizes putrescine, cadaverine and spermidine but does not oxidize L-amino acids (11). On the basis of the differences of reaction specificity and substrate specificity of both enzymes (Fig. 1), new enzymatic methods for the selective assay of L-lysine were developed utilizing L-Lys-DC/OD or L-Lys-DC/OD plus PUO, and that for differential assay of L-lysine and cadaverine was developed utilizing L-Lys-DC/OD and PUO as follows.

Selective assay of L-lysine with L-Lys-DC/OD The substrate solution containing 0–1.0 μmol of L-lysine was incubated at 30°C for 3 min with a color developing reagent I consisting of 0.12 μmol of 4-AA, 0.38 μmol of TOOS and 1.8 units of POD in 0.9 ml of 0.1 mmol of potassium phosphate, pH 6.0. Then, 0.1 ml of L-Lys-DC/OD solution (1.3 m units) was added into the above solution, and the hydrogen peroxide formed by L-lysine oxidation was followed at 30°C for 20 min by measuring the absorbance at 555 nm.

Differential assay of L-lysine and cadaverine with L-Lys-DC/OD and PUO In the first step, a substrate solution containing 0–30 nmol of L-lysine and 30 nmol of cadaverine was incubated at 30°C for 3 min with the color reagent I in 0.9 ml of 0.1 mmol of potassium phosphate, pH 7.0. Then, 50 μl of PUO solution (0.2 units) was added to the above solution, and the hydrogen peroxide formation was followed at 30°C for 30 min by measuring the absorbance at 555 nm (hydrogen peroxide is generated from cadaverine in this step, and a violet color develops). After finishing the cadaverine oxidation, 50 μl of L-Lys-DC/OD solution (2.3 m units) was added to the reaction mixture, and the hydrogen peroxide generated by L-lysine oxidation was further followed at 30°C for 30 min by measuring the absorbance at 555 nm (hydrogen peroxide is formed from L-lysine in this step, and a violet color develops).

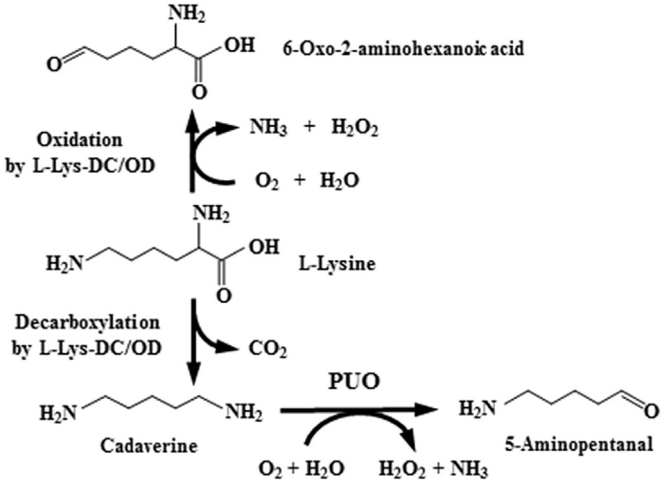

FIG. 1. Reaction of L-Lys-DC/OD and PUO.

Selective assay of L-lysine with L-Lys-DC/OD and PUO On the basis of the above differential assay method, a new enzymatic method for selective assay of L-lysine with high sensitivity was developed. In the first step, the substrate solution containing 0–60 nmol of L-lysine and 60 nmol of cadaverine was incubated at 30°C for 3 min with a reagent I-a consisting of 0.12 μmol of 4-AA and 1.8 units of POD in 0.9 ml of 0.1 mmol of potassium phosphate, pH 7.0. Then, 50 μl of PUO solution (0.2 units) was added to the solution, and the hydrogen peroxide generated by cadaverine oxidation was followed at 30°C for 30 min by measuring the absorbance at 555 nm (absorbance does not develop in this step, because the hydrogen peroxide formed is removed by the formation of a colorless compound, as described in Ref. 13). After finishing the above cadaverine oxidation, 50 μl of a reagent I-b consisting of 2.3 m units of L-Lys-DC/OD and 0.38 μmol of TOOS was added to the above reaction mixture, and the hydrogen peroxide generated from L-lysine was followed at 30°C for 30 min by measuring the absorbance at 555 nm (hydrogen peroxide, which is formed from L-lysine by the oxidation reaction of L-Lys-DC/OD and by the decarboxylation reaction of L-Lys-DC/OD plus the oxidation reaction of PUO, is recognized by a violet color).

Deviation of the selective assay method with L-Lys-DC/OD and PUO Solutions containing 60 nmol cadaverine and 10, 30 or 60 nmol of L-lysine were used as substrates. The deviation was evaluated by the procedure described above in the section on the selective assay method with L-Lys-DC/OD and PUO, using 10 samples for each concentration of L-lysine. Within-run precisions were calculated from the data at each concentration. The between-assay precision was analyzed using the newly prepared color development reagent and three different samples. The assay was carried out on five different days.

Correlation between the enzymatic method and HPLC method The L-lysine assay was carried out by HPLC, the selective assay method was performed using L-Lys-DC/OD and PUO, and five food samples were analyzed [two kinds of plain soup stock, milk, soy milk, and miso (fermented soybean paste)]. The sample solutions for L-lysine assay were prepared as follows. First, 10 g of each sample was dissolved in 10 ml of MilliQ and vigorously mixed. The solutions were then centrifuged to remove the precipitates. Proteins in the samples were further removed using a Microcon YM-10 filter (Millipore, Bedford, MA, USA) prior to the enzymatic assays and HPLC analyses. The filtrates were directly applied to the enzymatic assays. In the case of HPLC analysis, the filtrates were derivatized by an AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) according to the manufacturer's manual. Subsequently, the analytes were subjected to liquid chromatography by the UPLC system equipped with an AccQ-Tag Ultra column (1.7 μm, 100 × 2.1 mm i.d.; Waters) (12). AccQ-Tag Ultra eluents A and B were used at a flow rate of 0.7 ml/min under the gradient described in the manufacturer's manual. The elution of derivatized L-lysine was detected by monitoring the absorbance at 260 nm.

RESULTS

Effects of pH on enzyme activity and stability The effects of pH on the enzyme activity and stability of L-Lys-DC/OD and PUO were analyzed as follows. When the L-Lys-OD activity of L-Lys-DC/OD was assayed under standard assay conditions except that the pH was varied between 5.0 and 8.5, the oxidase activity was detected in these pH ranges and maximum enzyme activity was obtained at pH 6.0 (Fig. 2A). The effect of pH on the stability of L-Lys-DC/OD was investigated by incubation at 30°C for 30 min in the pH range from 5.0 to 8.5. After this incubation, more than 85% of enzyme activity remained in the pH range between 5.0 and 7.0 (Fig. 2A). Then, the effect of temperature on the L-Lys-OD activity of L-Lys-DC/OD was assayed under standard assay conditions, except that the reaction temperature was varied between 20°C and 60°C. The maximal oxidase activity for L-lysine was obtained at 50°C (Fig. 2B). When L-Lys-DC/OD was incubated at pH 6.0 for 30 min between 20°C and 60°C without substrate, approximately 90% of the enzyme activity remained below 45°C, but the activity was completely lost at 55°C (Fig. 2B).

It has been reported that PUO was stable in the pH range from 6.0 to 8.0, but the optimal pH for cadaverine oxidation of PUO was not studied. We therefore assayed cadaverine oxidase activity of the PUO in the pH range from 5.0 to 8.5. The oxidase activity for cadaverine was detected from pH 6.5 to pH 8.5, and the highest enzyme activity was obtained at pH 8.5 (Fig. 2A). These results indicated that reaction at pH 6.0 was desirable for the L-lysine assay using L-Lys-DC/OD and reaction at pH 7.0 was desirable for the L-lysine assay by the combination of L-Lys-DC/OD and PUO.