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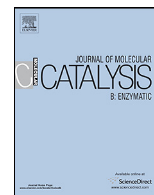

Application of diethyl ethoxymethylenemalonate (DEEMM) derivatization for monitoring of lysine decarboxylase activity

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ABSTRACT

Aliphatic diamines have been used as chemical reagents for the monomers of polyamides (*i.e.*, Nylon 46, Nylon 510, Nylon 66, Nylon 610 and so on), and can be made from lysine, arginine or ornithine by the metabolism of microbes through decarboxylases. However, the conventional derivatization methods for the HPLC-based quantitative analysis of aliphatic diamines exhibit poor sensitivity and efficiency for the monitoring of enzyme activity. In this study, a chemical derivatization method using diethyl ethoxymethylenemalonate (DEEMM) was applied to monitor lysine decarboxylase activity for the production of cadaverine by measuring the levels of intracellular and secreted lysine and cadaverine. The calibration graphs for the determination of 2,4-diaminobutyrate, 2,6-diaminopimelic acid, ornithine, arginine, lysine, 1,3-diaminopropane, putrescine dihydrochloride, cadaverine, hexamethylenediamine, and 1,7-diaminoheptane were measured by the highly sensitive method using DEEMM, and a linear relationship was observed for each dimaine compound, with limits of detection up to 0.001 mM. Application of this method will be useful for the sensitive monitoring of lysine decarboxylase reactions through the detection of substrates containing amine groups and products with diamines using HPLC and a UV detector.

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1. Introduction

Aliphatic diamines such as 1,3-diaminopropane (C3), 1,4-diamine (putrescine) (C4), 1,5-diamine (cadaverine) (C5), hexamethylenediamine (C6) and heptamethylenediamine (C7) are widely used as chemical reagents in the preparation of polyamides, polyimides and polyureas [1], with an additional use as chelating agents for analytical purposes [2]. They are made by petroleum-based methods, and have been found to have high potential for the production of polyamide, which can be made by reaction of aliphatic diamines and dicarboxylic acid [3]. However, the petrochemical route suffers from the drawbacks of limited supply and rising price of fossil fuels, as well as low eco-efficiency [4]. As an alternative, aliphatic diamines can also be made by enzymatic methods or by whole cells through the use of decarboxylases of ornithine, lysine, and arginine. Among these chemicals, cadaverine, produced by lysine decarboxylase with lysine as a substrate, is well known in the drug industry [7]. It is particularly relevant in the production of bio-polyamides derived from renewable feedstock, as a replacement for conventional polyamides derived from petrochemicals [8]. The existence of lysine decarboxylases in anaerobic bacteria and eukaryotes has been well-reported [9], and there are several different types and, for example, CadA is expressed in low pH environments, while LdcC is constitutively expressed in metabolic pathways to protect cells from the drop of pH [5,6].

To detect decarboxylase activities, pH indicators [10], enzyme coupling [11], and HPLC methods are commonly used [12,13]. Among them, HPLC generally gives the best results with repeatable and stable data, although it requires derivatization of the analytes

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[14], which also affects the separation and purification steps [15]. There are several derivatization methods available, which make use of derivatization molecules such as o-phthalaldehyde (OPA), dansyl chloride, and acetyl acetone [16–20] (Supplementary Table 1, Supplementary Fig. 1). However, all of these methods were reported to result in relatively poor derivatization efficiency of lysine, cysteine, and aliphatic amines, wihle sometimes requiring expensive mass spectrometry [21]. Even worse, the processing of such derivatized molecules in compounds containing amines through HPLC and UV detectors produced results with poor sensitivity and high background peaks.

For the monitoring of lysine decarboxylase activity, diethyl ethoxymethylenemalonate (DEEMM) was applied herein to detect the enzymatic production of cadaverine. Although DEEMM has been used in ESI–MS for metabolite detection in food [22,23] as well as for *in vivo* screening of biogenic amine production [24,25], there are few reports on its direct application for the monitoring of lysine decarboxylase activity through detection of its substrate and products. Application of DEEMM for the detection of lysine-related compounds herein revealed it to be a good derivatization molecule allowing the detection of various substrates and products with high sensitivity, without interference from excess reagent.

2. Materials and methods

2.1. Chemical reagents

The chemical reagents used in this study, including 2,4-diaminobutyrate, 2,6-diaminopimelic acid, ornithine, arginine, lysine, 1,3-diaminopropane, putrescine dihydrochloride, cadaverine, hexamethylenediamine, and 1,7-diaminoheptane, were purchased from Sigma Aldrich (USA). The derivatization reagent, diethyl ethoxymethylenemalonate, was purchased from Fluka (Japan), and the ortho-phthaldialdehyde reagent was purchased from Sigma Aldrich (Korea). HPLC-grade acetonitrile and water were purchased from Fisher. Sodium acetate, acetic acid, sodium borate and Trichloroacetic acid purchased from Biosesang (Korea).

2.2. Bacterial strain and media

The *Corynebacterium* strains used in this study were the wild type strain ATCC 13032 and *ldcC* overexpressing *Corynebacterium glutamicum*. The cadaverine producing strain, *ldcC* overexpressing *C. glutamicum*, was constructed by transforming *C. glutamicum* with the lysine decarboxylase (*ldcC*) gene, amplified by PCR from *Escherichia coli* K12, on pXMJ19. Strains were tested by plating on BHIS medium (brain heart infusion sorbitol) containing 15 μg/ml Kanamycin. In order to construct pXMJ19-*ldcC*, *ldcC* was amplified by PCR using the upstream primers *ldcC* (*XbaI*)-F and *ldcC* (*HindIII*)-R, and the PCR fragments were cloned into *C. glutamicum*, resulting in plasmid pXMJ19-ldcC, which was transformed by electroporation. Cultivation was carried out at 30 °C and 200 rpm in a shaking incubator (Han-Beak Science Co. Korea). The first pre-cultures (5 ml LB medium in 14 ml round-bottom tubes) were inoculated with single colonies from LB agar plates, made by adding Bacto-Agar (Difco) at up to 2% and incubating for 16 h. Cells were harvested by centrifugation (13,000 × *g* for 2 min at 4 °C), washed with distilled water, and used as inocula for the main cultivation. The main cultivation was carried out in 50 ml LB medium containing 15 μg/ml Kanamycin in 250 ml baffled flasks. The *ldcC* and *cadA* from *E. coli* and *Salmonella entertica* were prepared by cloning into the pET24ma vector for enzyme reaction, and those from *E. coli* BL21(DE3) were prepared by the induction of 0.1 mM iso-propyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 15 h. Whole cells were harvested by centrifugation (13,000 × *g*, 5 min, 4 °C) and washed twice with distilled water.

2.3. Reaction of lysine and cadaverine produced by biotransformation with whole cells

The assays were performed in a total volume of 500 μl, containing 500 mM sodium acetate buffer (pH 6.0), 10 mM L-lysine, 0.1 mM pyridoxal-5-phosphate, and 20 μl whole cells, at 37 °C in a water bath. The reaction was stopped after 24 h by the addition of 10 μl of ethanol. The reaction mixtures were centrifuged, and the 30 μl of supernatant was applied to derivatization to determine the amounts of residual lysine and product, *i.e.* cadavarine.

2.4. Derivatization reaction

Diamine derivatives were obtained by the reaction of 180 μl of borate buffer 0.05 M (pH 9), 60 μl of 100% methanol, 47 μl of distilled water, 30 μl of 10 mM sample and 3 μl of 200 mM diethyl ethoxymethylenemalonate without any pretreatment. The samples were heated at 70 °C for 2 h to allow complete degradation of excess DEEMM [22] and derivatization (Supplemenatry Table 2).

2.5. HPLC analysis

After derivatization with diethyl ethoxymethylmalonate, analyses were performed on a high performance liquid chromatograph (HPLC, YL-9100, Korea) consisting of a binary pump, an in-line degasser, an autosampler, and a column thermostat. Chromatographic separation was carried out by reverse-phase chromatography on a C18 column (Agilent ZORBAX SB-C18 column, 4.6 × 250 mm, 5 μm particle size), maintained at 35 °C. Mobile phase A was composed of 100% acetonitrile, and B was made up of 25 mM aqueous sodium acetate buffer (pH = 4.8). The flow rate of 1 ml/min was used, with the following gradient program: 0–2 min, 20–25% A; 2–32 min, 25–60% A; 32–40 min, 60–20% A. Detection was carried out at 284 nm.

2.6. Detection of intracellular and secreted lysine and cadaverine

For the detection of secreted lysine and cadaverine, the grown cells were first centrifuged (13,000 × *g*, 10 min, 4 °C). The supernatant was then obtained, and derivatization was carried out. For the detection of intracellular lysine and cadaverine, cells grown in LB medium were harvested by centrifugation and washed twice in 1 ml of phosphate-buffered saline. Next, 3 μl of 5% trichloroacetic acid (TCA) per mg of fresh cell weight was applied to lyse the cells, and the mixed solutions were incubated overnight at 4 °C. They were then centrifuged (13,000 × *g*, 10 min, 4 °C) and applied for derivatization.

3. Results and discussions

3.1. Monitoring of enzyme reaction for CadA and LdcC

Although OPA is one of the commonly used molecules for amino acid analysis, it typically performs poorly in the derivatization of lysine [21]. It also shows very small dynamic ranges, especially with lysine and diamines (Supplemenatry Fig. 1B). To avoid this problem DEEMM was applied herein, and it was found to have 43 fold higher sensitivity than OPA for lysine, and 15 fold higher for cadaverine (Fig. 1). It could be applied to monitor the enzyme reactions of

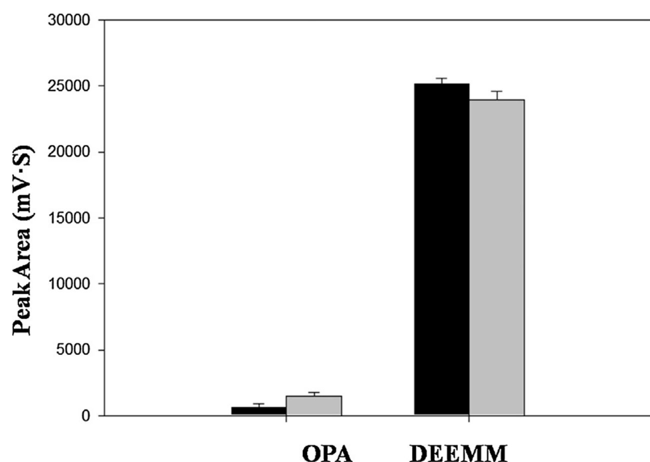

Fig. 1. Comparison of the detected peaks of lysine (black bar) and cadaverine (gray bar) when derivatized with OPA and DEEMM.

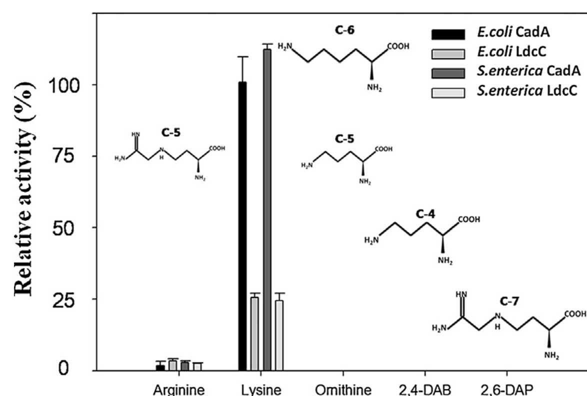

Fig. 3. Broad substrate specificity of CadA and LdcC from *E. coli* and *S. enterica* as examined by the DEEMM method. Abbreviations: 2,4-diaminobutyrate (2,4-DAB), 2,6-diaminopimelic acid (2,6-DAP).

lysine decarboxylases such as CadA and LdcC from *E. coli*, successfully detecting the decrease of lysine and increase of cadaverine in a 10 mM enzyme reaction (Fig. 2). In addition, DEEMM modification could also be applied to detect broad substrate specificities of lysine decarboxylase by monitoring the concentrations of various substrates, including 2,4-diaminobutyrate, 2,6-diaminopimelic acid, ornithine, and arginine, as well as products such as 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, and 1,6-diaminohexane (Supplementary Table 3). Based on this ability, the broad substrates of CadA and LdcC enzymes from *E. coli* and *S. entertica* could be measured with the employment of DEEMM, although they showed specific activity mainly on lysine (Fig. 3).

3.2. Detection of intracellular and secreted lysine and cadaverine produced by C. glutamicum *wild type and* ldcC *strains*

To examine application to the cell-based production of cadaverine, DEEMM was directly applied for the detection of lysine and cadaverine in cultured samples of *C. glutamicum* wild type and *ldcC* overexpressing strain. To realize this goal, an *ldcC* from *E. coli K12* strain was expressed in *C. glutamicum* [27], as explained in Section 2. DEEMM was then applied to monitor the intracellular production and secretion of lysine and cadaverine in the media of the *C. glutamicum* wild type strain and *ldcC* overexpressing strain. Sensitive monitoring of the intracellular and secreted lysine and cadaverine was achieved, which could not easily be measured with samples as small as 1 ml with the previous derivitization molecules due to their poor sensitivity and poor limit of detection (LOD) with HPLC and UV detectors. Using DEEMM, *C. glutamicum* was found to produce 3.75 mmol/wet weight cell (g), and to secrete 2.84 mmol/wet weight cell (g) (Fig. 4).

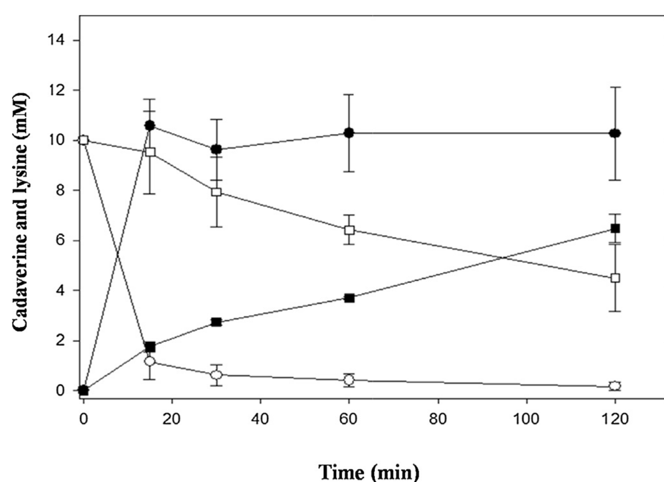

Fig. 2. Monitoring of lysine decarboxylase enzyme reaction through CadA and LdcC from *E. coli*. The concentration of lysine and cadaverine in the case of CadA and LdcC were shown. Lysine-CadA (○), cadaverine-CadA (●), lysine-LdcC (□), cadaverine-LdcC (■).

3.3. Application of DEEMM for the detection of various diamines

To further examine the efficiency of DEEMM derivatization, the performance of DEEMM was compared with that of OPA,

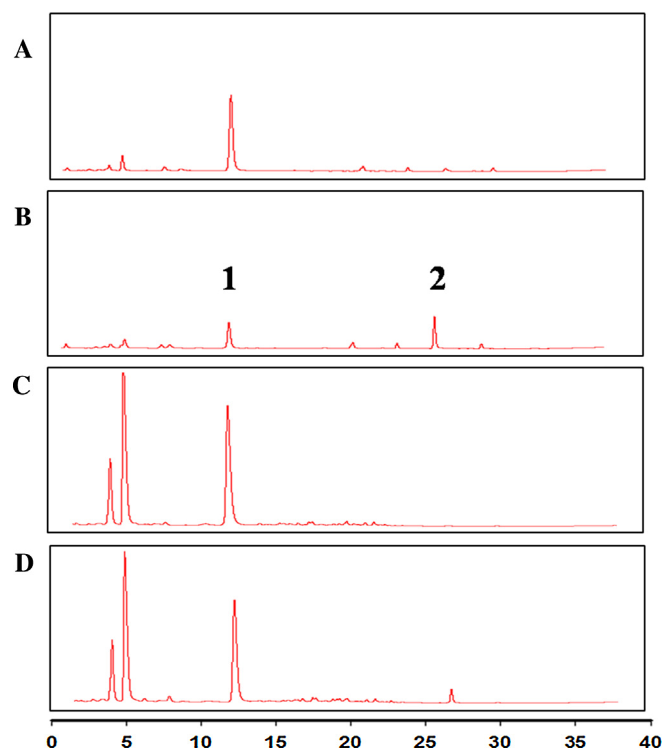

Fig. 4. HPLC analysis of intracellular metabolites in the whole cell culture media of *Corynebacterium glutamicum* (A: *wild type*, B: *ldcC*): 1 – lysine, 2 – cadaverine; and HPLC analysis of extracellular metabolites in the whole cell culture media of *C. glutamicum* (C: *wild type*, D: *ldcC*).

Table 1
Retention times and detection limits for determining diamine concentrations through reversed-phase HPLC.

Diamines	OPA		DEEMM		Relative intensity to OPA (Fold)
	Retention time (min)	Detection limit (mM)[a]	Retention time (min)	Detection limit (mM)	
1,3-Diaminopropane	29.0	>0.1	21.0	>0.001	26.6
1,4-Diaminobutane	32.5	>0.01	24.0	>0.001	15.1
1,5-Diaminopentane	34.0	>0.01	26.8	>0.001	15.4
1,6-Diaminohexane	39.0	>0.01	30.1	>0.001	19.2
1,7-Diaminoheptane	41.4	>0.01	33.2	>0.001	19.6

[a] Detection limit (DL): concentration at which the analytic peak above the peak area of 3 mV can be detected.

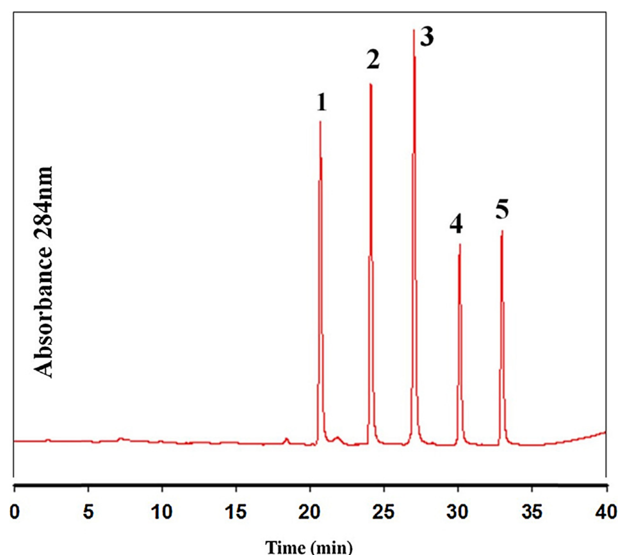

Fig. 5. HPLC profiles of diamines derivatized by DEEMM: 1 – diaminopropane, 2 – diaminobutane, 3 – diaminopentane, 4 – diaminohexane, and 5 – diaminoheptane.

one of the most well-known derivatization molecules for amino acids [28]. When comparing DEEMM with OPA for the derivatization of diamines 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, and 1,7-diaminoheptane, DEEMM showed at least 10-fold higher sensitivity than OPA with the use of very small amounts of samples, resulting in very low limits of detection (Table 1). Depending on the molecules, DEEMM had a detection limit of about 0.001 mM while OPA required 0.1–0.01 mM for detection, which suggests that DEEMM is a more effective molecule for the detection of diamines (Table 1 and Fig. 5). Correlation coefficients showed linear correlation from 0.001 to 1 mM of diamines with small standard deviation, suggesting that DEEMM derivatization can be used for the stable quantification of diamines (Supplementary Table 4). Derivatization with DEEMM is known to require a reaction time of up to 2 h [29]. However, when the reaction time of 5 min was compared to that of 2 h, the 5 min reaction produced 70% of the sensitivity of that shown by the 2 h reaction for derivatization with DEEMM (data not shown).

4. Conclusions

Highly sensitive derivatization methods were applied to monitor diamines and their substrates with an HPLC–UV detector using diethyl ethoxymethlenemalonate, through which the enzymatic reactions of lysine decarboxylases were monitored by measuring the intracellular and secreted lysine and cadaverine produced by *E. coli* and *C. glutamicum* strains. This will be a very useful method for the detection of industrially crucial diamines, and for the monitoring of decarboxylase reactions for the production of various polyamide monomers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.01.018.

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