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RAPID COMMUNICATION

Alkaliphilic lysine decarboxylases for effective synthesis of cadaverine from L-lysine

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Abstract−The enzymatic decarboxylation of L-lysine using lysine decarboxylase is a promising biological approach for producing cadaverine, a versatile platform chemical for bio-polyamides. However, due to the problem with elevated pH in the reaction solution during the enzymatic process, it is desirable to use lysine decarboxylases effectively active in alkaline conditions. In this study, the catalytic properties of three lysine decarboxylases from *Selenomonas ruminantium* (srLDC), *Vibrio vulnificus* (vvLDC), and *Geobacillus thermodenitrificans* (gtLDC) were characterized, and the applicability of the enzymes in alkaline conditions was investigated. Among the three enzymes, only vvLDC exhibited effective activity in alkaline pH conditions. The conversion rate of vvLDC was 1.5-fold higher than that of srLDC and 5.3-fold higher than that of gtLDC in pH 9.0. The results indicate that vvLDC is more advantageous than srLDC and gtLDC for the enzymatic conversion of L-lysine to cadaverine in alkaline conditions.

Keywords: Lysine Decarboxylase, L-Lysine, Cadaverine, pH Optimum, Alkaline Conditions

INTRODUCTION

Significant applications have been found for polyamide polymers (nylon) in a variety of commercial areas, including textile and automotive industries [1,2]. Nylon 66, which is an example of a polyamide, is widely used as an engineering plastic because of its superior elongation properties and abrasion resistance [3]. Nylon 66 is produced by co-polymerizing two 6-carbon monomers: adipic acid and hexamethylenediamine. One of the monomers, hexamethylenediamine, is a diamine compound that contains six carbons, which is produced from petrochemicals such as benzene, propylene, butadiene, or naphtha. Due to the toxicity and energy-consuming issues of petrochemical processes, the move to more moderate and environmentally benign approaches, such as microbial or enzymatic process, is gaining both social and commercial interests [3]. However, methods for the biological production of hexamethylenediamine have yet to be demonstrated [4]. In contrast, pentamethylenediamine, which is also called cadaverine, contains five carbons and is known to be produced from lysine using lysine decarboxylase (LDC) [5]. Cadaverine is a promising chemical platform that has a variety of applications, including the production of polyamides, polyurethanes, chelating agents, and additives. In particular, it is relevant for the production of bio-based nylon, which can be used to replace conventional polyamides from petrochemical routes [5]. Whereas Nylon 66 is a polymer composed of hexamethylenediamine and adipic acid, Nylon 56 is a polymer that is produced by the co-polymerization of cadaverine and adipic acid. Therefore, if nylon is produced by using a cadaverine as a raw material instead of hexamethylenediamine, it is then possible to provide a bio-polyamide, Nylon 56, which has comparable mechanical properties to Nylon 66 [4,6-8].

In biological systems, cadaverine is produced through the decarboxylation of L-lysine catalyzed by LDC [9]. The use of LDC for the enzymatic conversion of L-lysine to cadaverine has received much attention [5,10]. However, there is an intrinsic problem with this reaction scheme: the pH increases in the reaction solution during the enzymatic process. When L-lysine is converted to cadaverine by LDC, carbon dioxide is liberated from L-lysine and consumes equimolar amounts of protons from the reaction solution; hence, the increase in pH in the solution is inevitable during the reaction [11,12]. The change in pH conditions, in general, has deleterious effects on the catalytic function of enzymes. Therefore, to prevent the rise of pH and thus maintain the optimal pH for the enzymatic reaction, it is necessary to perform the reaction in a buffer that has a high concentration or to successively add an acid to the reaction system to neutralize the alkalinity. Conventionally, inorganic acids such as hydrochloric acid, sulfuric acid and phosphoric acid are used to neutralize the increased pH level. When alkalinity is neutralized with these acids, the cadaverine that is obtained from the reaction mixture exists in the form of a salt such as cadaverine hydrochloride, cadaverine sulfate and cadaverine phosphate. However, the cadaverine that is obtained through the neutralization process cannot be used directly in the polymerization process, and free cadaverine must be re-prepared from a salt of cadaverine. Therefore, additional separation steps are required, which make the process more complex and expensive.

One alternative approach to overcome the pH increase problem is the use of LDC, which is active at high pH levels and has an optimum pH in alkaline conditions. However, most of the LDCs

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reported to date have their optimum pH in neutral or acidic conditions [13-20]. In this study, three LDCs, i.e., *Selenomonas ruminantium* lysine decarboxylase (srLDC), *Vibrio vulnificus* (vvLDC), and *Geobacillus thermodenitrificans* (gtLDC), were prepared via heterologous expression in *E. coli*. The catalytic properties of the three LDCs, including optimum pH, were characterized, and the *in vitro* enzymatic conversion of L-lysine to cadaverine using the LDCs was performed to investigate the applicability of the enzymes in alkaline conditions.

MATERIALS AND METHODS

1. Materials

The pET-22b(+) vector and Bugbuster™ were purchased from Invitrogen (Carlsbad, CA, USA). Restriction enzymes were purchased from Takara (Tokyo, Japan). T4 DNA ligase was purchased from Gendepot (Barker, TX, USA). The *pfu* DNA polymerase was purchased from Bioneer (Daejeon, Korea). *Escherichia coli* strains of DH5α and BL21 (DE3) were purchased from RBC Bioscience (Taipei, Taiwan). Ni-NTA agarose was purchased from Qiagen (Valencia, CA, USA). The other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2. Expression of Recombinant LDC

Each encoding gene of *Selenomonas ruminantium* lysine decarboxylase srLDC (O50657.1, 13124043), *Vibrio vulnificus* lysine decarboxylase vvLDC (KFZ84912.1, 684019336), and *Geobacillus thermodenitrificans* lysine decarboxylase gtLDC (ABO66307.1, 134266112) was synthesized after codon optimization of their expression in *E. coli* (Bioneer, Korea). The synthetic genes were digested by NdeI and XhoI and cloned into the multicloning site of the pET-22b(+) vector to produce recombinant plasmids for the expression of proteins with C-terminal 6-histidine tags (Novagen, USA). The recombinant LDC plasmids were transformed into *E. coli* DH5α for plasmid amplification and DNA preparation. The amplified LDC plasmids were transformed into BL21 (DE3) for protein expression. Recombinant *E. coli* BL21 (DE3) was cultured in LB medium. Cells were induced by adding 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), cultured at 25 °C for 6 hr, and harvested by centrifugation (6,000 ×g, 20 min). Cell pellets were suspended in Bugbuster™ 5 ml per 1 g cell weight for cell lysis. The cell debris was removed by centrifugation (11,000 ×g, 20 min). The supernatants were incubated with Ni-NTA agarose and 10 mM imidazole in 4 °C for 1 hr. The solutions were loaded onto purification columns and washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The purified LDC was eluted using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Enzyme purity was estimated by 12% SDS-PAGE, and protein concentration was determined by Bradford assay.

3. Activity Assay and Enzymatic Conversion

The activity of LDCs was determined by measuring the conversion rate of L-lysine to cadaverine at 37 °C for 30 min for vvLDC and srLDC and at 60 °C for 12 hr for gtLDC because of its very low activity. The enzymatic reactions were performed via the addition of enzymes (final concentration of 0.2 μM) to the buffer solutions, which were composed of 100 mM L-lysine and 0.2 mM pyridoxal 5′-phosphate (PLP). The buffer solutions of 200 mM sodium acetate, 200 mM sodium phosphate and 200 mM sodium carbonate were used for pH 5.0, pH 6.0-7.0 and pH 8.0-9.0, respectively.

4. Substrate Specificity of vvLDC

The substrate specificity of vvLDC was investigated with five amino acids with various carbon numbers (2,3-diaminopropionic acid (C3); 2,4-diaminobutric acid (C4); L-ornithine (C5); L-lysine (C6); 2,7-diaminoheptanoic aid (C7)). The enzyme activity with the substrate candidates were determined by measuring the conversion rate of the decarboxylation reaction at 37 °C for 30 min. The enzymatic reactions were performed by the addition of vvLDC enzyme (final concentration of 1.2 μg/ml) in 20 mM sodium phosphate buffer containing 1 mM of the substrate candidates and 0.2 mM pyridoxal 5′-phosphate (PLP).

5. HPLC Analysis

Cadaverine modified by ortho-phthaldialdehyde (OPA) derivatization [21] was detected by HPLC (Agilent Technologies 1200 Series LC system, USA) using a Zorbax eclipse xdb-c18 column. Then, 40 mM acetate buffer (A), 100% acetonitrile (B) [A : B=6 : 4] was used as mobile phase with the flow rate of 0.6 ml/min. The column temperature was 40 °C, and the wavelength of the DAD detector was 338 nm for OPA-derivatizedcadaverine.

RESULTS AND DISCUSSION

1. Expression of LDC

Three recombinant LDCs were prepared by expressing each synthetic gene in *E. coli*. The protein amounts of three LDCs expressed in 1 L culture were estimated as follows: srLDC, 20.07 mg; vvLDC, 27.47 mg; and gtLDC, 27.77 mg. As shown in Fig. 1, the three LDCs were well expressed in *E. coli* as soluble forms.

2. Optimum pH of the Three LDCs

The catalytic activity of the three LDCs in various pH ranges (pH 5.0-10.0) was investigated by measuring the enzymatic conversion rate of L-lysine to cadaverine. Whereas the optimum pH val-

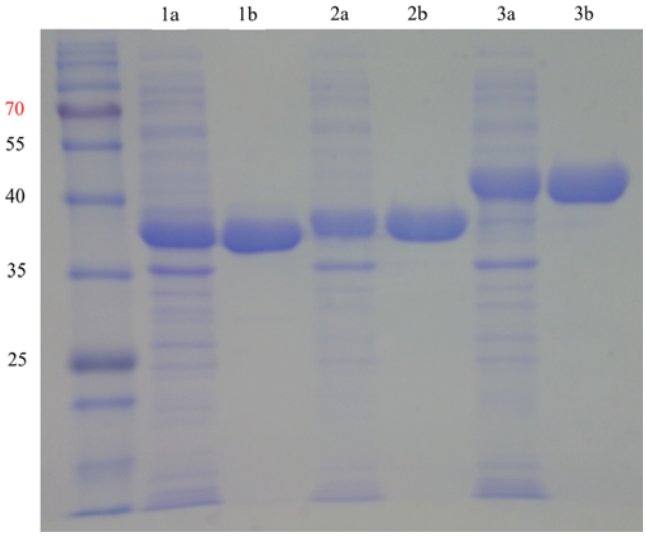

Fig. 1. SDS-PAGE of three LDCs: 1. srLDC (44.2 kDa), 2. vvLDC (44.7 kDa), 3. gtLDC (54.8 kDa), a: crude extract, b: purified enzyme, the unit of protein marker is kDa.

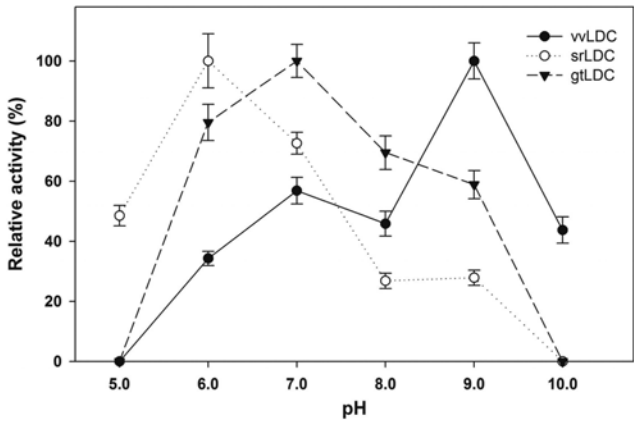

Fig. 2. Activity profiles in various pH conditions for vvLDC (●), srLDC (○) and gtLDC (▼). Relative activity was expressed based on the maximum activity of each LDC. The experiments were carried out in.

ues for srLDC and gtLDC were estimated as pH 6.0 and pH 7.0, respectively, the optimum pH of vvLDC was identified as pH 9.0 (Fig. 2). The vvLDC retained a relative activity of 45.8% and 43.7% in pH 8.0 and 10.0, respectively, and 0% and 34.2% in pH 5.0 and pH 6.0 (Fig. 2). The results indicate that vvLDC has higher activity for L-lysine decarboxylation in alkaline conditions than it does in acidic conditions. The optimum pH values of other LDCs from various sources are presented in Table 1. Because most LDCs have their maximal activity in neutral to acidic conditions, it is notable that the optimum pH for vvLDC is pH 9.0, which could be an important point for utilizing the enzyme in alkaline conditions.

3. Substrate Specificity of vvLDC

It has been reported that vvLDC has catalytic activity not only with L-lysine (C6) but also with L-ornithine (C5) [22]. In addition to these C5 and C6 compounds, in this study, the substrate specificities of vvLDC with l-2,3-diaminopropionic acid (C3), L-2,4-diaminobutyric acid (C4), and 2,7-diaminoheptanoic acid (C7) were investigated. However, it was found that vvLDC cannot catalyze the decarboxylation reaction with the C3, C2 and C7 compounds (Table 2). Also, the conversion rate of L-ornithine to putrescine is higher than that of L-lysine to cadaverine (Table 2). The results indicate that it is reasonable for vvLDC to be classified as part of the ornithine/lysine decarboxylase family.

Although vvLDC exhibits higher substrate preference toward L-orrnithine than L-lysine, it is no problem with the enzyme in the application of *in vitro* enzymatic production of cadaverine from L-lysine. In general, only the L-lysine is supplied as substrate, but no L-ornithine is required for the *in vitro* reaction. On the other hand, Lee et al. intensively investigated the substrate preference of nine LDC/ODC enzymes, including vvLDC [22]. According to their results, vvLDC showed the highest substrate preference toward L-lysine compared to the other LDC/ODC enzymes reported in the reference; the substrate preference ratio (Orn/Lys) for vvLDC was 42-670 times lower than the other eight LDC/ODC enzymes [22].

4. Enzymatic Conversion of L-lysine to Cadaverine in Alkaline Conditions

The enzymatic conversion of L-lysine to cadaverine using the three LDCs was performed in alkaline conditions of pH 8.0, 9.0 and 10.0 (Fig. 3). The conversion rate of srLDC (24.2%) at pH 8.0 was 1.4-fold higher than that of vvLDC (17.8%) and 2.8-fold higher than that of gtLDC (8.7%). In pH 9.0, however, the conversion rate

Table 1. Optimum pH values of lysine decarboxylases from various sources

Source of LDC	Optimum pH	Remarks	Reference
Lactobacillus saerimneri 30a	5.2		[13]
Vibrio parahaemolyticus	5.5		[23]
Escherichia coli	5.7		[14,24]
Selenomonas ruminantium	6.0		[16,18]
Selenomonas ruminantium	6.0	Recombinant expression in *E. coli*	This study
Burkholderia sp.	6.0		[17]
Escherichia coli	6.2	Second LDC in *E. coli*	[19]
Geobacillus thermodenitrificans	7.0	Recombinant expression in *E. coli*	This study
Glycine max	7.5		[20]
Vibrio vulnificus	9.0	Recombinant expression in *E. coli*	This study

Table 2. Substrate specificity of lysine decarboxylases from *Vibrio vulnificus*

Substrate candidates	Number of carbons	Relative activity (%)*
2,3-Diaminopropionic acid	C3	n.d.**
2,4-Diaminobutric acid	C4	n.d.
2,5-Diaminopentanoic acid (L-ornithine)	C5	100
2,6-Diaminohexanoic acid (L-lysine)	C6	58
2,7-Diaminoheptanoic acid	C7	n.d.

*Relative activity was expressed relative to the maximum activity of vvLDC with the substrate candidate tested in this study
**n.d.: not detectable

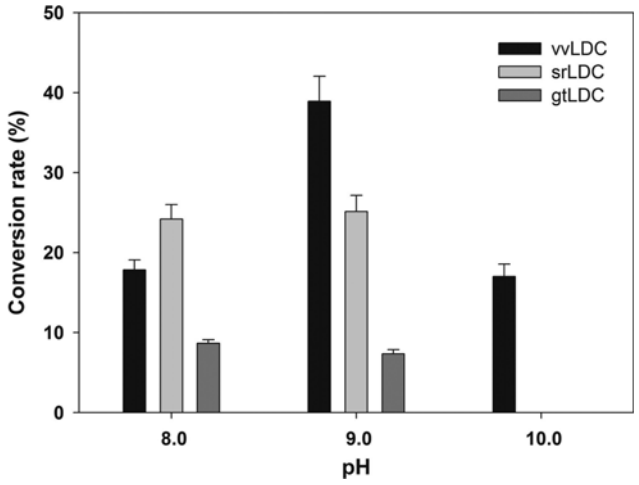

Fig. 3. Enzymatic conversion rate of L-lysine to cadaverine with vvLDC, srLDC and gtLDC in alkaline conditions. The conversion rate was measured from the molar ratio of cadaverine converted from 100 mM of L-lysine. The experiments were carried out in triplicate.

of vvLDC (38.9%) was 1.5-fold higher than that of srLDC (25.1%) and 5.3-fold higher than that of gtLDC (7.3%). Furthermore, only vvLDC retained catalytic activity in pH 10.0, whereas srLDC and gtLDC resulted in no conversion. The results indicate that vvLDC is more advantageous than srLDC and gtLDC in the enzymatic conversion of L-lysine to cadaverine in the alkaline conditions of pH 9.0 and 10.0. The use of LDCs, which exhibit maximal activity in alkaline conditions, could be a probable solution to the pH increase problem in the enzymatic conversion of L-lysine to cadaverine. Therefore, the discovery and application of alkaliphilic LDCs, such as vvLDC, could be a starting point to solve the problem of the increasing pH during the enzymatic reaction system.

Lee et al. previously investigated the catalytic performance of vvLDC by measuring the kinetic parameters of the enzyme [22]. They used a coupled enzyme system by indirectly measuring the concentration of CO_2 generated in the decarboxylation reaction. In this study, however, the concentration of the target product, cadaverine, was directly measured by HPLC, with which the applicability of vvLDC in alkaline conditions could be directly evaluated.

CONCLUSION

Cadaverine, which can be used as a monomer of bio-polyamides, can be produced from enzymatic decarboxylation of L-lysine by LDC. However, the increase in pH of the reaction solution during the enzymatic process could be problematic. In this study, the catalytic characteristics of three LDCs from *Selenomonas ruminantium*, *Vibrio vulnificus*, and *Geobacillus thermodenitrificans* were investigated. Whereas the optimum pH values for srLDC and gtLDC were determined as pH 6.0 and pH 7.0, respectively, vvLDC exhibited its maximal activity in pH 9.0. The enzymatic conversion of L-lysine to cadaverine using the three LDCs was performed in alkaline conditions, and it was found that vvLDC showed a higher conversion rate than did srLDC and gtLDC in alkaline conditions.

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