Journal of Molecular Catalysis B: Enzymatic xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Characterization of a new lysine decarboxylase from *Aliivibrio salmonicida* for cadaverine production at alkaline pH

Fengyu Kou[a,b,1], Jing Zhao[b,c,1], Jiao Liu[b,c], Jie Shen[c], Qin Ye[a], Ping Zheng[b,c], Zhimin Li[a,**], Jibin Sun[b,c,*], Yanhe Ma[c]

[a] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China*
[b] *Key Laboratory of Systems Microbial Biotechnology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*
[c] *Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*

ARTICLE INFO

Article history:
Received 10 August 2016
Received in revised form
29 November 2016
Accepted 29 November 2016
Available online xxx

Keywords:
Lysine decarboxylase
Cadaverine
Biotransformation
Characterization
Aliivibrio salmonicida

ABSTRACT

Lysine decarboxylases (LDCs) catalyze the conversion of L-lysine to cadaverine, a highly attractive building block for bio-based polyamides. Due to economic and environmental concerns, LDCs active at elevated pH are highly desirable. In this study, a new LDC from *Aliivibrio salmonicida* (AsLdc) was discovered, expressed, and characterized. Compared to the LDCs from *Escherichia coli*, LdcC and CadA, the latter was frequently used for cadaverine production, the purified AsLdc showed much higher activities at alkaline pH 7.0–8.5, for instance, 205.1 U/mg at pH 7.5 with 10 μg/mL enzyme, in comparison to 68.3 and 51.5 U/mg for CadA and LdcC, respectively. The activities of AsLdc and CadA correlated well with the proportions of decamers at the pH range of 5.0–8.5. AsLdc with a melting temperature of 79 °C was more thermostable than CadA (73.6 °C). When used for whole-cell biotransformation of L-lysine to cadaverine at pH 7.5, AsLdc completed the transformation within 7 h while the CadA did only 82.8%. These results indicate the high potential of the new AsLdc for the industrial production of cadaverine.

1. Introduction

Lysine decarboxylases (LDCs; EC 4.1.1.18) are the key enzymes that catalyze the decarboxylation of L-lysine to form cadaverine (1, 5-diaminopentane) [1]. These enzymes require pyridoxal-5′-phosphate (PLP) as a cofactor to decarboxylate the α-carbonyl group of L-lysine [2]. The role of LDCs in cell growth and acid tolerance response has been well studied [3–6]. Till now, several LDCs from different bacteria (*e.g. Escherichia coli* [7,8], *Hafnia alvei* [9], *Burkholderia* [10], *Selenomonas ruminantium* [11,12], and *Vibrio parahaemolyticus* [13]) have been purified and characterized. For instance, *E. coli* has two types of LDCs: inducible CadA, also called LdcI [14], and constitutive LdcC [15]. Lane et al. determined the optimum pH and temperature of CadA (pH 5.5; 52 °C) and LdcC (pH 7.6; 52 °C) using cell-free extracts [16]. Krithika et al. purified and characterized these two enzymes, and they focused on the kinetic parameters of CadA (K_m: 0.27 mM; V_{max}: 8.148 nmol cadaverine/min/μg) and LdcC (K_m: 0.84 mM; V_{max}: 27.21 nmol cadaverine/min/μg) [17].

In recent years, LDCs have attracted great interests due to their application in bio-synthesis of cadaverine, which is a highly promising building block for polyamides (nylon) production [18–20]. The mechanical properties of bio-polyamides, *e.g.* PA510, even surpass those of commercial PA6 and PA66 in some aspects [21]. Bio-based polyamides from renewable feedstocks could be an alternative and replacement for polyamides derived from petrochemicals [22].

The bio-production of cadaverine can be achieved through biotransformation of L-lysine by LDCs [23,24] or fermentation of glucose and other sugars with cell factories such as engineered *E. coli* or *Corynebacterium glutamicum* [25–28]. The biotransformation of L-lysine by LDCs has gained more attention in the last few years since the conventional production process of L-lysine is very well established, the price of lysine is very low and the serious overcapacity situation of lysine industry is eager to new application for lysine. In addition, the direct biotransformation process is expected more preferable than fermentative process since cadaverine is very toxic to the cells. Kim et al. reported that 80% lysine was converted to cadaverine with a recombinant *E. coli* strain overexpressing CadA when high concentration of L-lysine up to 1.75 M was applied [29]. Wang et al. constructed recombinant *E. coli* cells co-

* Corresponding author at: Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China.
** Corresponding author.
E-mail addresses: ZhiminLi@ecust.edu.cn (Z. Li), Sun_jb@tib.cas.cn (J. Sun).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.molcatb.2016.11.023
1381-1177/

overexpressing CadA and CadB (lysine/cadaverine antiporter), and a cadaverine titer of 221 g/L with a molar yield of 92% from lysine was finally obtained after optimization of gene expression and L-lysine feeding strategy [30]. Noteworthy, the direct fermentation of cadaverine from sugar with cell factories is often less efficient. For instance, Mimitsuka et al. reported that the cadaverine production was achieved at a titer of 2.6 g/L and molar yield of 9.1% (glucose to cadaverine) using a metabolically engineered *C. glutamicum* in which the L-homoserine dehydrogenase gene (*hom*) was replaced with *cadA* from *E. coli* [31]. The highest level achieved by fermentation process was 103 g/L from xylose and molar yield of 32%, significantly lower than biotransformation process [32]. Non-specific inhibition of the cells by cadaverine is at least partially the reason. It is still a long way to engineer cell factories to sustain the high concentration of cadaverine and economically produce it. Biotransformation of L-lysine is still the industrial choice for cadaverine production.

Since cadaverine is a strong base which is continuously released during the biotransformation production of L-lysine, acid is usually added to maintain the reaction condition close to the optimal pH of the enzyme. Such process is economically unfavorable due to the use of inorganic acids and environmentally unfriendly due to the production of inorganic salts. Nishi et al. proposed an interesting alternative by adding an organic dicarboxylic acid which can be co-crystalized and used for co-polymerization process later together with cadaverine [33]. However, due to the difficulties in removal of tiny organic acid impurities by this approach, the conventional inorganic acid neutralization/distillation process is still dominant. In addition, industrial enzymes often require good thermostability to reduce the dosage of enzyme and prolong their effective lifetime [34,35]. Thus, a LDC with high activity at alkaline solution and high thermostability would be highly desirable. Unfortunately, most of the existing LDCs cannot satisfy these requirements. For instance, Lemonnier and Lane reported that the widely used CadA from *E. coli* has an optimum pH of 5.5, and it loses almost all the activity at pH 8.0 [16]. LdcC has a relatively higher pH optimum (7.6), but it was inhibited as temperature increases and progressively inactivated with incubation above 37 °C. They have reported that LdcC retains less than 60% of its activity when incubated at temperatures higher than 60 °C for 15 min, while CadA was very stable after heating for 15 min at 60 °C [16]. In this study we decided to find stable LDCs with higher activity at alkaline pH to meet the industrial demand for the green and economic production of cadaverine.

2. Materials and methods

2.1. Strains and chemicals

Chemically competent *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from TransGen Biotech (Beijing, China). Plasmid pET-21a(+) for expression was obtained from Genewiz (Suzhou, China). All chemicals were at analytical grade or higher and purchased from Sigma-Aldrich or AppliChem unless specified. Phusion DNA polymerase, T4 DNA ligase and restriction enzymes were obtained from New England Biolabs, and all oligonucleotides were from Genewiz (Suzhou, China) in salt-free, lyophilized forms. The DNA gel extraction kit and plasmid extraction kit were from Tiangen (Shanghai, China). Bis-Tris protein gels and BCA protein assay kit were from Thermo Scientific. His-trap column was obtained from GE Healthcare Life Sciences (Tianjin, China).

2.2. Cloning lysine decarboxylase-encoding gene

The genomic DNA of *E. coli* K12 MG1655 was extracted and purified using the TIANamp Bacteria DNA Kit (Tiangen, Shanghai). The *cadA* and *ldcC* genes were amplified by polymerase chain reaction (PCR) using primers flanked with the *Nde*I and *Xho*I restriction sites and Phusion DNA polymerase. The amplified DNA fragments were digested and inserted into pET-21a(+) expression vector. Twenty-four putative LDC genes (Table S1) discovered by genome mining were synthesized chemically (Genewiz, Suzhou, China), and ligated to pET-21a(+) expression vector at the same site. All the 26 gene products fused with His-tag at their C-terminal. All the resulted plasmids were then transformed into *E. coli* BL21 (DE3) or *E. coli* Rosetta (DE3) cells for expression.

2.3. Expression and purification of LDCs

The positive transformants were cultivated in LB medium supplemented with ampicillin (100 μg/mL) at 37 °C, 220 rpm. When OD_{600} of the culture reached 0.6–0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the temperature was lowered to 20 °C for 20 h. The cells were then harvested by centrifugation (4 °C, 4000*g*, 20 min), and used for enzyme study or biotransformation. The cell pellets were re-suspended in lysis buffer A (20 mM phosphate buffer, 150 mM NaCl, 1 mM dithiotreitol (DTT), 0.1 mM PLP, 10 mM imidazole, pH 7.4) prior to disruption by sonication (scientz-IID, Ningbo Scientz Biotech. Co. Ltd). After cell disruption, the cellular debris was removed by centrifugation at 13,000*g* and 4 °C for 30 min. The resulting supernatant was loaded onto a Ni-NTA column (5 mL, GE Healthcare Corp) equilibrated with buffer A, and the retained protein was eluted with an increasing gradient from 100 to 500 mM imidazole in buffer A at a flow rate of 1 mL/min. The purity of the collected fractions was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the pure enzyme were combined and desalted using Amicon Ultra-4 centrifugal concentrator (10 kDa) at 4 °C. The purified LDCs were stored in storage buffer (20 mM phosphate buffer, 150 mM NaCl, 0.1 mM PLP, 5% glycerol, pH 7.4) in small aliquots at −80 °C, and each aliquot was used for test only once after thawing.

2.4. Enzyme activity assay

The activities of LDCs were measured as described previously with some modifications [36]. The reaction mixture in microtiter plate (MTP) per well was prepared by addition of 50 μL of buffer B (100 mM phosphate buffer, 0.1 mM PLP, 1 mM DTT, 150 mM NaCl) containing 10 μg/mL LDC to 50 μL of substrate solution (buffer B plus 20 mM L-lysine). The MTP was then incubated at 37 °C for 5 min at 900 rpm. To stop the reaction, 30 μL of reaction mixture was mixed with 70 μL of 1 M Na_2CO_3. Subsequently, 50 μL of 2, 4, 6-trinitrobenzenesulfonic acid (TNBS; 20 mM) was added, and samples were incubated at 42 °C for 5 min to allow the formation of *N*,*N*′-bistrinitrophenyl-cadaverine (TNP-cadaverine) and *N*,*N*′-bistrinitrophenyl-lysine (TNP-lysine) adducts. TNP-cadaverine was extracted using 500 μL toluene, and the absorbance at 340 nm was measured. One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 μmol of cadaverine per minute under the assay conditions. The specific activity of a LDC was calculated by dividing the activity by the milligrams of enzyme. Protein concentration was determined with the BCA Protein Assay Kit (Thermo Scientific).

2.5. Effect of pH on the activity and stability of LDCs

The effect of pH on enzyme activity was determined with buffer B with various pH values in the range of 5.0–8.5 at 37 °C. The specific activities of LDCs were measured using the standard enzyme assay. The pH stability of LDCs were investigated by incubating the purified enzymes (10 μg/mL) in the buffer B at various pH values

Please cite this article in press as: F. Kou, et al., Characterization of a new lysine decarboxylase from *Aliivibrio salmonicida* for cadaverine production at alkaline pH, J. Mol. Catal. B: Enzym. (2016), http://dx.doi.org/10.1016/j.molcatb.2016.11.023