Process Biochemistry 44 (2009) 460–465

Contents lists available at ScienceDirect

Process Biochemistry

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

Biochemical and molecular characterization of a novel high activity creatine amidinohydrolase from *Arthrobacter nicotianae* strain 02181

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ARTICLE INFO

Article history:
Received 29 July 2008
Received in revised form 12 November 2008
Accepted 17 December 2008

Keywords:
Creatinase
CODEHOP PCR
Genome walking
Nucleotide sequence
Arthrobacter

ABSTRACT

A high activity creatine amidinohydrolase (creatinase) from *Arthrobacter nicotianae* 02181 (a strain newly isolated from soil which may utilize creatinine as the unique organic source) was purified, characterized and the creatinase gene was cloned and analyzed in this study. Cells were cultivated under optimized condition for enzyme yield and creatinase was purified by the DEAE-cellulose and hydroxylapatite (HA) chromatography. The creatinase was found to be a dimmer formed by two identical subunit of 46.4 kDa, and the specific activity of the purified creatinase reached 124.44 U/mg protein, which was about 13 folds of the maximum value ever reported. The enzyme was found to be most active at 37 °C (pH 7.0), and it was found to be relatively stable bellow 45 °C around pH 7.0 by fluorescence spectroscopy and circular dichroism (CD) analysis. The activity of this creatinase could be significantly inhibited by Cu^{2+}, Hg^{2+}, $Fe^{3+}$and SDS, and it could be improved by Ca^{2+} and NaN_3.The creatinase gene was cloned by the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) PCR and the genome walking method. Nucleotide sequence analysis of this gene revealed an open reading frame (ORF) of 1254 base pair (bp) encoding a 417 amino acid (aa) protein. The primary amino acid sequence alignment search in the database revealed a moderate homology between the deduced amino acid sequence and other creatinase. The sequence has been submitted to Genbank with the accession number EU004199.

1. Introduction

Creatine amidinohydrolase (creatinase, EC 3.5.3.3) is an important enzyme for creatinine concentration determination in biological fluids to evaluate renal damage. It was first characterized in 1950 by Roche et al. [1], who made the finding of the phenomenon that the creatine was metabolized to urea in two *Pseudomonads* strains (*Ps. eisenbergii* and *ovalis*). This phenomenon was explained by Appleyard and Woods [2] through the illumination of the creatine catabolism pathway in *Ps. pvalis.* Yoshimoto et al. [3] purified and crystallized the creatinase from *Ps. putida*, and later on the creatinase active sites were deduced on base of the crystal model [4,5]. The active sites were found to be conserved in creatinases from different microorganisms, while residues in the N terminal was found to determine the specific activity level. The highest specific activity of purified native creatinase ever reported was about 9 U/mg protein, which may be improved up to 16 U/mg protein by a modified cell split method in *Escherichia coli* (*E. coli*) [6]. Recent researches mainly focused on the detection of creatinine by biosensors [7], while reports about newly discovered creatinase was only a few.

As an enzyme for clinical applications, activity is an important property. In previous work, an *Arthrobacter nicotianae* strain 02181 that produced high activity creatinase was isolated in our laboratory. We purified and characterized the creatinase from *A. nicotianae* 02181 in the present study, and the creatinase gene was cloned for a better understanding of its high activity. To our best knowledge, the activity of this creatinase is much higher than those from other microorganisms ever reported, and this research may contribute to both clinical applications and deeper understanding of the mechanism involved in enzyme activity.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strain 02181 was isolated from soil using creatinine as the unique organic source. Sample collected were plated onto solid agar plate containing (g/l): creatinine 5, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 1.0, KCl 5.0, glucose 20 and agar 15. Plates

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doi:10.1016/j.procbio.2008.12.014

Table 1
Mediums used to determine essential organics for cell growth and enzyme yield.

Mediums	Components(1 l)
MA	Basic salts[a] + creatinine 5.0 g
MB	Basic salts + peptone 5.0 g
MC	Basic salts + creatinine and peptone 5.0 g each
MD	Basic salts + creatinine and sodium nitrate 5.0 g each
ME	Basic salts + creatinine 5.0 g and glucose 20.0 g
MF	Basic salts + sodium nitrate 5.0 g and glucose 20.0 g
MG	Basic salts + creatinine and peptone 5.0 g each, glucose 20.0 g

[a] Basic salts consist of $MgSO_4{\cdot}7H_2O$ 0.5 g, K_2HPO_4 1.0 g and KCl 5.0 g.

were incubated at 28 °C until colonies reached 2 mm. Single colonies were inoculated into liquid medium (M1, as same as solid plate but agar) separately for 24 h incubating at 28 °C and those that may produce urea were picked out for another round of screening. Finally, a strain with the highest urea production (02181) was obtained. Based on morphological and physiological characteristics and 16S rRNA gene homologies, it was identified to be a member of the genus *A. nicotianae*.

E. coli DH5α strain (Novagen) and plasmid *pGEM*-T (Promega) were used for gene cloning.

2.2. Cultivation and media

A. nicotianae 02181 was cultivated in medium M1 under temperatures ranged from 25 to 35 °C to determine the optimized temperature for enzyme production. It was also cultivated in mediums (MA–MG, Table 1) consisted of different organic sources to determine what was essential for enzyme activity and productivity.

2.3. Chemicals and reagents

Creatine, creatinine, lysozyme, *Pfu* DNA polymerase and streptomycin were purchased from Promega. Restriction enzymes were from TOYOBO. Glutathione, 2-diethylaminoethyl-cellulose (DEAE-cellulose) was purchased from Sigma; hydroxylapatite (HA) was purchased from Shanghai Bio Life Science and Technology Ltd. GSTrap™FF column was a product of Amersham. Oligonucleotide primers were synthesized by Invitrogen Co., Ltd. (China).

2.4. Creatinase activity assay

Specific enzyme activity of creatinase was determined according to the method set up by Yoshimoto et al. [3] 0.1 ml enzyme solution was added into a 0.9 ml 50 mM potassium phosphate buffer (PBS, pH7.0) containing 100 mM creatine as substrate. After 10 min incubation at 37 °C, 2.0 ml *p*-dimethylaminobenzaldehyde solution (2.0 g *p*-dimethylaminobenzaldehyde dissolved in 100 ml dimethylsulfoxide, then 15 ml HCl stock added) was added to stop the reaction by incubation at 25 °C for 20 min and absorbance was measured against the blank at 435 nm. Protein concentration was determined by the method described by Lowry et al. [8]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the synthesis of 1 μmol of the urea per minute under the conditions described in this section.

2.5. Purification of native creatinase

After cultivation, cells were resuspended with a buffer containing 3 mM EDTA-Na_2, 23 mM K_2HPO_4 and 7.6 mM NaN_3 (pH 7.2) and lysed by lysozyme and a subsequent supersonic operation. Cell lysate was centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was removed into a new tube. Then streptomycin was added to 200 mg/ml. To remove nucleic acids, polycoses and somatic proteins, and supernatant after a centrifugation at 13,000 rpm for 30 min at 4 °C was loaded onto a DEAE-cellulose column preequilibrated with binding buffer (50 mM PBS, pH 7.2). The column was washed with NaCl in linear gradient (0–1 M) and creatinase activity of eluted fractions was tested. Fractions showing creatinase activity were mixed together and applied to the HA column preequilibrated with 10 mM PBS (pH 7.2), and then the column was washed with K_3PO_4 in linear gradient (20–400 mM). Eluted fractions were collected and analyzed by sodium dodecyl sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and PAGE, and specific creatinase activities were tested separately.

2.6. Kinetics parameters

The purified enzyme was incubated with different concentrations of creatine (10–100 mM) in 100 mM PBS (pH 7.2) at 37 °C, and enzyme activity was measured at different spots of time. Each reaction was performed in triplicate and the reaction rates obtained were fitted to Michaelis–Menten kinetics.

2.7. Effect of pH and temperature on enzyme activity and stability

The purified creatinase was dialyzed against 50 mM citrate buffer (pH 3.0–6.0), 50 mM PBS (pH 6.0–8.0) or 50 mM borate buffer (pH 8.0–10.5), and then enzyme activities were tested according to the method described in Section 2.4 with pH adjusted correspondingly to determine the optimum pH. Enzyme stability in these buffers was evaluated by fluorescence spectroscopy and circular dichroism (CD) assay. In the fluorescence spectroscopy assay, the creatinase was diluted to 5 μg/ml with corresponding buffers and then the fluorescence emissions were scanned from 300 to 500 nm under the excitation wavelength of 280 nm. In the CD assay, the concentration of creatinase was 50 μg/ml and the wavelength was 190–260 nm.

The optimum temperature for creatinase activity was determined by testing enzyme activity at temperatures from 15 to 60 °C according to Section 2.4, and effect of temperatures on enzyme stability was studied by fluorescence spectroscopy and CD assay. Briefly, the purified creatinase was diluted with 50 mM PBS (pH 7.0) to 5 and 50 μg/ml for fluorescence spectroscopy and CD assay, respectively, and tests were taken using the same wavelength as that in pH assay after having incubated for 15 min from 4 to 80 °C.

2.8. Effect of inhibitors and metal ions on enzyme activity

The effect of enzyme inhibitors were studied in 50 mM PBS (pH 7.0) containing SDS, Brij35, Triton X-100 or NaN_3 et al at a final concentration of 5 g/l or EDTA at 1 mM, respectively. Purified enzyme was pre-incubated with inhibitors at 37 °C for 30 min and the residual activity was measured by the method described in Section 2.4. The influence of various metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, Fe^{3+}, Hg^{2+} and Cu^2) was studied by testing residual enzyme activity after incubating for 30 min in 50 mM PBS (pH 7.0) containing 1 mM metal ion at 37 °C.

2.9. Cloning of creatinase gene by CODEHOP PCR and genome walking

Genomic DNA of *A. nicotianae* 02181 was prepared by the CTAB method [9]. Oligonucleotide primers in CODEHOP PCR (P1-1, P1-2, P2-1, P2-2, P3-1 and P3-2, Table 2) were designed according to conservative gene blocks by the program CODEHOP[2] following the method described by Rose et al. [10]. The PCR procedure was as follows: initial denaturation at 95 °C for 2 min and 30 cycles of 90 s at 95 °C, 60 s at 55 °C, 60 s at 72 °C, followed by additional 5 min at 72 °C. DNA fragments were recovered, subcloned in to the *pGEM*-T vector and sequenced. After a partial creatinase gene obtained, specific primers (GSP1u, GSP1d, GSP2u and GSP2d, Table 2) were designed for subsequent genome walking [11–13] using the BD GenomeWalker™ Universal Kit (Takara) and BD Advantage™ II PCR Kit (Takara). Genomic DNA of 02181 was digested by restriction enzymes DraI, PvuII, EcoRV or StuI, respectively to serve as templates for the primary and the secondary PCR amplification. The primary PCR procedure was as follows: initial denaturation at 95 °C for 2 min, 7 cycles of 25 s at 95 °C, 3 min at 72 °C, and 32 cycles of 25 s at 95 °C, 3 min at 67 °C. Primary PCR products were diluted 50 times as templates for the secondary PCR. The secondary PCR was similar to the primary PCR except for a reduction of 2 and 12 in cycle numbers. All PCR products were analyzed by electrophoresis in 1.5% agarose gels and DNA fragments of interest were cloned into *pGEM*-T vector and sequenced.

2.10. DNA sequence analysis

DNA sequencing was carried out with the dideoxy-chain termination method [14] by using an ABI Prism 377 Genetic Analyser (Applied Biosystems). DNAStar (Lasergene), Vector NTI 8.0 (InforMax Inc.) and Primer Premiers 5.0 (PREMIER Biosoft international) software were employed to analyze the DNA sequence.

2.11. Nucleotide sequence accession number

The nucleotide sequence reported in this study has been submitted to the GenBank database with the accession number EU004199.

3. Results

3.1. Effect of cultivation conditions on enzyme activity

Studies were first carried out to optimize the medium for the growth and the production of enzymes, and the most appropriate medium (MC) for the protease production is composed of (g/l): $MgSO_4{\cdot}7H_2O$ 0.5, K_2HPO_4 1.0, KCl 5.0, creatinine 5.0 and peptone 5.0. It was very interesting to notice that *A. nicotianae*

[2] The program was provided on http://bioinformatics.weizmann.ac.il/blocks/blockmkr/www/make_blocks.html.