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Cloning, expression and characterization of D-aminoacylase from *Achromobacter xylosoxidans* subsp. *denitrificans* ATCC 15173

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ABSTRACT

D-Aminoacylase catalyzes the conversion of *N*-acyl-D-amino acids to D-amino acids and fatty acids. The aim of this study was to identify the D-aminoacylase gene from *Achromobacter xylosoxidans* subsp. *denitrificans* ATCC 15173 and investigate the biochemical characterization of the enzyme. A previously uncharacterized D-aminoacylase gene (*ADdan*) from this organism was cloned and sequenced. The open reading frame (ORF) of *ADdan* was 1467 bp in size encoding a 488-amino acid polypeptide. *ADdan*, with a high amino acid similarity to *N*-acyl-D-aspartate amidohydrolase from *Alcaligenes* A6, showed relatively low sequence similarities to other characterized D-aminoacylases. The recombinant *ADdan* protein was expressed in *Escherichia coli* BL21 (DE3) using pET-28a with a T7 promoter. The enzyme was purified in a single chromatographic step using nickel affinity gel column. The molecular mass of the expressed protein, calculated by SDS–PAGE, was about 52 kDa. The purified *ADdan* showed optimal activity at pH 8.0 and 50 °C, and was stable at pH 6.0–8.0 and up to 45 °C. Its activity was inhibited by Cu^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and Hg^{2+}, whereas Mg^{2+} had no significant influence on this recombinant D-aminoacylase. This is the first report on the characterization of D-aminoacylase with activity towards both *N*-acyl derivatives of neutral D-amino acids and *N*-acyl-D-aspartate. The characteristics of *ADdan* could prove to be of interest in industrial production of D-amino acids.

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1. Introduction

D-Amino acids are important intermediates in the preparation of semi-synthetic antibiotics (Tournaire et al., 1994), therapeutic drugs (Ferraris and Tsukamoto, 2011) pesticides (Tang et al., 1998), and bioactive peptides (Yukio and Suzuki, 1981). They occur in microorganisms, plants and animals and their physiological roles have been investigated (Wakayama et al., 2003). It has been reported that D-amino acids can be introduced into the bacterial cell wall and have regulatory roles in the bacterial kingdom (Lupoli et al., 2011; Cava et al., 2011). D-Amino acids, such as poly-D-leucine and poly-D-lysine, can be used in the production of bioactive peptides composed entirely of these isomers (Martínez-Rodríguez et al., 2010). Recently, an increasing number of studies on natural D-amino acid-containing compounds have been reported. For example, nateglinide, a D-phenylalanine derivative lacking either a sulfonylurea or benzamido moiety, is used in diabetes type-2 treatment (Chachin et al., 2003). *N*-acyl-D-amino acid amidohydrolase (D-aminoacylase, EC 3.5.1.81) is an important enzyme for industrial applications and liberates D-amino acids from the corresponding *N*-acyl-D-amino acids. They have been found in a variety of microorganisms including *Alcaligenes* (Moriguchi and Ideta, 1988; Moriguchi et al., 1993), *Pseudomonas* (Sakai et al., 1991), *Variovorax* (Lin et al., 2002), *Stenotrophomonas* (Muniz-Lozano et al., 1998), *Streptomyces* (Sugie and Suzuki, 1978), *Bordetella* (Cummings et al., 2009), *Defluvibacter* (Kumagai et al., 2004) and *Microbacterium natoriense* (Liu et al., 2005, 2012).

N-Acyl-D-amino acid amidohydrolase, which possesses strict substrate recognition, is classified into three types according to substrate specificity (Wakayama and Moriguchi, 2001). D-Aminoacylase is capable of catalyzing the hydrolysis of *N*-acyl derivatives of neutral D-amino acids. *N*-Acyl-D-glutamate amidohydrolase and *N*-acyl-D-aspartate amidohydrolase are specific for *N*-acyl-D-glutamate and *N*-acyl-D-aspartate, respectively. Interestingly, L-aminoacylases from *P. maltophila* Bl, *B. Stearothermophilus*, and porcine kidney catalyze the hydrolysis of *N*-acyl derivatives of both various neutral L-amino acids and L-glutamate while the enzymes from *Aspergillus oryzae* and *Bacillus thermoglucosidius* were only specific for *N*-acyl derivatives of various neutral L-amino acids (Wakayama et al., 1997).

In the present study, the gene (*ADdan*) encoding for D-aminoacylase from *Achromobacter xylosoxidans* subsp. *denitrificans* was cloned and sequenced. *ADdan* displayed a high sequence identity (78%) to *N*-acyl-D-aspartate amidohydrolase from *Alcaligenes*

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Table 1
Primers used in this study.

Primer	Sequence(5′→3′)
danF	GGCTTCATCGACTCGCACACSCA
danR	CATSACYTTGTGGTGCGARATMA
danF1	GACGGCTACACSRTCGGCTACGGCAAC
danR1	GCGTTTCCACCGGCACCTTCTAC
danF2	GCGCCAGGCTGATCCCGCAGTTG
danR2	GTCGGGRATGCGSGGRTCKTYGC
dan1	CATATGACCGCATCCCCCCTGCAGGCAGAC
dan2	AAGCTTTTATTCACTCCGAGGTAATGCC

Restriction sites were underlined.

A6 (Wakayama et al., 1995) and only low sequence similarities to all other D-aminoacylases which are specific for neutral D-amino acids. The work reported here also describes the successful expression of active *ADdan* from *A. xylosoxidans* subsp. *denitrificans* ATCC 15173 in *Escherichia coli* to characterize the enzymatic properties such as substrate specificity and stability. The purified recombinant *ADdan* displayed the highest preference for *N*-acyl-D-methonine, and unlike many other D-aminoacylases, it showed activity towards *N*-acyl-D-aspartate, a *N*-acyl derivative of acidic D-amino acid. The enzymatic properties of *ADdan* from this strain were of great importance because there have been no reports of D-aminoacylase which acted on *N*-acyl-D-aspartate thus far.

2. Materials and methods

2.1. Materials and regents

Restriction enzymes, r*Taq* polymerase and T4 DNA ligase, were purchased from TaKaRa Biotech Co., Ltd (Dalian, China). Isopropyl-*b*-D-thiogalactoside (IPTG) was purchased from Sigma (USA). The molecular weight marker for SDS–PAGE was purchased from TaKaRa Biotech Co., Ltd. (Dalian, China). Substrates and standards were purchased from Sigma. All other chemicals were analytical grade.

2.2. Bacterial, plasmids and conditions

A. xylosoxidans subsp. *denitrificans* ATCC 15173 was used as a source for genomic DNA. It was grown at 30 °C in medium containing 1% peptone, 0.3% beef extract, 0.5% NaCl (pH 7.0). *E. coli* DH5α and *E. coli* BL21 (DE3) grown at 37 °C in Luria–Bertani medium were employed for gene cloning and expression. Luria–Bertani medium containing 100 μg/mL ampicillin or 50 μg/mL kanamycin (Sigma, USA) was used for plasmid maintenance. Two plasmids, pEASY^{TM}-T1 Simple Cloning Vector (Transgene, China) and pET-28a (Invitrogen, USA), were used as gene cloning and expression vectors, respectively. For protein expression, *E. coli* BL21 (DE3) containing recombinant plasmids was grown in Luria–Bertani medium supplemented with 50 μg/mL kanamycin. Isopropyl-b-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM for induction when an OD_{600} of 0.8 was reached.

2.3. Cloning and sequencing of D-aminoacylase gene

Different D-aminoacylases were aligned using DNAMAN (Lynnon Biosoft, USA) and primers danF and danR (Table 1) were designed from the conserved regions of the genes (Lin et al., 2002). A DNA fragment of the open reading frame (ORF) of D-aminoacylase was first amplified from the genomic DNA of *A. xylosoxidans* subsp. *denitrificans* ATCC 15173 with the following PCR conditions: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 54 °C for 40 s and 72 °C for 2 min, followed by an elongation at 72 °C for 10 min. Recombinant DNA technology was carried out as described elsewhere (Sambrook and Russell, 2001). For sequencing purpose, the amplified product was examined by electrophoresis, purified from gel then ligated into pEASY^{TM}-T1 Simple Cloning Vector (Transgene, China) and sequenced by Invitrogen Co., Ltd. (Shanghai, China). To obtain the remaining 5′ portion of D-aminoacylase gene (*ADdan*), primers danF1 and danR1 (Table 1) were designed from the partial *ADdan* sequence and the conserved regions of the upstream of the ORF. The amplified PCR product was then subcloned as previously described. The remaining 3′ portion of *ADdan* gene was obtained by primers danF2 and danR2 (Table 1) designed from the partial *ADdan* sequence and the conserved regions of the downstream of the ORF. The amplified PCR product was then subcloned as described above. The entire *ADdan* sequence was obtained by PCR with primers dan1 and dan2 (Table 1) designed from the beginning and end regions of the open reading frame. PCR was performed according to the following protocol: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 63 °C for 40 s and 72 °C for 2 min, followed by an elongation at 72 °C for 10 min. The PCR product was subcloned and sequenced as mentioned above. The plasmid pEASY^{TM}-T1 Simple Cloning Vector carrying the whole of the *ADdan* coding sequence was named p*EASY*-dan.

2.4. Nucleotide and amino acid sequence analysis

The sequence analysis was performed using the software package DNAMAN 5.0 (Lynnon Biosoft, USA) and homology was analyzed in the GenBank with the BLAST program (Altschul et al., 1990). The nucleotide sequence data of the isolated D-aminoacylase gene from *A. xylosoxidans* subsp. *denitrificans* ATCC 15173 were deposited in NCBI GeneBank database with the accession number JX993347.

2.5. Expression of D-aminoacylase in E. coli

A 1.5 kb fragment was released from p*EASY*-dan digested with *Nde*I and *Hin*dIII restriction enzymes, and then ligated with pET-28a(+) digested with *Nde*I and *Hin*dIII. The recombinant plasmid was designated as pET-dan and transformed into *E. coli* BL21 (DE3) for expression. Recombinant *E. coli* BL21 cells containing pET-dan were subcultured at 37 °C for 12 h in 10 mL of Luria–Bertani (LB) medium supplemented with 50 μg/mL kanamycin. The subculture was transferred into fresh medium and cultured until OD_{600} reached 0.6, and IPTG (final concentration 0.1 mM) was then added for induction. The bacterial cells were cultured with vigorous shaking at 20 °C for a further 20 h and then harvested by centrifugation at 8000 rpm for 10 min at 4 °C

2.6. Purification of D-aminoacylase

For D-aminoacylase purification, all operations were performed at 4 °C unless otherwise mentioned. The cells were harvested by centrifugation, washed with cold saline, and suspended in buffer A (50 mM Tris–HCl buffer, pH 7.8). Cells were homogenized by ultrasonic treatment. Supernatant was obtained as crude enzyme solution by centrifugation at 12 000 rpm and 4 °C for 20 min. The crude enzyme solution was loaded onto Ni-NTA agarose affinity resin (Qiagen, USA) pre-equilibrated with buffer A. The weakly bound protein was washed away from the column after extensive washing of the resin with buffer B (300 mM NaCl; 50 mM Tris–HCl buffer, pH 7.8; 20 mM imidazole). Then the recombinant fusion protein was eluted with buffer C (300 mM NaCl; 50 mM Tris–HCl buffer, pH 7.8; 100 mM imidazole) at a flow rate of 0.5 mL/min. The fractions were collected and the D-aminoacylase activity was analyzed. Fractions containing higher enzyme activity were pooled.

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