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Expression, purification, and characterization of arginine deiminase from *Lactococcus lactis* ssp. *lactis* ATCC 7962 in *Escherichia coli* BL21

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Abstract

The *arcA* gene that encodes arginine deiminase (ADI, EC 3.5.3.6)—a key enzyme of the ADI pathway—was cloned from *Lactococcus lactis* ssp. *lactis* ATCC 7962. The deduced amino acid sequence of the *arcA* gene showed high homology with the *arcA* gene from *Lactobacillus plantarum* (99%) and from *Lactobacillus sakei* (60%), respectively. The *arcA* gene from *Lc. lactis* spp. *lactis* ATCC 7962 was expressed in soluble fraction of recombinant *Escherichia coli* BL21. ADI produced from *Lc. lactis* spp. *lactis* ATCC 7962 (LADI) in *E. coli* BL21 (DE3) was purified using sequential Q-Sepharose anion exchange and Sephacryl S-200 gel filtration column chromatography. The final yield of LADI in the purification procedure was 63.5%, and the specific activity was 140.27 U/mg. The presence of purified LADI was confirmed by N-terminal sequencing and determination of the molecular mass. The LADI had a molecular mass of about 140 kDa, and comprised a homotrimer of 46 kDa in the native condition. LADI exhibited only 35% amino acid sequence homology with ADI from *Mycoplasma arginini*. However, LADI shared a similar three dimensional structure. The K_M and V_{max} values for arginine were 8.67 ± 0.045 mM (mean ± SD) and 344.83 ± 1.79 μmol/min/mg, respectively, and the optimum temperature and pH for the production of LADI were 60 °C and 7.2.

Keywords: Arginine deiminase; Arginine depletion enzyme; *Lactococcus lactis*; Lactic acid bacteria

Lactococcus lactis species are commonly used in the production of dairy products [1], and are the best characterized species among lactic acid bacteria (LAB)[1]. *Lc. lactis* comprises the three subspecies *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris*, and *Lc. lactis* ssp. *hordniae*, with only the first two being identified and used in dairy manufacture. These two subspecies are similar in phenotype and genotype, with one of their differences being in arginine utilization. *Lc. lactis* ssp. *lactis* hydrolyzes arginine because it has an arginine deiminase (ADI, EC 3.5.3.6) pathway [2], which *Lc. lactis* ssp. *cremoris* lacks [3]. The ADI pathway involves three enzymes: (1) arginine is hydrolyzed to citrulline and ammonia by ADI, (2) the citrulline is then converted to carbamoyl phosphate and ornithine by ornithine transcarbamylase (EC 2.1.3.3), and (3) finally the carbamoyl phosphate is used to phosphorylate ADP by carbamate kinase (EC 2.7.2.2), producing a single ATP molecule [4].

Arginine is involved in several biosynthetic pathways that significantly influence carcinogenesis and tumor biology (e.g., nitric oxide (NO) generation, creatine production, and polyamine synthesis). Therefore, ADI-degraded arginine has been regarded as a potential anticancer agent [5]. ADI from *Mycoplasma arginini* (MADI) has been suggested as a inhibitor of cell proliferation in hepatocellular, melanoma, leukemia, and prostate cancer cell lines [6–9] that are auxotrophic for arginine [10]. In addition, ADI has been shown to affect apoptosis and inhibit NO synthesis (i.e., antiangiogenic effects), and exert effects against tumor necrosis factor-α (TNF-α) and neutralize endotoxin [6,7,11–13]. Recently, the effects of the pegylated form of

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[1] *Abbreviations used:* LAB, lactic acid bacteria; NO, nitric oxide; TNF-α, tumor necrosis factor-α; LB, Luria–Bertani.

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ADI on hepatocellular carcinoma and melanoma have been investigated in phase I and II clinical studies [14,15].

We have previously found that the cytoplasmic fraction of *Lc. lactis* ssp. *lactis* exhibited the strongest antiproliferative activity in a human stomach cancer cell line (SNU-1)—and the strongest ADI activity—among six types of LAB tested [16], which was attributable to the induction of apoptosis. These results suggest that ADI was responsible for the antiproliferative activity. Until now, ADI from *Lc. lactis* (LADI) has not been studied using molecular biology. In this study, the gene that encodes ADI was cloned from *Lc. lactis*, and characterized on the basis of the enzyme activity.

Materials and methods

Strains and culture media

Lactococcus lactis ssp. *lactis* ATCC 7962 was used as the source of the *arcA* gene, and was cultured in M17 broth (Difco, USA) containing 0.5% glucose for 24 h at 30 °C. *Escherichia coli* BL21 (DE3) used as the expression host, and was incubated in LB (Luria–Bertani) broth at 37 °C for 12 h.

Cloning and sequence analysis of arcA

Genomic DNA of *Lc. lactis* ssp. *lactis* ATCC 7962 was obtained according to the method of Johansen and Kibenich [17]. The *arcA* gene was amplified from *Lc. lactis* ssp. *lactis* ATCC 7962 by PCR using primers (forward, 5′-gtaaagtgtagcataagtgc-3′; reverse 5′-cctaagctatcaatgaactt acg-3′) based on the *arcA* gene (NCBI Accession No. AE006433.1) of *Lc. lactis* ssp. *lactis* IL1403. PCR amplification comprising 30 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 2 min, and elongating at 72 °C for 3 min was performed using Ex *Taq* DNA polymerase (TaKaRa, Japan) using a commercial PCR system (2700, GeneAmp, USA). The amplified fragment was inserted into pGEM-T Easy (Promega, USA), with the resulting plasmid designated as pLADI. The nucleotide sequence was determined by the dideoxy chain-termination method [18]. The homologies of the nucleotide sequence and amino acid sequences were analyzed based on information from NCBI obtained using the BLAST program.

Expression of LADI

Escherichia coli BL21 (DE3) harboring pLADI was cultured at 37 °C in 500 ml of LB broth containing ampicillin (50 μg/ml) until the optical density at 600 nm (OD_{600}) was 0.5. LADI was induced by incubation with IPTG (1.0 mM) for 3.5 h at 30 °C, and the cells were harvested by centrifugation (Hanil Science, Korea) at 4000*g* for 15 min at 4 °C. The pellets were washed twice with 50 ml of 50 mM Tris buffer (pH 7.0). The washed pellets were resuspended in the same buffer, and disrupted by an ultrasonic oscillator (Sonics and Materials, USA). The cell debris was removed by centrifugation (14,000*g*, 30 min, 4 °C). Cell lysates were stored at 4 °C.

Purification of LADI

Cell lysates were subjected to anion exchange chromatography on a HiTrap-Q FF column (5 ml; Amersham, Sweden), which had been pre-equilibrated with 50 mM Tris buffer (pH 7.0). Two milliliters of cell lysates was loaded. Fractions were eluted by a NaCl gradient in 50 mM Tris buffer (pH 7.0) at a rate of 2 ml/min, with the obtained fraction dialyzed in water and lyophilized to concentrate it. The lyophilized fraction was resuspended in 1 ml of 50 mM Tris buffer (pH 7.0) containing 0.15 M NaCl, and 0.5 ml of it was subjected to gel filtration column chromatography (HiPrep 16/60 Sephacryl S-200 HR, Amersham). The column was calibrated with the following proteins of known molecular mass: chymotrypsinogen A (25 kDa), bovine serum albumin (BSA; 66 kDa), and lactate dehydrogenase (140 kDa). All of the purifications were carried out using an ÄKTA explorer (Amersham).

SDS–PAGE and native gel electrophoresis

The expression and purification levels of LADI were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Native PAGE was performed by Novex® 4–20% Tris–Glycine Gel (Invitrogen, USA) for molecular mass estimation under nondenaturing conditions. The protein bands were visualized by staining with Coomassie brilliant blue.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry of LADI was performed using a Voyager-DE STR Biospectrometry workstation with delayed extraction and linear capability (Applied Biosystems, USA) at the Seoul National University National Center for Inter-University Research Facilities (Seoul, Korea). It was equipped with a 337-nm nitrogen laser and a 2-m flight tube. Mass spectra were obtained in the positive ion mode, with an accelerating voltage of 25 kV. One microliter of the protein was mixed with 1 μl of matrix (10 mg of sinapinic acid and 0.1% 4-hydroxy-α-cyanocinnamic acid in 1 ml of distilled water), and 0.3 μl of this mixture was applied to the Teflon-coated plate. BSA was used for calibration.

Amino acid sequence analysis

N-terminal sequences of LADI were identified with a protein sequencer (model 491 Procise, Applied Biosystems) after desalting with a sample preparation cartridge (Prosorb, Applied Biosystems) at the Korea Basic Science Institute (Seoul, Korea).