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Purification of a dimeric arginine deiminase from *Enterococcus faecium* GR7 and study of its anti-cancerous activity

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ABSTRACT

The arginine deiminase (ADI, E.C 3.5.3.6) – a key enzyme of ADI pathway of *Enterococcus faecium* GR7 was purified to homogeneity. A sequential purification strategy involving ammonium sulfate fractionation, molecular sieve followed by Sephadex G-100 gel filtration was applied to the crude culture filtrate to obtain a pure enzyme preparation. The enzyme was purified with a fold of 16.92 and showed a final specific activity of 76.65 IU/mg with a 49.17% yield. The dimeric ADI has a molecular mass of about 94,364.929 Da, and comprises of hetrodimers of 49.1 kDa and 46.5 kDa as determined by MALDI-TOF and PAGE analysis. To assess anti-cancerous activity of ADI by MTT assay was carried out against cancer cell lines (MCF-7, Sp2/0-Ag14 and Hep-G2). Purified ADI exhibited the most profound antiproliferative activity against Hep-G2 cells; with half-maximal inhibitory concentration (IC_{50}) of 1.95 μg/ml. Purified ADI from *E. faecium* GR7 was observed to induce apoptosis in the Hep-G2 cells by DNA fragmentation assay. Our findings suggest the possibility of a future use of ADI from *E. faecium* GR7 as a potential anti-cancer drug.

1. Introduction

Lactic acid bacteria (LAB) are widely used in food industry and are enlisted as generally recognized as safe (GRAS). LAB are commonly known to possess several health promoting attributes, including anti-mutagenic activity [1], anti-carcinogenic activity [2,3], hypocholesterolemic properties [4], antagonistic actions which can restrain the intestinal and food-borne pathogens [5] and have immunomodulation effects [6,7]. *Enterococcus faecium* GR7, a Gram-positive and catalase-negative bacteria; which is a natural member of the human and animal micro flora. This microorganism plays important role in food industry as this bacterium involved in ripening process and in aroma development of diverse cheeses [1–3].

ADI pathway involves three enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC), and carbamate kinase (CK) in most of the prokaryotes. Arginine deiminase (ADI, E.C 3.5.3.6) belongs to a guanidine group modifying enzyme superfamily and catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia [8]. One mole of ATP is yielded by phosphorylation of ADP for each mole of arginine degraded in the ADI pathway; thus, arginine is major source of energy in many microorganisms such as *Mycoplasma*, *Pseudomonas*, *Bacillus*, *Streptococcus*, etc. [9]. In most animals, including humans, the citrulline produced by ADI can be converted back into arginine by two enzymes of the urea cycle, argininosuccinate synthetase and argininosuccinate lyase.

Arginine is a "semi-essential" amino acid for adult humans, i.e. nearly 50% of arginine is synthesized *de novo*. The metabolic differences between normal cells and tumor cells have provided opportunities for developing novel approaches for the diagnosis and treatment of cancer. Tumor cells such as hepatocarcinomas, melanomas, renal and pancreatic carcinomas and some types of leukemia, are unable to synthesize arginine due to defects in ornithine carbamoyltransferase, OCT (EC 2.1.3.3) and/or argininosuccinate synthetase, ASS (EC 6.3.4.5) which occur most probably due to defects in transcription of the corresponding structural genes [10–15]. Starvation for arginine of auxotrophic cancer cells leads selectively to tumor cell death [16,17]. These observations have

Abbreviations: ADI, arginine deiminase; ASS, argininosuccinate synthetase; CK, carbamate kinase; CF, cytoplasmic fractions; DMSO, dimethylsulfoxide; GRAS, generally recognized as safe; HKC, heat-killed cells; Hep-G2, Human hepatocellular carcinoma cell line; IC_{50}, half-maximal inhibitory concentration; LAB, lactic acid bacteria; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MCF7 ATCC-HTB-22, Human Breast carcinoma cell line; OTC, ornithine transcarbamylase; OCT, ornithine carbamoyltransferase; RSM, response surface methodology; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Sp2/0-Ag14 ATCC-CRL-1581, spleen lymphoblast cell line of Mus musculus.

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been used by many laboratories for the development of cancer treatments based on arginine deprivation achieved by systemic injection of arginine degrading enzymes, arginine deiminase or arginase [18,19].

In our previous work, arginine catabolizing LAB were screened and isolated from milk and dairy products in our laboratory. The isolate GR7 shown to degrade arginine through ADI pathway was identified as *E. faecium* GR7 on the basis of morphological, biochemical tests and 16S rRNA gene sequence analysis [20]. Genetic organization of arginine catabolizing enzymes in *E. faecium* GR7 was further studied which revealed a partial ADI operon of 3906 bp consisting of *arc* CBA genes localized on the anti-sense strand of the genomic DNA encoding ADI pathway enzymes namely *arcA* (arginine deiminase), *arcB* (ornithine transcarbamylase), and *arcC* (carbamate kinase) (20). To enhance ADI specific activity of the native strain *E. faecium* GR7, *Enterococcus* ADI fermentation media (EAPM) was optimized using single factor independent experiments which resulted in 4-fold increase in specific ADI activity. Further a statistical tool of response surface methodology (RSM) was used on selected variables to find out optimum values of significant response factors, which resulted in 15-fold increase in ADI production in the native strain [21].

ADI is a promising anticancer drug active against melanoma, hepatocarcinoma and other tumors. Recombinant strains of *Escherichia coli* that express ADI from *Mycoplasma*, *Lactococcus lactis* and *Pseudomonas* have been developed [22,23,9]. However, production costs of heterologous ADI are high due to use of an expensive inducer and extraction buffer [24], as well as using diluted culture for enzyme induction [25]. So, an attempt to reduce number of steps involved in ADI purification from the whole cell culture lysate has been made. A novel method for purification of the native producer of ADI from *E. faecium* GR7 has been developed. Further, *in vitro* cytotoxicity assays were performed to assess the inhibition of cell proliferation in some cancer cell lines using crude and purified ADI of *E. faecium* GR7.

2. Materials and methods

2.1. Materials

L-Arginine and ammonium sulphate were provided by Hi-Media Pvt. Ltd. Bugbuster (Protein Extraction Reagent, Novagen), Slide-A-Lyzer G2 Dialysis Cassettes, 20 K (Thermo Scientific, Pierce Biotechnology, USA) and Sephadex G-100 (Pharmacia, Uppsala, Sweden) were employed to purify ADI from crude cell lysates. All of the other chemicals were of analytical grade.

2.2. Bacterial strain and culture medium used

E. faecium GR7 isolated from an indigenous dairy product (Patiala, India) was used in this study [20]. The stock culture was maintained on MAM medium (g/L) consisting of tryptone 10.0; glucose 5.0; yeast extract 5.0; arginine 3.0; KH_2PO_4 0.5; $MgSO_4$ 0.2; $MnSO_4$ 0.05; Tween-80 1.0 ml/L and Agar 2.0; pH 6.0 (26). Enzyme was produced by cultivation of bacterial strain in RSM optimized medium (g/L) consisting of tryptone 15.0; lactose 10.0; arginine 20.0; NaCl 1.0; K_2HPO_4 0.5; $MgSO_4$ 0.2; $MnSO_4$ 0.05 and Tween-80 1.0 ml/L, pH 6.0 at 30 °C for 24 h. Optical density of the inoculum was adjusted to 1.0 and 2% *v/v* culture after 2nd subculturing was used in each experiment. Cultures were incubated at 30 °C for 24 h [21]. The cells were harvested by centrifugation at 4 °C. Cell pellet was suspended in lysis buffer (Bug Buster Protein Extraction Reagent, Novagen). This suspension was centrifuged at 10,000*g*, 4 °C for 10 min and cell free supernatant (crude extract) was used to purify native ADI.

2.3. Procurement and maintenance of cancer cell lines

The Hep-G2 (Human hepatocellular carcinoma cell line) was procured from NCCS, Pune, India, MCF7 ATCC-HTB-22 (Human Breast carcinoma cell line) and Sp2/0-Ag14 ATCC-CRL-1581 (spleen lymphoblast cell line of Mus musculus) were gifted by Mrs. Bharti Mittu, Niper, Mohali. All cell lines were maintained in DMEM and RPMI 1640 medium with (10% *v/v*) fetal bovine serum, (2% *v/v*) antibiotic solution (streptomycin (100 mg/ml) and penicillin (10,000 U/ml) in a humidified incubator (Star Microtek devices, SMD, New Delhi) with 5% CO_2 at 37 °C. Cells (1.0×10^6) were cultured for measuring antiproliferative activity and DNA fragmentation assay.

2.4. ADI activity determination

ADI activity was determined by following standard method of De Angelis [26]. Briefly, under standard conditions, the reaction mixture consisted of 150 µl of 50 mM arginine, 2.3 ml of 50 mM acetate buffer (pH 5.5), 50 µl of cell wall or cytoplasm preparation, and 3.6 µl of sodium azide (final concentration, 0.05% *w/v*). Controls without substrate and without enzyme were included. After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.5 ml of a solution of 2 N HCl, and precipitated protein was removed by centrifugation. According to standard method of Archibald [27], citrulline content of the supernatant was determined. One milliliter of supernatant was added to 1.5 ml of an acid mixture of H_3PO_4-H_2SO_4 (3:1 *v/v*) and 250 µl of diacetylmonoxime (1.5% 2,3 butanadionamonoxime) in 10% (*v/v*) methanol, mixed and boiled in dark for 30 min. After cooling for 10 min, the absorbance at 460 nm was measured. One enzyme unit was defined as the amount of enzyme required to catalyze formation of 1 µmol citrulline per min De Angelis [26]. Total protein was estimated by measuring absorption at 280 nm and quantified using standard curve of BSA. Finally, specific ADI activity was calculated as international enzyme units present per mg (IU/mg) of protein [21].

2.5. Purification of arginine deiminase

The purification was carried out at 4 °C using crude cell extract, according to the modified method of Li et al. [28] following an ammonium sulphate fractionation, ultrafiltration and Sephadex G-100 gel filtration step approach as described below.

2.6. Ammonium sulphate fractionation

Finely powdered ammonium sulphate was added to 0–20%, 20–40%, 40–60%, 60–80% and 80–100% saturation level in 25 ml of crude extract. The mixture was left for 12 h at 4 °C. The mixture was left for 12 h at 4 °C, followed by centrifugation at 8000 rpm for 20 min at 4 °C. The precipitate was dissolved in 5 ml of 20 mM sodium phosphate buffer saline (pH 7.2). The collected ammonium sulphate precipitate was assayed for protein and ADI specific activity using standard procedures of De Angelis [26] and Archibald [27].

2.7. Ultrafiltration

Molecular sieve separation was carried out using Slide-A-Lyzer G2 Dialysis Cassettes (Thermo Scientific, Pierce Biotechnology, USA), 20 K MWCO (Molecular weight cut off). 2 ml of ammonium sulphate fraction was injected in the dialysis cassette. Keeping at approximately >1/3 of the cassette's maximum volume after hydrating membrane by immersing cassette in dialysis buffer for 2 min. The sample was dialyzed overnight at 4 °C with decreasing concentration (5–0.1 M) of sodium chloride buffer with mild