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Arginase purified from endophytic *Pseudomonas aeruginosa* IH2: Induce apoptosis through both cell cycle arrest and MMP loss in human leukemic HL-60 cells

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ABSTRACT

Arginase is a therapeutic enzyme for arginine-auxotrophic cancers but their low anticancer activity, less proteolytic tolerance and shorter serum half-life are the major shortcomings. In this study, arginase from *Pseudomonas aeruginosa* IH2 was purified to homogeneity and estimated as 75 kDa on native-PAGE and 37 kDa on SDS-PAGE. Arginase showed optimum activity at pH 8 and temperature 35 °C. Mn^{2+} and Mg^{2+} ions enhanced arginase activity while, Li^{+}, Cu^{2+}, and Al^{3+} ions reduced arginase activity. *In-vitro* serum half-life of arginase was 36 h and proteolytic half-life against trypsin and proteinase-K was 25 and 29 min, respectively. Anticancer activity of arginase was evaluated against colon, breast, leukemia, and prostate cancer cell lines and lowest IC_{50} (0.8 IU ml^{-1}) was found against leukemia cell line HL-60. Microscopic studies and flow cytometric analysis of Annexin V/PI staining of HL-60 cells revealed that arginase induced apoptosis in dose-dependent manner. Cell cycle analysis suggested that arginase induced cell cycle arrest in G0/G1 phase. The increasing level of MMP loss, ROS generation and decreasing level of SOD, CAT, GPx and GSH suggested that arginase treatment triggered dysfunctioning of mitochondria. The cleavage of caspase-3, PARP-1, activations of caspase-8, 9 and high expression of proapoptotic protein Bax, low expression of anti-apoptotic protein Bcl-2 indicated that arginase treatment activates mitochondrial pathway of apoptosis. Purified arginase did not exert cytotoxic effects on human noncancer cells. Our study strongly supports that arginase could be used as potent anticancer agent but further studies are required which are underway in our lab.

1. Introduction

Cancer is a deadly disease of uncontrolled cell division and has the potential to invade or spread to other parts of the body and affects normal functioning of the vital organs. Nowadays cancer is considered as leading cause of human death in all over the world and its burden will continue to increase due to continuous growth and ageing of world's population [1]. Leukemia is one of the major types of cancer, affecting significant segments of the human population, especially children and adolescents. Based on diagnosis the most common forms of leukemia are acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia and chronic lymphocytic leukemia [2]. Previously, it has been well documented that amino acids serve as regulatory molecules that modulate numerous cellular functions and provide substrates for protein synthesis in both cancer as well as normal cells [3]. Several auxotrophic cancers including leukemia nutritionally depend on host to fulfil their amino acids requirements. Therapeutics selectively targeting deprivation of these amino acids are currently used in effective treatment strategy for some cancers and numerous are still under intensive clinical investigations [4]. Arginase (L-Arginine amidinohydrolase, E.C. 3.5.3.1) is a metallo-enzyme hydrolyses L-arginine into non-proteinogenic amino acid L-ornithine and urea. Since last few years, this enzyme is widely investigated for starvation therapy of hepatocellular carcinoma and melanoma. In the recent past, researchers have paid much attention and also explored arginase against other arginine-auxotrophic cancers such as pancreatic cancer [5], prostate cancer [6], leukemia [7],

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glioblastoma [8], breast cancer [9] and non-Hodgkin's lymphoma [10].

Many arginine auxotrophic cancers have shown lack expression of argininosuccinate synthetase-1 (ASS1) due to which they are unable to synthesize their own arginine. Indeed, they required massive amount of arginine for malignant proliferation and metastasis [11]. Therapeutic application of arginase hydrolyses L-arginine of circulating system into L-ornithine and urea [12]. L-Arginine depletion leads nutritional starvation, resulting in inhibition of biosynthesis of DNA, RNA, and proteins which causes apoptosis of cancer cells. In the contrary, normal cells remain unaffected due to endogenous biosynthesis of arginine [13]. Beside in cancer chemotherapy, arginase is also involved in the treatment of hyper-argininemia; a genetic metabolic disorder occurs due to the deficiency of liver arginase [14,15]. In recent years, many scientists have reported purification and characterization of arginase from bacteria [16], fungi [17], plants [18] and animal tissues [19]. But relative higher antigenicity, short serum half-life, rapid proteolysis and lower thermal stability of currently available arginases are restricted its clinical applications. Previously, several efforts have been made to mitigate these therapeutic limitations but results are still unsatisfactory. Therefore, a new arginase which possesses potent and selective anticancer activity with prolonged serum half-life and strong proteolytic tolerance is essentially required for successful therapeutical applications.

Endophytes are the microorganisms that live inside the plants and exert many advantageous effects in host plants. Now a day's, endophytes are explored to discover new compounds having extensive biotechnological and pharmaceutical applications [20,21]. Although, several researchers have evaluated terrestrial microbes for therapeutically active arginase but in existing literature, no such reports are available which have explored endophytes for arginase enzyme with potential chemotherapeutic application. Therefore, in the present communication, we report purification and characterization of an extracellular arginase from endophytic *P. aeruginosa* IH2. Further, purified arginase has been evaluated for anticancer activity against panel of human cancer cell lines. Purified enzyme showed the highest anticancer activity against human leukemic cell line HL-60 and no substantial cytotoxicity was observed with human noncancer cells (HEK-293 and FR-2). Therefore, further antileukemic potential of purified arginase was investigated against human promyelocytic leukemia HL-60 cells, using various cellular and sub-cellular assays.

2. Material and methods

2.1. Chemicals and reagents

All the chemicals and reagents were purchased from Sigma Chemical Co. USA, Bio-Rad Lab. USA, and Himedia, Mumbai, India. Antibodies used in this study were procured from Santa Cruz Biotechnology, USA.

2.2. Isolation and screening of bacterial strains

For the isolation of endophytic bacteria, samples were collected from the various agronomic (*Zea mays*, *Glycine max*, *Triticum aestivum*, *Pisum sativum*, *Capsicum annuum*, and *Cicer arietinum*) and medicinal plants (*Allium sativum*, *Cymbopogon citrates*, *Asparagus racemosus*, *Catharanthus roseus*, *Argyreia speciosa* and *Rosa indica*) from State Forest Research Institute and various sites of agriculture fields of Jabalpur, Madhya Pradesh, India. All samples were surface sterilized, cut into small pieces and used for isolation of endophytic bacteria. All isolates were subcultured and purified on Luria-Bertani (LB) agar plate (pH 7) and maintained on LB slant. For primary screening of the arginase activity, basal semi-synthetic broth medium (pH 7) was used with slight modification (instead asparagine, arginine was used) [22]. In brief, a 24 h old isolated bacterial colony was aseptically transferred in Erlenmeyer flask containing 20 ml pre-autoclaved basal semi-synthetic broth. Flasks were incubated at 37 °C and 180 rpm for 24 h in orbital shaking incubator. The culture was centrifuged at 10,000 rpm for 5 min (4 °C) and supernatant was taken for estimation of arginase activity.

2.3. Enzyme assay and protein determination

The arginase activity was measured colorimetrically by detecting liberated urea according to the thiosemicarbazide-diacetylmonoxime urea method [23]. One international unit (IU) of arginase is defined as the amount of enzyme that released 1 μmol of urea min^{-1} at 37 °C. Specific activity of arginase is expressed as U mg^{-1} protein. The total protein content was measured colorimetrically [24], using BSA as standard.

2.4. Identification of bacterial isolates

Taxonomic identification of arginase producing potent strain IH2 was done on the basis of cultural, morphological and biochemical characteristics and 16S rRNA gene sequencing as described previously [22]. The identified strain was submitted in Bacterial Germplasm Collection Centre (BGCC), Rani Durgavati University, Jabalpur, Madhya Pradesh, India.

2.5. Condition for production of arginase

Basal semi-synthetic broth was used to produce arginase. For the preparation of primary inoculum, a 24 h old of logarithmic phase culture was aseptically transferred in flask containing 20 ml aforementioned broth medium. The flask was incubated at 37 °C and 180 rpm till the growth attained optical density (A_{600}) 0.6–0.8. The 2% of this inoculum was aseptically inoculated in 50 ml of semi-synthetic broth medium and incubated in rotatory shaker at 37 °C for 24 h and 180 rpm. The broth containing culture was centrifuged at 10,000 rpm for 5 min at 4 °C and supernatant was used as crude enzyme.

2.6. Purification and quantification of arginase

Purification of extracellular arginase was performed by ammonium sulphate precipitation, ion exchange and gel-filtration chromatography. After each step, arginase activity was determined as described previously [23]. In brief, crude arginase was salted out by gently adding powdered ammonium sulphate with constant stirring for 4 h at 4 °C. The maximum arginase activity was achieved at 30–60% ammonium sulphate saturation fraction. The precipitate was collected by centrifugation at 10,000 rpm for 30 min (4 °C) and dissolved in least volume of 50 mM Tris-HCl buffer (pH 8). The protein was dialyzed with the membrane of 12–14 kDa cut off (Hi-Media, India) in 10 mM Tris-HCl buffer (pH 8.0) at ice cold conditions for 24 h. Further, the dialysate was lyophilized and loaded on pre-equilibrated (50 mM Tris-HCl buffer, pH 8) diethylaminoethyl (DEAE) cellulose column (2.5 × 25 cm, Sigma). The absorbed protein was eluted with a linear gradient of NaCl (0–1 M), prepared in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 2 ml min^{-1}. Protein concentration in each chromatographic fraction was measured at 280 nm. The maximum arginase activity exhibiting fractions were pooled, dialyzed in 10 mM Tris-HCl buffer (pH 8.0) and lyophilized. Then, lyophilized sample was loaded on Sephadex G-100 column (2 × 22 cm, Sigma) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The protein was eluted with the aforementioned buffer at