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Applicability of Native L-Arginase produced by *Streptomyces plicatus* KAR73 as Antineoplastic Agent

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Most of the cancer cells require high quantity of arginine for sustaining their fast-metabolic rates. Limiting supply of arginine to cancer cells using arginase may prove to be of great therapeutic value. The arginase produced by a micro organism isolated from soil has been used in industrial production of ornithine, however its use in anticancer activities is scarcely studied. This study optimized soybean meal supplemented basal media for Arginase production. Arginase was purified using ammonium sulphate precipitation, sephadex G-100 column chromatography and DEAE chromatography achieving 79.2% purification fold and 24.26% yield. It had 23 KD molecular weight as determined using Native PAGE and was active at considerably wide pH range of 6–10 and temperature 30–50°C. Whereas the maximum arginase activity was noticed with Mn^{2+} ions followed by polyvinyl pyrrolidine (PVP) at 70 mM substrate concentration, the maximum inhibition of activity was caused by CuC. *Streptomyces plicatus* KAR 73 produced arginase on mouse mammary cell line (CID 9) was not inhibited by the arginase upto 6.5 U/mL. Significant ($p< 0.001$) inhibition in Mouse mammary tumor (C1271) cell lines was observed with IC_{50} 5.2 U/mL. The ornithine has been produced earlier with *Mycoplasma* and *Clostridium* by other researchers but production of native arginase from *Streptomyces* specifically for anticancer activities has not yet been reported. The present study infers that Arginase produced from native *Streptomyces* has shown promising results thereby enabling feasibility assessment towards cost effective industrial production of arginase.

Keywords: Anticancer, Apoptosis, Arginase, Cytotoxicity, Mammary tumor

Introduction

Cancer, with its high morbidity and mortality, poses serious threat to the sustenance and quality of life. Hence, vigorous, coordinated and evidence-based efforts are needed to prevent and treat carcinomas. It is noticeable that normal cells die during various developmental and stress processes. Since, apoptotic processes remain inactive in cancer cells, effective strategies are required to counter tumor propagation.[1] Normal adult cells synthesize adequate levels of arginine to maintain cellular metabolic functions.[2] It becomes conditionally indispensable during tissue injury, metabolic disorders and disease conditions demanding increased supply of arginine from neighbouring tissues or through diet. Rapidly growing tumor and cancer cells require a nutrient-rich environment to maintain their growth. Arginine serves as an intermediate in the urea cycle as well as a precursor for protein, polyamine, creatine and nitric oxide. Therefore, restricting access to arginine by arginase may cause death of malignant cells resulting in defective cell cycle check-point control.[3] Normal healthy cells, by contrast, become inactive and remain viable under arginine starvation, displaying full recovery upon return to arginine rich conditions.[4] Some arginine hydrolysing enzymes including mycoplasma derived arginine deiminase and human recombinant arginase have been developed for therapeutic purposes.[5]

Arginases (EC 3.5.3.1), also known as arginine amidinase, L-arginase, canavanase and arginine transamidinase, are manganese containing enzymes converting arginine into ornithine and urea. Apart from mammalian tissues, these have also been isolated and characterized from worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae and plants. Among microorganisms actinomycetes, *Entamoeba histolytica*e, *Plasmodium falciparum*, *Bacillus anthracis*, *Rummeliibacillus pycnus*,

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Agaricus bisporus, *Enterococcus faecalis*, *Evernia prunastri*, *Xanthoria parietina*, *Peltigera canina*, *Fasciola gigantica* and *Squalus acanthias* are reported to produce arginase that differ widely in their molecular structure.[6–7] Use of native arginine deiminase (ADI) for therapeutic purposes has also shown antigenicity.[8] The pegylated form of ADI has shown its strong anticancer activities against HCC and malignant melanoma.[9,10] However, unfavorable effects under *in vivo* conditions remain to be thoroughly explored before clinical application of such conjugated enzyme system.[11]

Precise role of arginine in cancer may include influence tumour initiation, promotion, progression, apoptosis, tumour-cell adhesion, angiogenesis, differentiation, and immunosuppression.[12] The efficiency, effectiveness and applicability of native L-arginase produced by *Streptomyces plicatus* KAR73 as anti-neoplastic agent are illustrated in this paper. In addition, economic feasibility for production of microbial therapeutic enzymes may be accentuated using *Streptomyces plicatus* KAR73. Hence, the objective of this study included production, characterization and anticarcinogenic-potential assessment of native arginase derived from *Streptomyces plicatus* KAR73.

Materials and Methods

Chemicals and Reagents

The chemicals and reagents were procured from Himedia Laboratoties Pvt Ltd, Mumbai, India; Thermo Fisher Scientific India Pvt Ltd, Mumbai, India and used as per manufacturer's instructions. *Streptomyces plicatus* KAR73, an isolate of cultivated field soil of district Bhopal, Madhya Pradesh, India (identified using 16S RNA and biochemical test reported elsewhere) was maintained on International *Streptomyces* Protocol medium-2 (ISP-2). The broad range mol wt marker of 29 KDa, 45 KDa, 66 KDa, 97 KDa, 116 KDa and 200 KDa mol wt were obtained from Sigma Chemical Company. Mouse mammary tumor cell line C-1271 was obtained from Cell line Repository, National Centre for Cell Sciences, Pune, India. The generally used media, buffers and reagents were obtained from HiMedia Pvt. Ltd.

Microorganism

The actinomycetes *Streptomyces plicatus* KAR73 used in the study was islaoted from cultivated field soil of karond area of Bhopal, soil characteristics include black colour with pH 7.8, the strain was selected for further studies after primary screening of 231 strains of Actinomycetes for production of arginase enzyme.[13]

Production of Arginase

Production of L-arginase by *Streptomyces plicatus* KAR73 was done on pre-optimised media containing (g/L) Glucose, 10.0; K_2HPO_4, 0.40; $MgSO_4.7H_2O$, 0.05; NaCl, 0.01; $FeSO_4.7H_2O$, 0.01 supplemented with L-arginine 0.1 (w/v%). Inoculum density equivalent to 0.500 Optical density (2×10^6 Colony forming units/mL) was added to the medium in a ratio of 5:95 (v/v). The variation in pH of medium and incubation temperature was done to assess optimum condition for production of arginase under shake conditions (150 rpm) for a period of eight days. A set of flasks were terminated after every 24 h and whole culture broth was centrifuged at 3,000 × g for 10 min at 4°C. Arginase activity and protein content were measured in supernatant, while pellet was used to measure growth. Growth (biomass) was quantified in terms of dry weight of biomass after overnight drying at 100°C. All the experiments were conducted in triple sets throughout this study.

Assay for Arginase Activity and Protein Quantification

Arginase activity was measured by the modified method of Roman and Ruy's Roman.[14] Reaction mixture contained 20 mM Tris HCl buffer (pH 8.0), 2 mM $MnCl_2$, 100 mM arginine (pH 7.5) and enzyme sample in total volume of 2 mL and incubated at 37 °C for 1 h and stopped by adding ice cold trichioroacetic acid 10% (w/v) followed by centrifugation at 10,000 × g for 10 min at 4°C. Blank was processed in similar manner except that addition of ice cold trichioroacetic acid 10% (w/v) was done prior to addition of enzyme sample. Amount of L-ornithine present in supernatant obtained from above was measured using HPLC Yu.[15] The analysis of ornithine level was performed after filtering the supernatant through 0.45 µm PVDF filter, followed by derivatizing with 6-aminoquioly-N-hydroxy–succinimidyl carbonate. The derivatives were separated with a Nova-Pak$_{TM}$C 18 column. Content of Ornithine was calculated by referring to the standard curve. One unit of enzyme was defined herein as the amount of arginase that produces 1 µmol of ornithine per minute at 37°C. The protein content was also determined in enzyme samples according to the method of Bradford Bradford[16] using Bovine Serum Albumin (Himedia, India).

Purification of Arginase

Ammonium Sulphate Precipitation

Purification of arginase from cell free culture broth was carried out in sequential ascending manner. Cell free culture broth was filtered twice with 0.22 µm millipore filters. The resultant broth was saturated with solid ammonium sulphate with continuous stirring at 4°C. Salt addition was done in an aliquot of 1gm per 100 ml broth until complete dissolution of salt. The saturated solution was centrifuged at 10000 × g for 15 min at 4°C. The pellet containing enzyme was dissolved in minimum amount of 10 mM Tris-HCL buffer (pH 7.5) containing 0.5 mM $MnCl_2$, 0.5 µM arginine and 5% glycerol, stored at −80°C. The precipitate was dialysed thrice using dialysis membrane overnight at 4°C against 0.1 mM Tris-HCL buffer (pH 7.5).

Sephadex G-100 Chromatography

Arginase was purified using Sephadex G-100 (2 × 50 cm) column equiliberated with 20 mM HEPES and 0.5 mM $MnCl_2$. Washing of column was done twice. Dialysed, concentrated protein mixture was applied to column and eluted with same buffer with a flow rate of 1.0 ml per minute.

DEAE Cellulose Chromatography

Active fractions obtained from above were pooled and applied on DEAE-cellulose column (2 × 10 cm) equilibrated with 10 mM Tris-HCL buffer (pH 7.5) containing 10% glycerol. The column was washed twice with buffer and enzyme elution was done using a linear gradient of sodium chloride (0.1–0.5 M) prepared in 10 mM Tris-HCL buffer (pH 7.5). The elute was then dialysed using dialysis membrane at 4°C against 0.01 mM Tris-HCL buffer (pH 7.5). The excess buffer from dialysate was removed by keeping the dialysis membrane containing sample in sucrose bed for 24 h at 4°C.

Polyacrylamide Gel Electrophoresis

Molecular weight was determined on 12% (w/v) SDS gel electrophoresis.[17] The purified enzyme was treated with loading dye containing SDS 1% (w/v) and β-mercaptoethanol and boiled for 2–3 min at 100°C and loaded in wells. Sample staking was done at 4% gel. Resolution was carried out at 110 V. The mol wt was determined using broad range mol wt marker (Sigma Chemical Co.) of 29 KDa – 200 KDa mol wt. The gel was carefully removed after electrophoresis and stained with 0.25% (w/v) Coomassie brilliant blue (R-250) dissolved in 50% (w/v) methanol and 10% (w/v) acetic acid followed by destaining with 10% (w/v) acetic acid. Zymography of purified arginase was also performed with native PAGE.

Characterisation of Arginase Activity

Purified arginase was appropriately diluted before characterization studies. The characterization was performed in sequential manner and the optimum conditions of previous experiment were used for successive characterization studies. Buffers systems used for determining pH dependence of arginase activity were 0.01 M acetate buffer (pH 5), 0.01 M phosphate buffer (pH 6–7), 0.01 M Tris HCl buffer (pH 8–9) and 0.01 M borate buffer (pH 10). Effect of temperature on the arginase activity was studied at 20−50°C. Effect of substrate concentration (10–100 mM) on arginase activity was studied at optimum temperature and pH elucidated from above experiment. Activity of arginase in presence of inhibitors 10 mM SDS, 10 mM EDTA, 10 mM PMSF, 10 mM DMSO, 10 mM DTT, 20% (v/v) Triton X-100 and 20% (v/v) Tween-20 was carried out at herein identified optimum pH and temperature. In addition, the effect of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}) on enzymatic activity was also determined.

Study of Effect of Purified Arginase on Mouse Mammary Tumor (C-1271) Cell Lines

Preparation of Cell Lines for Cell Cytotoxicity Assay

Mouse mammary tumor cell line C-1271 was obtained from National Centre for Cell Sciences, Pune, India. These were cultured in complete growth medium Dulbecco's Modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 0.1m M non-essential amino acids, and 1 mM sodium pyruvate 100 µL/mL, penicillin 100 µL/mL, streptomycin 100 µl/mL and 0.2 mM L-glutamine. These cell lines were maintained at 37°C in humidified atmosphere of 95% air and 5% CO_2. Sub-culturing was performed every 24 h with cells from subconfluenet cultures after treated with Trypsin-EDTA solution (Himedia Pvt. Ltd.). After trypsinization, cells were counted using nebular cell counter and diluted appropriately. The cell viability was checked using trypan blue assay. The 99% viable cells were used for further experimentation.

MTT cell Cytotoxicity Assay

Quantitative cell cytotoxicity assay under different treatment conditions was determined with

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) assay.[18] Cell density of 1×10^4 cells (ml^{-1}) were plated into 96 well tissue culture plates. The cells were allowed to reach confluence for 24 h at 37°C. The purified arginase was added in increasing concentration of 0.1 to 6.5 U/mL and incubated for 24 h. Subsequently, cytotoxicity was determined by MTT Assay using EZ count™ MTT Cell Assay Kit. The optical density was measured at 570 nm (ELISA reader, Multiskan Ascent, Thermo Scientific, India). The half maximal inhibitory concentration (IC_{50} value) was defined as 50% decrease in cell viability.

Trypan Blue Assay

Cells with density 10^4 cells/mL were seeded on plates and were allowed to multiply and adhere for 24 h at 37°C and 5% CO_2 in a humidified atmosphere. Successive sequential concentration of Arginase was added to all the cell lines C-1271 in different Sets once they reached confluency. After 1 h of treatment, cells were trypsinized and to appropriate cell density of cell line (s) Trypan blue dye was mixed and incubated for 1 min.[19] Thereafter, 10 µl of this cell-dye suspension was loaded in Neubauer chamber and observed under low (100x) and high (400x) power of an inverted microscope. Number of viable (bright cells) and non-viable cells (stained blue) were counted.

Colony Forming Unit Assay Using Crystal Violet Staining

Cell density (10^4 cells/ml) was seeded on plates and allowed to adhere and multiply for 24–48 hours at 37°C and 5% (v/v) CO_2 in a humidified atmosphere. The used up medium was discarded and fresh medium was added to each plate. Arginase enzyme was added as mentioned before; a set of plates untreated with arginase was labeled as control. On completion of desired incubation period plates were washed twice with Phosphate Buffer Saline followed by fixing with 10% formalin for 15 minutes. Staining was done with Crystal Violet for 10 minutes,[20,21] Excess stain was removed by washing with Phosphate Buffer Saline (pH 7.2). Colony forming units were counted using stereo and compound microscope.

Apoptosis Assay using Annexin V- FITC and PI Assay

The cells were cultured in tissue culture plates and treated with arginase, after 6 hours and 24 hours of incubation number of viable cells, apoptotic and necrotic cells were analysed.[18] Briefly, the cells after treatment with purified arginase were washed with PBS (pH 7.2) and then trypsinized with Trypsin-Ethylene diamine tetraacetic acid (EDTA) solution for 1 minute. These cells were thereafter collected and mixed with Dulbecco's Modified Eagle's medium (DMEM) at 1600 rpm. The pellet was washed and stained with Annexin V-FITC antibody and PI. The cells were scanned for fluorescence intensity using Flow Cytometer (Lab Quanta). The control was also processed in same manner except that it was not treated with arginase.

Statistical Analysis

Statistical analysis was done using Epi Info™ Software available online from Division of Health Informatics & Surveillance (DHIS), Center for Surveillance, Epidemiology & Laboratory Services (CSELS), Centers for Disease Control and Prevention (CDC). The analysis was done for experimental and control groups. Graphical representations of the data were performed using GraphPad Prism.

Results and Discussion

Production Media for Arginase

Streptomyces plicatus KAR73 was found to produce extra-cellular arginase with 450.5 U/mL activity and 3.821 mg/mL protein (Table 1). The production of arginase by *Streptomyces plicatus* KAR73 is found in all temperature and pH range tested. The overall peak effectiveness is best amongst three different temperature constants (28°C, 37°C and 42°C) at pH range of 5–10. The most suited one with best results is observed at pH 7.0.

Optimization of Temperature and pH Tolerance Favor Production Potential

Across temperature range of 28–48°C, there is homogeneity in peak observance for production of arginase to be highest at pH 7 both for individual

Table 1 — Purification of L-arginase produced by *Streptomyces plicatus* KAR73

Purification steps	Volume (ml)	Enzyme activity (U/mL)	Total units	Protein content (mg/ml)	Specific activity	Recovery percentage (%)	Purification fold
Culture filtrate	100 ml	450.5	45050	3.821	117.90	100	—
Precipitated by ammonium sulphate	17 ml	1974.84	33572.38	2.209	894	74.52	7.58
DEAE-Cellulose chromatography	14 ml	2134.8	29887.2	1.15	1856.34	66.34	15.74
Sephadex G-100	7.8 ml	4306.43	32728.86	0.832	5176	72.65	43.90

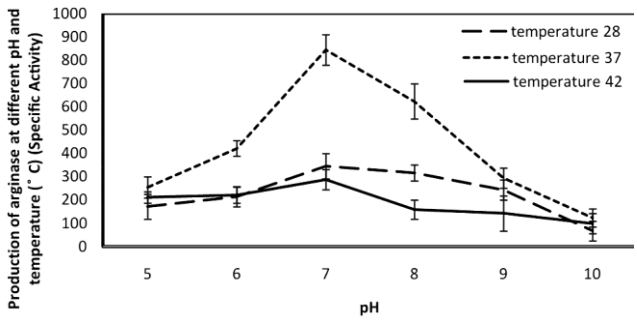
temperature constant and variation in the temperatures for the same pH. This implies three fold increases in production of enzyme at temperature 37°C as compared to temperature 28°C (Fig. 1). Similar results have been observed with *Alcaligenes faecalis*.[22] The extracellular production of arginase was pH dependent at 37°C which might be due to alteration in permeability of membrane.

Purification of Arginase

Complete recovery of protein from culture broth of *Streptomyces plicatus* KAR73 was achieved on 65% (w/v) ammonium sulphate saturation. The purification fold increased three times (15.74 to 43.90) when the purification step was raised from DEAE- cellulose chromatography to Sephadex G-100 with respective yields of 66.34% and 72.65% (Table 1). It is hence inferred here that purification upto Sephadex G-100 level may be commercially useful. It is noteworthy that the completion of designated steps of purification provides better results including gain in protein contents and purification fold as depicted by highest specific gravity in DEAE- cellulose chromatography.

Molecular Weight of Arginase

Studies revealed that *Streptomyces plicatus* KAR73 produced only one type of arginase with its molecular weight being 23 KDa. While ensuring purity of arginase by SDS PAGE, the zymography has clearly indicated enzymatic action with noticeable impact. Purified arginase of molecular weight 75 KDa, has been isolated and studied from *Pseudomonas aeruginosa* IH2.(18) The molecular weight (23 KDa) of derived arginase from *Streptomyces plicatus* KAR73 in this study is comparable with that of human Arg 1 having molecular weight in the range of 35–105 KDa.[23,24] Metabolic and genetic diversity of *Streptomyces* sp. is vast and require extensive studies



Fig, 1 — Effect of pH and temperature on production of Arginase by *Streptomyces plicatus* KAR-73 grown on medium containing glucose and arginine as carbon and nitrogen source under shake conditions (150 rpm)

to completely define them. Also, limited availability of the relevant published literature on *Streptomyces plicatus* produced arginase is although not self speaking about the similarity of size of arginase obtained herein with that of human arginase but the experimental study revealed it to be in same range. This range is further verified by a study from Warszawa, Poland observing human heart arginase to be 30 KDa[25] and Arginase II from *Heteropneustes fossilis* showing 96 KDa band on native PAGE in another study from Varanasi, India.[26]

Characterization of Arginase Activity

The arginase was found to be active in broad range of pH from 6 to 10 with optimum activity at pH 7.0–8.0 (Relative activity 99.14%–100%) (Fig. 2a). The range of pH 6.0 to pH 10.0 observed in our study on activity of purified arginase produced is in resonance with the cellular environmental pH and hence anticarcinoma potential has wide ranging applicability in on site anticarcinoma action. The maximum activity of arginase produced by *Streptomyces plicatus* KAR 73 as pH (7.5 pH) in this study is in near absolute resonance with human physiological pH (7.2–7.4 pH), whereas other studies show maximal activity in outlier pH viz., *Bacillus anthracis* arginase activity occurred with nickel at an alkaline pH 9.0 in a study from University of South Alabama, USA.[27] Also, it is well known fact that cancerous cell have slightly acidic pH and thus the arginase obtained from *Streptomyces plicatus* KAR 73 may prove to be more suitable for cancer treatment as compared to those obtained from Buffalo liver which was optimally active at pH 9.2.(28) Wheatley and his co-workers[5] have reported that bovine liver arginase is remarkably heat resistant enzyme with a long life on storage at 4°C in lyophilized form and is active at pH 7.2 than at pH 9.9 while arginase derived herein was maximally active at pH 7.5.

Arginase from *Streptomyces plicatus* KAR 73 was active and stable at 25−42°C with 100% relative activity at 37°C (Fig. 2b). It is noteworthy that relative activity of arginase was almost stable in the temperature range 30–37°C with progressive difference of 0.002%. Cai and coworkers[8] have reported production of thermostable arginine deiminase by *Enterococcus faecalis* SK23.001. Thermostable arginase from *Geobacillus thermodentrificans* NG80-2 was found to be optimally active at pH 9 and temperature of 80°C.[21] In the present study, the purified arginase from

Streptomyces plicatus KAR 73 is found active in a broad range of temperature and tolerated even 50°C. The purified arginase was found to be active at temperature range of 30–40°C thereby making it suitable for therapeutic purposes under physiological conditions. Recently, purification of arginase from Camel liver done using heat denaturation, ammonium sulphate precipitation, DEAE-cellulose, SP-Sepharose and Sephadex G 100–120 chromatography columns has shown its optimum temperature activity at 70°C.[29] The maximum arginase activity was noticed at 70 mM Arginine concentration (Fig. 2c).

All the inhibitors and detergents used in the study caused reduction in enzyme activity, EDTA caused 59.7% inhibition and PMSF caused 21.8% inhibition, respectively. Arginase activity was found to be even higher than that of control in presence of Mn^{2+}, while only 15.7% residual activity was observed with Cu^{2+} metal ion as compared to control (Fig. 2d). Arginase activity inhibition was noted with other metal ions as well. Interestingly, presence of PVP did not influence arginase activity under *in vitro* conditions. The observed inhibition of arginase by CuC, FC and CaC may assist in the controlled production, activity and interaction of arginase with concurrent use of drugs, chemicals and other therapeutic interventions. In this study, enhancement in the activity of arginase was observed in presence of Mn^{2+} and PVP. It is noticeable that Arginases Arg I and Arg II from *Fasciola gigantica* have also been observed activated by Mn^{2+} and inhibited by Fe^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+} and Mg^{2+} ions.[30] Similarly, recombinant *Plasmodium falciparum* arginase activity has been found to be dependent on Manganese.[31] D-arginase from *Arthrobacter sp* KUJ8602 revealed an optimum pH of 9.5 and requirement of Zn^{2+} for activation instead of manganese ions.[31] Activity of *Saccharomyces cerevisiae* arginase was found to be dependent on Mn^{2+} ions, and removal of ions via dialysis caused significant changes in its spectra.[32]

Lytic Potential Confirmed by Early Apoptosis, Late Apoptosis and Dead Cells

The Mouse mammary tumor cell line (C1271) was found to be inhibited by different concentrations of arginase (0.1 to 6.5 U/mL) with IC_{50} 5.2 U/mL (Fig. 3). Dead cells were confirmed post staining and treatment with purified arginase under visualization through Trypan Blue staining of C1271. The disseminated fragmentation of CFU assay, as

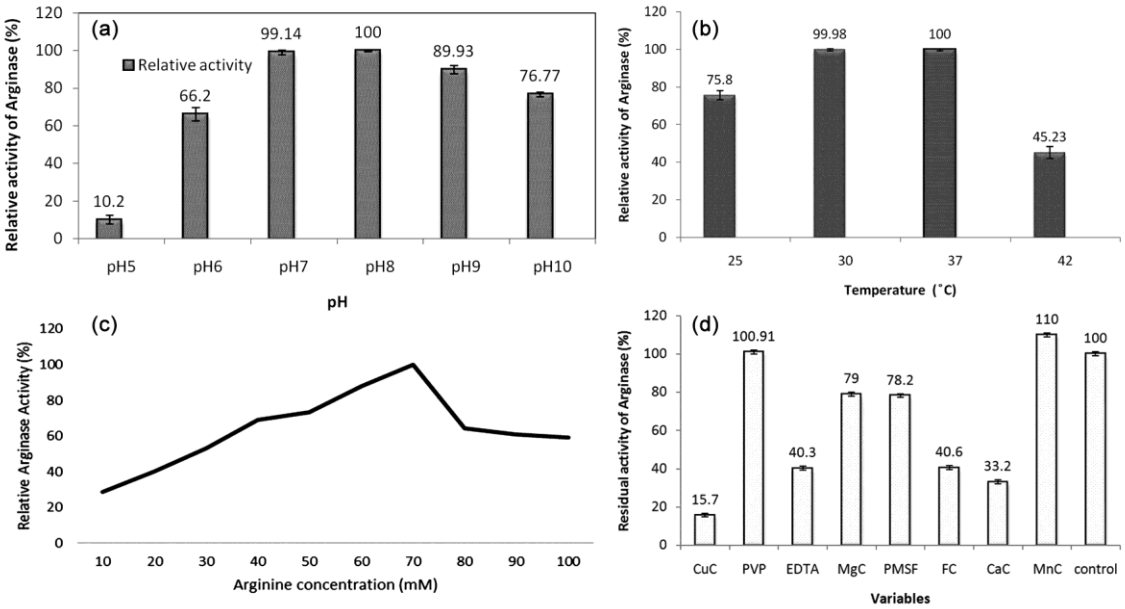


Fig. 2(a–d) — (a) Relative activity of purified arginase produced by *Streptomyces plicatus* KAR 73 at different pH under *in vitro* conditions; Assay conditions temperature 30°C and buffers (pH 5–10), (b) Relative activity of purified arginase produced by *Streptomyces plicatus* KAR 73 at different temperature under *in vitro* conditions; Assay conditions pH 7.5 and temperature (25°C–42°C). **Copper chloride (CuC), PVP, EDTA, magnesium chloride (MgC), PMSF, ferric chloride (FC), calcium chloride (CaC) and manganese chloride (MnC)*, (c) Effect of substrate concentration on arginase activity; The arginase activity is expressed as relative activity the assay conditions were pH 7.5 and 37°C temperature and (d) Effect of activators and inhibitors on arginase activity; The results are expressed in terms of residual activity remaining after treatment with activators and inhibitors under in vitro conditions; Assay conditions pH 7.5 and temperature (37°C)

observed through crystal violet staining of arginase treated plate in comparison to same staining for control plate illustrates the non-viability of cells including clumps of dead cells (Fig. 4). The confirmation was met by Annexin V FITC staining showing live apoptotic and dead cells through morphological visualization. The observance of 90.44%, 3.63%, 5.26% and 0.68% being apoptotic, early apoptotic, dead cells, and viable cells respectively, underlines that there is high lytic potential of arginase for carcinoma cells (Fig. 5).

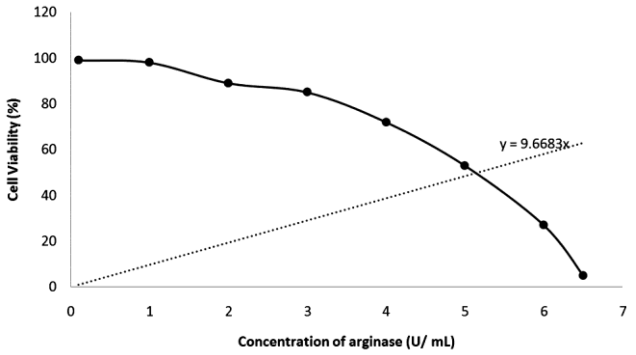


Fig. 3 — IC_{50} of Arginase against C1271 cell lines determined using MTT assay

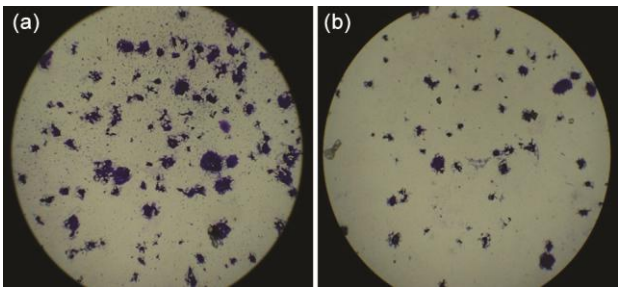


Fig. 4 — Colony forming unit assay: C1271 cell line were cultured in complete growth medium DMEM with 5% FBS, 24 h incubation period (a) Microscopic observation of control plate (first quadrant) after Crystal violet staining; (b) Microscopic observation of arginase treated plate (first quadrant) after Crystal violet staining

Arginine deiminase (ADI) has been reported to exert inhibitory effects on cancer cells under *in vitro* and *in vivo* conditions as depicted in earlier studies for Pegylated enzymes.[11] It is further approved by the present study even for native enzyme(s). The result of cell cytotoxicity assay (Fig. 5) illustrates the acceptability of arginase for use in the human tissues with unhindered and un-interfered biological activity required for normal cell functioning and maintenance of homeostasis of body systems.

It is evidenced by apoptosis and cell death even in the presence of Arginosuccinate synthetase enzyme in this study, whereas it was hypothesized till now that its absence is essential for any effective internvention.[12,23,24] Resonance of observations of this study with natural occurrences in cancer cell growth and development is found unique for cellular milieu. It therefore makes the arginase borne of *Streptomyces plicatus* most suited for effective interventions throughout the spectrum of cancers and their stages. However, the ADI has two major disadvantages. Firstly, ADI is not produced by mammals and must be derived from microbes. As a consequence, nascent ADI is strongly antigenic in mammals.[33] Secondly, ADI has a short circulating half-life in mammals (approx. 5 h) and is to be essentially administered in large daily dose to inhibit tumors. The ADI has also been formulated with polyethylene glycol to produce ADI-SS PEG 20,000 MW, which is found safe and non toxic in mice.[11] It has also been stated to be arginine specific and has no role for other

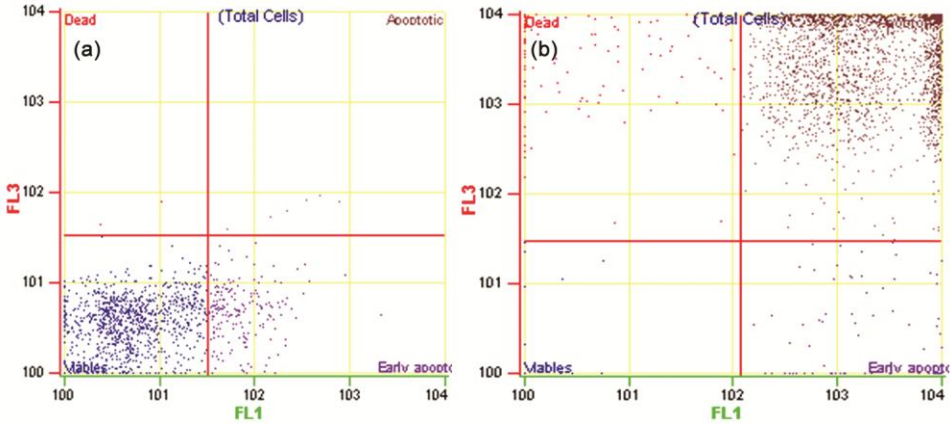


Fig. 5 — Dose and time dependent cycle of early apoptosis- apoptosis- death among C1721 mammary carcinoma cells using purified arginase derived from *S. plicatus* KAR 73; Flow cytometric analysis was conducted using Annexin V-FITC/PI for (a) After 1 h showing number of viable cell, early apoptotic and apoptotic cells (b) After 24 h showing quantum of early apoptotic, apoptotic and dead cell

Arginosuccinate synthase thereby proving anticancer activity of arginase derived from *S. plicatus* KAR 73 in this study.

Conclusions

The arginase derived from *Streptomyces plicatus* KAR 73, as illustrated vide this study, is facilitatory to anticancer research and its industrial applications. The arginase produced herein is within the size range of human arginase thus assuring it to be bioacceptable with special reference to antigenicity. Another highlight of this study is physiologically ambient similarity of *Streptomyces* sp. borne arginase with human arginase as urea and ornithine are byproduct of both these unlike commercially available pegylated arginine deiminase having outcome as citrulline and ammonia. It is hence concluded that *in vitro* effect of arginine deprivation on adenocarcinoma cell lines through native arginase is a promising way forward to the carcinoma therapy. The ray of hope for the public health based utility of arginase in cancer treatment is indeed bright since autophagy targeting drugs in combination with standard chemotherapies will add to the multipronged approach for cancer therapy.

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