Entrapment of L-Arginase in Alginate Beads: A Promising Way Forward as Anticancer Agent

Original Research Article

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ABSTRACT

Multiple applicability of therapeutic potential obtained from L-arginase, especially highly purified ones, is being widely appreciated these days in scientific fields. Due to lesser physiological stability and its non acceptance in human body due to allergic reactions, its use is however hampered. Hence, this study, while hypothesizing that enabling entrapment of L-arginase in adequate matrix may be biologically acceptable, aimed to immobilise the L-rginase produced by Actinomycete and to assess its stability at varied pH, temperature and in serum under *in vitro* conditions. The alginate beads formed herein were of uniform size (4.00 mm in external diameter) and have shown entrapment efficiency of 84.23±0.63 %.The stability of entrapped L-arginase at different pH and temperature was found to be significantly increased/ high (p value 0.001). The enzyme retained its cent percent activity even after 5 hours of preincubation of alginate beads under spectrum of conditions. No loss in activity of L-arginase occurred upon its incubation in commercially available fetal bovine serum. This study hence opens vistas of further research opportunities in alleviation of suffering of cancer patients as alginate entrapment has proven longer bioavailability and hence greater efficiency cum effectiveness of L-arginase herein as anticancer agent.

KEYWORDS: alginate beads, anticancer, arginase, immobilization.

INTRODUCTION:

L-arginase also known as L arginine amidinohydrolase, (EC 3.5.3.1) is being reported from several microorganisms, plants and animals[1-3]. Production and use of microbial enzymes have gained importance as higher yield of enzymes can be achieved in lesser time, limited space and possibility of genetic manipulation in them. Also, enzymes with desired properties can be obtained from them. L-arginase is produced by a variety of microorganisms such as *Aspergillus nidulans*, *Penicillium chrysogenum* KJ185377.1, *Helicobacter pylori Bacillus licheniformis, Bacillus subtilis* KY 3281, *Bacillus caldovelox* and *Rhodobacter*, *Streptomyces calvuligerus*[4-6].

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It has been demonstrated that purified enzyme has remarkable anticancer activity as evidenced through experimentation on carcinoma cell lines. The molecular aspects, cytotoxicity assays, antigenicity profiling, tolerance and clinical effects of L-arginine depletion induced starvation have also been analysed at various researchers[7]. However, the sources of L-arginase have not been mostly from Actinomycetes and similar genera. The abundance, availability and saprophytic characteristic of Actinomycetes makes the commercial viability enhanced by multifold and thus this study.

L-arginase production, purification and downstream processing is labour intensive thereby requiring high cost inputs. Immobilization of L arginase in biocompatible substance like alginate may offer a promising solution for its application in several industries involved in research cum application of new initiatives for cancer treatment. In view of the above, the present research aimed to entrap L-arginase of Actinomycete and thereafter, evaluate the effect of entrapment on its activity thereby devising a

strategically competent tool enabling biomechanical and biochemical intervention in biological settings.

MATERIALS AND METHODS:

Media and culture condition. The Actinomycete isolate number 21 was grown on Soyabean meal broth for production of arginase. Inoculum (5% V/V) density 2 x 10^6 spores/mL (equivalent to 0.5 OD at 700nm) was inoculate into the medium and incubated at 28±2˚C for 8 days under shake conditions (150 rpm).

Preparation of L-arginase for immobilization L-arginase for immobilization process was obtained by centrifugation of fermented broth at 5000 rpm for 10 minutes at 4˚C followed by filtration of supernatant through Millipore syringe filters (0.22µm). The crude enzyme was concentrated by acetone precipitation method at 4˚C. The complete precipitation was achieved at 80% saturation. The precipitate was recovered by centrifugation at 10000rpm for 10 minutes at 4˚C. The precipitate was dialysed and used for further analysis.

Immobilization of L-Arginase on calcium alginate: The preparation of beads was done using sodium alginate (3.4% w/v) dissolved in phosphate buffer (0.02M) followed by autoclaving at 121 lbs for 15 minutes. The solution of 0.08M $CaCl_2$ was also prepared and autoclaved. To sodium alginate solution appropriate amount of chilled concentrated crude enzyme was added and mixed well to form homogenous solution. The chilled $CaCl_2$ was taken in clean glass beaker and dropwise addition of sodium alginate –crude arginase mix was done, Beads (small sphere) of uniform size having external diameter 4.0 mm were formed. For curing beads were incubated at room temperature for 30 minute. The Calcium alginate beads thus prepared were transferred and stored at 4˚C for further use. The enzyme activity of immobilized enzyme was compared with free enzyme.

Entrapment efficiency:

Entrapment efficiency was determined by dissolving the enzyme-loaded beads in a magnetically stirred PBS without enzyme for 45 minutes at 4˚C. The resulting solution was centrifuged at 5000 rpm for 10 minutes. The supernatant was assayed (n=3) for enzymecontent. The entrapment efficiency was calculated as described below : Entrapment efficiency= (Enzyme loaded/ Theoretical enzyme loaded) x 100

Arginase activity:

Buffered arginine (pH 7.0) was mixed with appropriate fixed amount of enzyme (free/ immobilized) and incubated at different experimental temperature and duration in water bath. On completion of incubation period reaction mix was centrifuged at 1000 rpm for 5 min at 4˚C. 1 ml of supernatant was dispensed in clean glass tube and to it 1 ml of TCA (10% w/v) solution was added to stop the reaction followed by centrifugation at 10,000 rpm for 10 minutes at 4˚C. To 1 ml of supernatant 3ml of chromogenic solution (acidified Diacetylmonoxime (damo)- thiosemicarbazide (TSC)) was added and boiled for 15 minutes at 100˚C. The absorbance was read at 525 nm using Picodrop spectrophotometer. Reaction blank was processed as above, with addition of TCA before incubation. One unit of enzyme activity was defined as that produced 1µmole of urea in 1 hour at 37˚C. The results were calculated in terms of relative activity.

Comparison of free and immobilized L-arginase at different conditions

a. pH

The immobilized L-arginase were incubated at different pH 5-10 at temperature 37˚C for 1 followed by estimation of L-arginase activity taking free enzyme as control. The results are expressed in terms of relative activity.

b. Temperature

The immobilized L-arginase were incubated at different temperature 30˚C, 35˚C, 37˚C, 40˚C, 45˚C and 50˚C for 1 and 5 hours pH 7.0 followed by estimation of L-arginase activity as described before, taking free enzyme as control. The results are expressed in terms of relative activity and residual activity. Study of stability of free and immobilized L-arginase at different conditions

To assess stability of L-arginase at different pH and temperature exposure was given at conditions for 5 hours thereafter residual activity was measured at 37°C and pH 7.0.

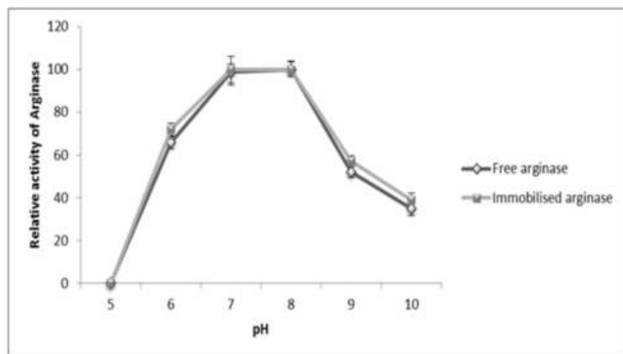
c. Serum stability

The immobilized arginase were mixed with filter sterilized 5ml of commercially available fetal bovine serum (Himedia Pvt. Ltd). The mixture was homogenized vigorously and incubated at 37˚C, for 1 and 5 hours. The L-arginase activity was measures as described previously, taking free enzyme as control.

The results are expressed in terms of relative activity and residual activity.

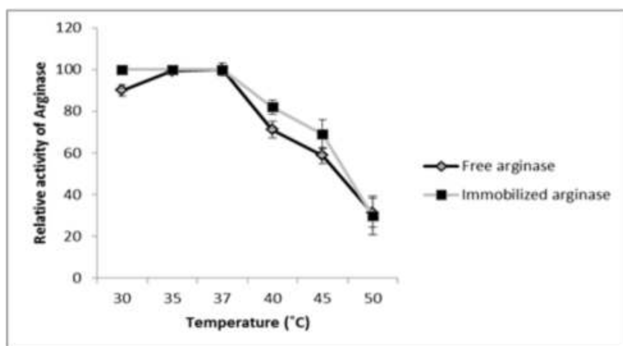
RESULTS:

The calcium alginate beads (4.0 mm diameter) have shown 84.23±0.63% entrapment efficiency of L-arginase enzyme produced by Actinomycete. Crude Enzyme (150 IU) was used for immobilization. No significant change in activity of arginase at different pH (Figure 1) and temperature (Figure 2) upon immobilization was observed, while comparing activities of immobilized L-arginase and free L-arginase. Its worth notice here that there was no loss in L-arginase activity on immobilization.



* *Activity of L-arginase expressed in term of percentage of relative activity*

Figure 1: Comparison of free and immobilised L-arginase activity at different pH at 37°C.

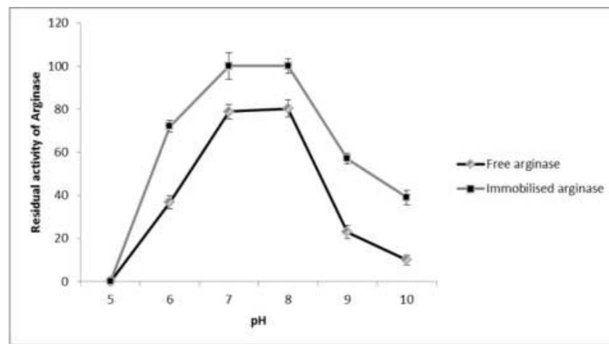


* *Activity of L-arginase expressed in term of percentage of relative activity*

Figure 2: Comparison of free and immobilised L-arginase activity at different temperatures at pH 7.0

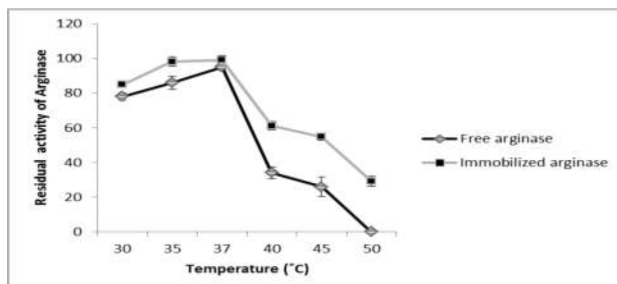
The significant increase in stability of immobilized L-arginase was observed at different pH (Figure 3) although the optimum pH of activity remained similar to that of free enzyme.

Stability of L-arginase under immobilized condition and its comparison with free arginase (Figure 4) was noticeable. It is found that there is sustained stability of arginase in immobilized condition. The studies have shown that complete denaturation of un-immobilized enzyme occurred at 50°C, while more than 20% activity remained with immobilized enzyme.



* *Residual activity of L-arginase measured at 37°C and pH 7 after 5 hours of incubation at different pH*

Figure 3: Stability of free and immobilised L-arginase activity at different temperatures.



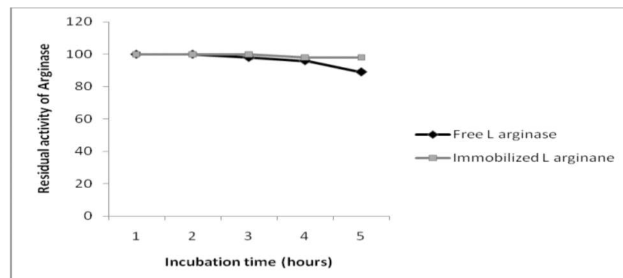
* *Residual activity of L-arginase measured at 37°C and pH 7 after 5 hours of incubation at different temperature.*

Figure 4: Stability of free and immobilised L-arginase activity at different pH.

Both immobilized and free arginase have shown retention of 100% activity in serum under *in vitro* conditions (Figure 5).

DISCUSSION:

The mechanism of action of alginate gels is favorable to the intra and extra cellular ambience thereby ensuring accelerated and targeted bioavailability of the drugs, chemicals and active materials used on medical, dental and biological sciences[8-11]. There is undisputed importance of biocompatibility for the natural products obtained from biological sources or their dependant interventional design based outcome products[12-14]. This is supposed to be so because of their range of applicability, replicable nature and long term use



** Residual activity of L-arginase measured at 37°C and pH 7 after 5 hours of incubation at*

Figure 5: Stability of free and immobilised L-arginase activity in Fetal Bovine Serum under *in vitro* conditions.

especially by industrial settings having a range of products derived through use of such materials[15-17]. Finding of this study, as illustrated herein, affirm that immobilization of L-arginase using alginate is beneficially applicable without loss in activity. The immune-protection obtained from the purified arginases is helpful in ensuring inactive bio-response for the desired reaction through a foreign body for therapeutic, research or other applied use[15].

Even the animal experiments have shown that the commercially available alginate is safe for use and does not produce any antigenic reaction[18]. Alginate beads, formed through addition of calcium chloride with alginate, is biologically acceptable material with longer stability due to its gel nature. The use of alginate, viz as wound healer, drug transport and biomedical engineering, is identified as a precursor, facilitator and enhancer of transport, structural integrity and thereby enhanced potentialities of applicable *in vivo* use[19].

Hence, the alginate provide a multi dimensional platform for smooth flow of designed bio reactive mechanism dependant activity cycles. Its properties and qualities enable a bio-environment suited to continued and un-interfered laboratory friendly settings for series of biochemical and biological activitie[9]. The immobilized L-arginase retained its activity and stability at physiological pH and temperature conditions and may prove beneficial for treatment purposes. This may, in addition to other intrinsic microcellular environmental and precursor enabling factors, be attributed to its property of being converted to gel under simple conditions in the presence of divalent metal ions thereby making this material suitable for immobilization of enzymes, drugs, cells and proteins [20-21]. The intracellular enzyme drug action is dependent on several factors including serum constituents and presence of other enzymes. In addition, the effective enzyme should possess greater and stable half life in serum.

In view of the above, it is worth to consider the augmented research cum industry friendly potential of arginases through interactive platform of multidisciplinary settings for human welfare oriented replicable applications.

CONCLUSION:

Entrapment of Actinomycete L-arginase in alginate beads resulted in enhanced stability of enzyme with retention of activity thus alginate entrapped L-arginase offers easy, inexpensive, portable system with improved shelf-life for treatment of cancer. Treatment involving such systems may prove to be of high commercial and therapeutic value although essentially post preclinical and clinical trials.As a suitable example, this immobilized enzyme maybe better alternative in pharmaceutical industry producing L-ornithine. Likewise, Food Processing Industry may also use it for reducing the amount of acryl-amide formed. Thus, themultidisciplinary platform of stable L-Arginase is a potent promising tool as commercial outcome enhancer.

ACKNOWLEDGMENT:

The authors are thankful to Madhya Pradesh Council of Science and Technology, Bhopal for approving Project proposal and sanctioned grant vide No. 1118/CST/R&D (BS)/2016 dated 13.07.2016 to carry out this study. We also thank People's University, Bhopal, Madhya Pradesh for all the technical and other support provided.

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Cite this article as: Jain R, Gumashta R, Gumashta J, Pandey A, Jain A:Entrapment of L-arginase in Alginate Beads: A Promising Way Forward as Anticancer Agent . PJSR ;2018:11(2):59-63.
Source of Support : Nil, Conflict of Interest: None declared.