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Research Article

Study on Pegylation of Therapeutic Enzyme L-Arginase and its Physiochemical Properties for Improving its Pharmaceutical Characteristics

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ABSTRACT:

Aim: The aims of the present work were to carry out comparative studies on free and immobilized l-arginase obtained from *Idiomarina sp.* **Background:** Therapeutic proteins of microbial origin, though they have good pharmacological activities, were found to be associated with problems of short half –life, immunogenicity and degradation by proteolytic enzymes etc. Pegylation was found to be one of the effective techniques for delivering therapeutic proteins in medicine. Hence in the present study the therapeutic enzyme thus produced was pegylated and evaluated for the presence (retention) of its activity. **Method:** Purified l-arginase isolated from *Idiomarina sediminium H1695* was used for the present study. The enzyme immobilization was carried out using different concentrations of PEG and l-arginase. The enzyme thus obtained was purified, characterized and evaluated for the presence of anti-proliferative activity against A375-C6 and HCT-113 cells by MTT assay. **Results:**The optimum l-arginase: PEG ratio (molar) and reaction period were found to be 1: 50 and 3 h, respectively for effective pegylation of the enzyme. The pegylated enzyme retained 92.75% of the initial arginolytic activity and was highly stable at pH 7.5.0, which is quiet close to physiological pH and at a temperature of 35°C. **Conclusion:** The enzyme remained effective even after pegylation. Animal studies must be carried out to develop the enzyme in the form of chemotherapeutic agent.

KEYWORDS: *Idiomarina sediminium H1695, ornithine carbamoyl transferase, argininosuccinate synthetase, A375-C6 and HCT-113*.

[I] INTRODUCTION:

L-Arginase (EC 3.5.3.1) is a metallo enzyme that catalyzes the irreversible hydrolysis of l-arginine to l-ornithine and urea [1]. It is a final enzyme involved in urea cycle. It is a multi-therapeutic enzyme found to play important role in the treatment of various physiological disorders such as rheumatoid arthritis, allergic asthma, acute neurological disorders, liver injury etc [2-5]. It can also be used to detect the concentrations of l-arginine in the biological fluids for clinical analysis [6].Currently its importance has been increased due to its anti-proliferative activities against wide range of cancer cells. L-Arginase is widely distributed in all kingdoms of life ranging from unicellular prokaryotes to multicellular eukaryotes. But its use for degrading l-arginine dependent tumors is also limited due to its low substrate specificity, short circulatory half-life, lower thermostability and higher antigenicity. Several attempts had been made to overcome the problems associated with therapeutic proteins. Pegylation method is one of the promising technique for delivering bioactive agents such as therapeutic proteins. Coupling of polyethylene molecule to the protein drug increases the molecular size of the drug, decreases the

ultrafiltration through kidneys with increase plasma half-life. It brings about charge modification and epitope shielding and thus decreases the immunogenicity. It also increases the pharmacological properties of the proteins [7]. For example, PEG conjugated recombinant human megakaryocyte growth and development factor, a polypeptide related to thrombopoietin is approximately ten times more potent in-vivo than the unconjugated polypeptide and is active in humans [8]. The present studies aimed at development of suitable therapeutic protein with better pharmacological and physiochemical properties. In our preliminary studies we have isolated l-arginase with good substrate specificity from potential strain [9].Same microorganism was used for the enzyme production. The enzyme thus obtained was purified and was pegylated to improve the therapeutic as well as pharmaceutical properties. It was noticed that most of the therapeutic enzymes looses its activity upon pegylation. Hence the pegylated protein was further evaluated for the presence of its characteristics. PEG was selected as a polymer for covalent modification of the enzyme because of non-toxicity, non-immunogenicity, higher water solubility etc [10].

[II] MATERIALS AND METHODS:

Chemicals used in the present study for the preparation of the media were obtained from Hi-Media laboratories. Remaining chemicals were purchased from Sigma Aldrich, Bengaluru, India.

2.1. Production of l-arginase:

Production of l-arginase was carried using*Idiomarina sediminium H1695* under optimal conditions. The enzyme thus obtained was purified by ammonium sulphate fractionation followed by ion exchange and gel filtration chromatography. The purity was further confirmed by SDS PAGE [11].

2.2. Modification of enzyme

Pegylation of l-arginase and its characterization : L-Arginase immobilization was carried out using different molar ratios of drug and the polymer (1:100, 1:50 and 1:20) at two different reaction times (24h and 3h) to ensure maximal immobilization.One international unit of l-arginase is defined as the amount of enzyme that can produce 1 mol urea/minute at 30°C, pH 8.5.

Purification of PEG –arginase

The mixture was concentrated by using amicon ultra -50 and further purified by anion exchange chromatography.

Molecular weight analysis of l-arginase and PEG-arginase

The products of pegylation were analyzed by automated gel electrophoresis.

L-Arginase activity and enzyme measurement

The arginase activity was measured by estimating the levels of urea formed by the assay described by Archibald [12]. And the protein concentrations were determined by Lowry's method [13].

2.3.Characterization of PEG- arginase

The immobilized enzyme was subjected to the following tests - effect of p H, temperature, half-life and K_m value.

2.4.In vitro cytotoxicity studies

In vitro cytotoxic assay was carried out by MTT assay method using cell lines A375-C6 and HCT-116 [14].

[III] RESULTS AND DISCUSSIONS:

3.1. Characterization of pegylated Arginase

Moelcular weight of free arginase was found to be 37 k Da in our previous studies. After pegylation, the mixture of pegylated arginase with varied molecular weights were obtained (smear on the SDS PAGE) due to the size distribution of PEG molecules. Less non-pegylated arginase was observed from the molar ratio of 1: 100 and 1: 50 (arginase: mPEG-SS) than 1: 20 (Figure 1 ,lane 3 and 4 refers to 1:20). Furthermore, different reaction times of 3 h and 20 h did not influence the yield of pegylation (Figure 1, lane 1 compares to lane 2, lane 5 compares to lane 6). From these results, the optimum arginase: PEG ratio (molar) and reaction period were found to be 1: 50 and 3 h, respectively.

Non-pegylated arginase was still detectable on SDS-PAGE. Therefore, the solution of arginase after pegylation was concentrated (through a 50 k Da Amicon Ultra-50) and further purified by anion-exchange chromatography. Pegylated arginase did not bind to the column due to the covered charges by PEG molecules; thereby it was obtained from the flow through. The pegylated arginase and arginase were tested by gel electrophoresis system. Pegylated was well separated from the mixture by anion-exchange chromatography, because there was no non-pegylated arginase noticed from the sample of purified pegylated Arginase. The average molecular weight of PEGylated l-arginase was calculated by automated gel electrophoresis system. Predicted number of PEG molecules attached to Lys were calculated by this equation: Number = [MW pegylated arginase) – MW of (arginase, 37 K Da)] / MW of (mPEG-SS, 5 K Da). The mean molecular weight of pegyalted arginase was found to be 67 k Da. The predicted number of PEG molecules attached to lysine were found to be 6. In previous studies of bioconjugation of a recombinant arginase I from *Pichia pastoris GS115* as the host strain, Xue Zhang *et al* [15] immobilized l-arginase I on the crosslinked chitosan particlesand found out that the enzyme complex exhibited the remarkable thermal and long-term stability as well as broad adaptability to pH . In another study, by Sam-Mui Tsui *et al* [16] on bioconjugation of recombinant human arginase 1 expressed in *Escherichia coli* with linear PEG (Mol Wt 5 k Da), the residual activity was found to be more than 90%.

From the data obtained through our present experimental results, retention of high residual activity, as high as 92.75% even after conjugation is also significant, as possession of high arginolytic activity is desired for an enzyme drug to be suited for administration.

Thus linking the protein drugs to a polymers such as PEG is a promising technique for a targeted drug delivery.

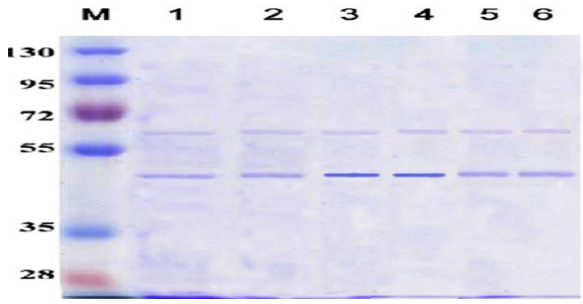

Figure 1: SDS PAGE results of pegylated l-arginase

Literature reports suggest that drugs weighing less than 40 k Da, unless bound to plasma protein, are cleared rapidly into urine, whereas drugs weighing more than 40 k Da usually have long t ½. However, accumulations of drugs should be kept in mind if used for long durations. Moreover, drugs > 40 K Da are taken inside the cell by endocytosis, transported to lysosomes and the active component is released by proteolytic enzyme at lower p H [17]. Newly formed tumour vessels are abnormal in form and architecture of fluid transport dynamics (especially tumour tissues lack effective lymphatic drainage), which results in enhanced permeability and retentions of macromolecules in the tumour cells. Lack of tumour selectivity is the major limitation inherent to most conventional anticancer chemotherapeutic agents. Pegylated drugs can target solid tumours selectively by exploiting these abnormalities of tumour vasculature.

Effect of p H on the enzyme stability

For the development of an effective chemotherapeutic drug, the main criteria is its stability at physiological p H and temperature. The effect of various p H on the enzyme activity is given in the following figure 2. The enzyme retained its maximal activity at physiological pH even after pegylation. Change in the pH did not show much effect on the activity and the stability of the pegylated enzyme.

Moreover optimum pH stability of native and PEG-Arg between 6.0–10, seems to be favored criterion, since the pH of blood plasma around 7.0–7.5. L-Arginase from *Rhodobactercapsulatus* has pHstability at 8.5–9.0 [18]. The slight alkaline

stability of theenzyme seems to be comparable to its relative basic aminoacids constitution.

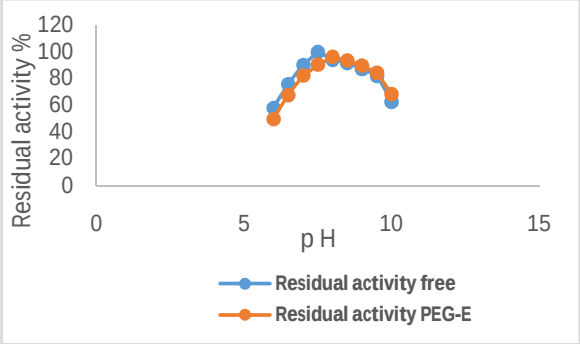

Figure 2: Effect of p H on activity of pegylated arginase

The effect of various temperatures on the stability of the pegylated arginase is shown in the figure 3. The thermal stability of the enzyme increased after pegylation. More over the immobilized enzyme retained 92.74 % of residual activity at 37^0C which is suitable for a therapeutic drug.

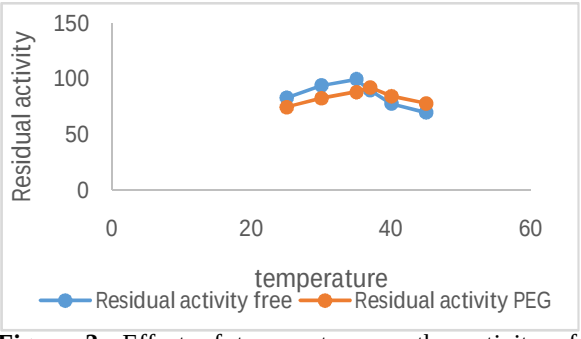

Figure 3: Effect of temperature on the activity of pegylated l-arginase.

Determination of half-life of pegylated l-arginase

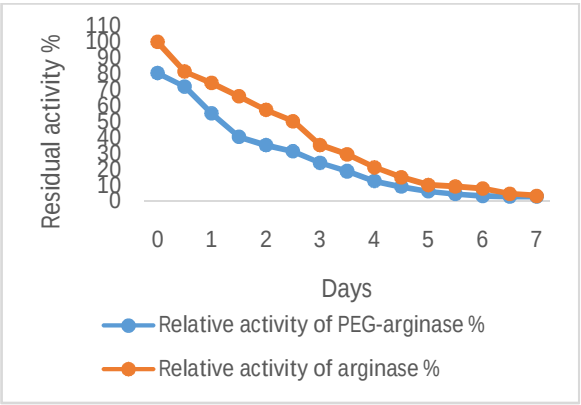

Figure 4: $T_{½}$ of pegylated arginase.

Solutions of both the enzymes were prepared in the PBS buffer and were incubated at 37^0 C to measure the half -life activity of both the enzymes. Half-life values of non-PEGylated arginase and pegylated arginase in PBS buffer were found to be 1.5 and 2.5 days respectively. Pegylation of the enzyme increased its half life.

In the studies carried out by R. Philip et al on arginase [19], pegylation resulted in a 40 % drop in the specific activity, although the enzyme remained more stable and active for more than 8 days.

Enzyme	$T_{½}$ Half values in PBS buffer (days)
Non-pegylated-arginase	1.5
Pegylated l-arginase	2.5

Table 1: The half life of the free and pegylated arginase.

K_m and V_{max} values of pegylated l-arginase

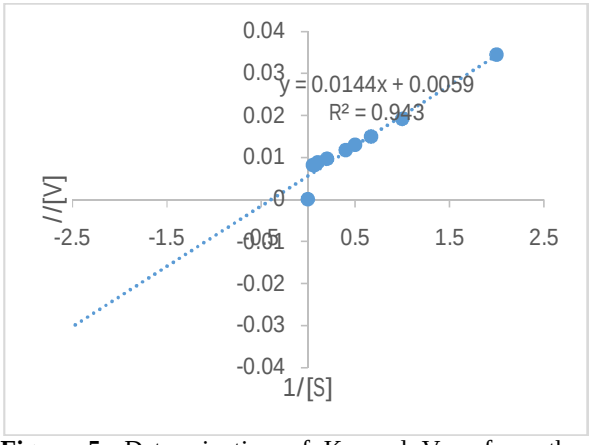

Figure 5: Determination of K_m and V_{max} from the Lineweaver-Burkedouble reciprocal plot for immobilized enzyme

K_m and V_{max} values were found to be 2.44mM and 169.49 U/ml/min respectively. The K_m value of the l-arginase was found to be slightly increased upon pegylation, which suggested that the attached PEG molecules on the surface of the enzyme did not significantly reduce the affinity for the substrate. Free arginase was found to have high substrate affinity when compared to immobilized arginase. The results obtained in our study coincides with that of Ashraf el sayed et al [20].

3.2.In vitro anti-proliferative activity: The results of anti-proliferative activity are given under the table 2

Cancer cell line	IC_{50}
A375-C6	4.24±0.11
HCT-116	5.12±0.21

Table 2: Results of MTT assay

The antiproliferative efficacy of the PEG l-arginase was reduced slightly when compared to free enzyme. Same type of results were obtained in the studies carried out by A. S. E-Sayed et al. They showed that the antiproliferative efficiency of the L-Arginase was reduced by two and three fold for HEPG-2 and A549, respectively, compared to free enzyme upon pegylation .

Pegylation studies carried out by Paul Ning-Man Cheng et al on Recombinant human Arginase expressed in *B. subtilis* showed that pegylated rhArg (rhArg-peg5,000mw) gave similar anticancer efficacy in vitro [21]. Furthermore, they showed that the growth of the OTC-deficient Hep3B tumor cells (ASS-positive and ADI resistant) in mice was inhibited by treatment with rhArgpeg5,000mw, which is active alone and is synergistic in combination with 5-fluorouracil. Thus, our data suggest that rhArg-peg 5,000mw is a novel agent for effective cancer therapy.

Arginine auxotrophy and sensitivity to arginine deprivation have been demonstrated in a number of tumor types including melanoma, hepatocellular carcinoma (HCC), renal cell carcinoma (RCC), prostate cancer and acute lymphoid leukemia (T-ALL). Several studies have shown that these tumor types are sensitive to arginine depletion-induced cytotoxicity following treatment with human arginase or with bacterial arginine deiminase demonstrating that amino acid deprivation in auxotrophic tumors is a novel targeted therapy [22]. Sensitivity of cells to arginine deprivation may be due solely to complete arginine auxotrophy (total lack of ASS-1 expression) or to a combination of partial arginine auxotrophy (low expression of ASS-1) coupled to a high cellular proliferation rate, rendering cells dependent on extracellular sources of arginine.

[IV]CONCLUSION:

The experimental results obtained by this work showed an increase in the molecular weight of the conjugate in comparison to that of the unmodified arginase, indicating formation of PEG 5K-l-arginase conjugates, which were highly stable at pH 7.5, which is quiet close to the physiological pH, and at temperature of 35°C. This conjugate can be further tested for its pharmacodynamic and pharmacokinetic properties through clinical trials for its therapeutic usage.

FINANCIAL DISCLOSURE: NIL

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REFERENCES:

1. Esch, F., Lin, K.I., Hills, A., Zaman, K., Baraban, J.M., Chatterjee, S., Rubin, L., Ash, D.E. and Ratan, R.R. Purification of a multipotent antideath activity from bovine liver and its identification as arginase: nitric oxide-independent inhibition of neuronal apoptosis. *J Neurosci.*, 1998;18(11):4083-4095.
2. Corraliza, I. and Moncada, S. Increased expression of arginase II in patients with different forms of arthritis. Implications of the regulation of nitric oxide. *The Journal of Rheumatology*, 2002; 29(11):2261-2265.
3. Esch, F., Lin, K.I., Hills, A., Zaman, K., Baraban, J.M., Chatterjee, S., Rubin, L., Ash, D.E. and Ratan, R.R. Purification of a multipotent antideath activity from bovine liver and its identification as arginase: nitric oxide-independent inhibition of neuronal apoptosis. *J Neurosci.*, 1998;18(11):4083-4095.
4. Munder, M. Role of arginase in asthma: potential clinical applications. *Expert Review of Clinical Pharmacology*, 2010; 3(1):17-23.
5. Morris, C.R., Poljakovic, M., Lavrisha, L., Machado, L., Kuypers, F.A. and Morris, S.M. Jr. Decreased Arginine Bioavailability and Increased Serum Arginase Activity in Asthma. *American Journal of Respiratory and Critical Care Medicine*, 2004; 170:148-153.

6. Kuldeep Kumar, Teena Phathak and Shefali Walia. L-arginase Based Biosensor for Detection of L-arginine in Juice Samples. *J. Nat. Prod. Plant Resour*. 2012, 2 (4):494-499.
7. Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly (ethyleneglycol) protein conjugates. Adv. Drug Deliv. Rev. 2003; 55: 1261–1277
8. Fanucchi, M., Glaspy, J., Crawford, J., Garst, J., Figlin, R., Sher- idan, W., Menchaca, D., Tomita, D., Ozer, H., and Harker, L.(1997) Effects of polyethelene glycol-conjugated recombinant human megakaryocyte growth and development factor on platelet counts after chemotherapy for lung cancer. *New Engl. J. Med*.**336,** 404–409.
9. Rahamat unissa, M. Sudhakar and a. Sunil kumar reddy. Selective isolation and molecular identification of l-arginase producing bacteria from marine sediments. World journal of pharmacy and pharmaceutical sciences,2015; (4), Issue 06, 998-1006**.**
10. Giri Tapan kumar et al. Biotechnology based drug delivery by pegylation method.IJRAP.2000; 2(1):95-102.
11. Rahamat unissa, M. Sudhakar and A. Sunil kumar reddy optimized production of l-arginase: a tumour inhibitor Isolated from marine bacteria Int J Pharm Bio sci 2015 July; 6(2): (p) 506 – 517.
12. Archibald. R.M. Colorimetric determination of urea. J. Biol Chem, 157:507- 518, (1945).
13. Lowry OH, Rosebrough NN, Farr AL, Randall RY. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193:265-275.
14. Mosmann T. *J Immunol Methods*. 1983; 65:55-63.
15. Xue Zhang et al. High-level expression of human arginase I in *Pichia pastoris* and its immobilization on chitosan to produce L-ornithine. Zhang et al. BMC Biotechnology (2015) 15:66.
16. Sam-Mui Tsui et al. Pegylated derivatives of recombinant human arginase (rhArg1) for sustained in vivo activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics *in vivo* and *in vitro* and action upon hepatocellular carcinoma cell (HCC). *Cancer Cell International* 2009, **9**:9.
17. Greish K, Fang J, Inutsuka T, Nagamitsu A and Maeda H. Macro molecular therapeutics advantages and prospects with special emphasis on solid tumor targeting . Clin Pharmacokinet 2003; 42(13): 1089-105.
18. Moreno-Vivian, C., G. Soler, and F. Castillo. 1992. Arginine catabolism in the phototrophic bacterium *Rhodobacter capsulatus E1F1*. Purification and properties of arginase. European Journal of Biochemistry 204(2): 531–537.
19. R Philip1, E Campbell1 and DN Wheatley. Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures. British Journal of Cancer (2003) 88, 613 – 623.
20. Ashraf S. El-Sayed et al. Purification and immobilization of L-arginase from thermotolerant *Penicillium chrysogenum KJ185377.1*; with unique kinetic properties as thermostable anticancer enzyme. Arch. Pharm. Res. DOI 10.1007/s12272-014-0498-y.
21. Cheng PN, Lam TL, Lam WM, et al. Pegylated recombinant human arginase (rhArg-peg 5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res.* 2007; 67(1):309-317.
22. Kim, J.E., D.W. Jeong, and H.J. Lee. 2007. Expression, purification, and characterization of arginine deiminase from *Lactococcus lactis ssp. lactis ATCC 7962* in *Escherichia coli BL21*. Protein Expression and Purification 53(1): 9–15.
23. Cheng P, Wheatley DN: Arginine deprivation addresses the Achilles Heel of Cancer: urea cycle deficiencies in melanoma, HCC and other cancers.*Oncology News* 2008, 2(4):6-8.