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Enhanced Plasma Persistence of Enzyme with Therapeutic Potential Using Polyethylene Glycol as a Coupling Agent

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ABSTRACT

Therapeutic proteins of microbial origin though possess good pharmacological activities, gets quickly broken down into amino acids by the action of proteases in the body. Hence to protect its activity and integrity the enzyme L-arginine deiminase (ADI) was coupled with a compatible polymer methoxypolyethylene glycol-succinimidyl succinate (mPEG-SS) and was further evaluated for the presence of its activity. Enzyme production was carried out by culturing *Vibrio alginolyticus* under optimal conditions and was purified by standard chromatographic techniques. The purified enzyme obtained was coupled with mPEG-SS 20k (1:50 mM) and purified. Coupling increased the molecular weight of the enzyme to about 112 kDa. The K_m and V_{max} values of immobilized enzyme were found to be 2.94 ± 0.13 mM and 129.87 ± 1.24 U/mL/min, respectively. Though there was a slight change in K_m and V_{max} values, it still retained its activity. Further, there was two-fold increase in the plasma half-lives of the enzymes. L-Arginine deiminase of *Vibrio alginolyticus* remained effective even after pegylation and showed enhanced plasma persistence in normal as well as tumour bearing mice. Hence it can be a promising candidate in treatment of L-arginine auxotrophic cancers. Further studies must be carried out to develop the enzyme in the form of chemotherapeutic agent.

Introduction

Enzymes of microbial origin though possess good pharmacological activities; their usage and application as therapeutic agents are still limited due to their short plasma half-life.[1] As these enzymes are proteinaceous, they get easily broken down by the action of other enzymes present in the blood and suffered from short plasma half-life. Several approaches for extending their retention in the systemic circulation have been tried, out of which pegylation was found to be one of the promising techniques.[2, 3]

In 1975, Vegarud and Christensen showed that various bacterial enzymes had increased stability towards thermal and proteolytic degradation *in vitro* when immobilized with polysaccharides, and heparin was shown to have a prolonged half-life *in vivo* when linked to the sucrose polymer.[4,5] Hence plasma half-life of the enzymes could be prolonged by coupling the enzymes with suitable carrier like chitosan, dextran and polyethylene glycol (PEG).

The process of linking an enzyme to polyethylene glycol is known as pegylation. It is usually preferred over other conventional techniques due to its ease of manufacture, ease of administration, prolonged retention in the body, decreased degradation by metabolic enzymes and reduction of protein immunogenicity.[1] Polyethylene glycol (PEG) at present is considered as the best polymer for protein conjugation.[6]

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]

In our previous studies, we isolated l-arginine deiminase (E.C.3.5.3.6) (a metalloenzyme of molecular weight 48 kDa) from marine isolates, optimized enzyme immobilization and evaluated its characteristics.[9,10] In order to extend its plasma half-life, in the present study we have immobilized the enzyme with PEG and further tested for its activity.

Materials and Methods

Chemicals and reagents

The reagents employed in the current investigation were of high grade and quality. Media components used for the preparation of bacteriological medium were of Hi- Media Labs. Remaining chemicals belongs to Sigma Aldrich, Bangalore.

Animals

Female mice (6-week-old) (25-30 g) were obtained from the departmental animal facility where they were housed under standard husbandry conditions (25 ± 2°C temp., 60-70% relative humidity and 12 h photoperiod) with standard mice feed and water *ad libitum*. Both normal and tumour induced mice were used in the present study. Some of the healthy mice were infected with the tumour cells by injecting 1 x 10^6 P815 mastocytoma cells (per animal) intraperitoneally. Experiments were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the Institutional Animal Ethics Committee (CPCSEA/1217/2008/a).

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Enzyme immobilization

L-arginine deiminase (ADI) from *Vibrio alginolyticus* 1374 was purified to near homogeneity and confirmed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was coupled to mPEG-SS 20 kDa.

Protein solution (0.5 mg/mL) was prepared in 50 mM phosphate buffer. The resultant solution was treated with PEG reagents in a molar ratio of 1:50 and mixed for 3 h at 4ºC. The mixture was washed thrice with phosphate buffer saline using an ultracentrifugal filter. The purity of the immobilized enzyme was checked using automated gel electrophoresis. Protein and enzyme contents of the eluted fractions were determined by the methods of Lowry *et al.* and Oginsky.[11,12]

Assay of ADI activity

ADI activity was assayed at 37ºC with a reaction time of 30 min in a 200 µL reaction mixture (25 µL of 80 mM diacetyl monoxime and 25 µL 2 mM thiosemicarbazide in deionized water and 50 µL 3 M phosphoric acid, 50 µL 6 M sulphuric acid and 50 µL 2 mM ferric ammonium sulphate dodecahydrate) containing 60 µL of test sample mixture (40 µL extract and 20 µL of 10 mM arginine solution). The release of l-citrulline was monitored using a spectrophotometer at a wavelength of 530 nm based on the method of Oginsky.[12] The enzyme protein content was determined by Folin's reagent.[11]

Enzyme persistence in vivo

Four groups of four 6-week-old female mice were inoculated intravenously with 1 mL of enzyme (two groups with 30 units of native enzyme and two groups with 36 units of PEG -linked enzyme). Fifth group (control group) were given phosphate-buffered 0.9% NaCl. Blood samples (10 µL) were taken from individual animals at regular intervals (12 hours) and assayed for enzyme activity.

In a second experiment, four groups of three tumour bearing mice were inoculated intravenously with 1 mL of the enzymes (first two groups with 30 units of native ADI and last two with 36 units of PEG-ADI). Fifth group was taken as control group. Blood samples were taken at regular intervals and tested for enzyme activity using a spectrophotometer at a wavelength of 530 nm.

Results and Discussion

Molecular weight of free ADI was found to be 48 kDa in our preliminary study. [9] N-hydroxylsuccinimide (NHS) active esters, such as mPEG-SS (mPEG-Succinimidyl Succinate) are used in common for the pegylation of therapeutic enzymes. Therefore, the mPEG-SS was used for the immobilization of ADI in the present study. The products of immobilization of ADI were resolved by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis), which showed that the size of ADI after PEGylation (112 kDa) was increased compared to non-PEGylated ADI (48 kDa).

Non-PEGylated ADI was still detectable on SDS-PAGE. Therefore, the solution of enzyme after PEGylation was concentrated (through a 50 kDa Amicon Ultra-50 centrifuge) and further purified by anion-exchange chromatography. PEG-ADI did not bind to the anionic column due to the covered charges by PEG molecules, thereby it was obtained from the flow through (Figure 1a). The PEG-ADI and ADI were tested by gel electrophoresis system. PEGylated ADI was well separated from the mixture by anion-exchange chromatography, because there was no non-PEGylated ADI noticed from the sample of purified PEGylated ADI.

The approximate molecular weight of the native ADI (mol. wt. 48 kDa) and coupled ADI (from the anionic exchange chromatography) on the SDS-PAGE calibrated with standard marker proteins were found to be 48 kDa and 112 kDa, respectively (Figure 1b). Molecular-weight data from the SDS-PAGE indicated that 3.1 of PEG molecules were bound to 1 mol of ADI respectively. Fraction collected from the anion exchange column was further purified by passing through the column of Sephacryl S-200 by gel column chromatography (Figure 1c) and lyophilized. The immobilized enzyme was shown to retain 95% of the enzyme activity.

Experiments with mice showed that the clearance rates of the pegylated enzyme preparations from both normal and tumour-bearing animals were two-fold slower (ADI) compared with tumour-bearing animals given the native enzyme and were considerably slower than normal animals given native enzyme (Tables 1). The observation that the native enzymes are more rapidly cleared from normal mice than from those bearing a tumour by a factor of approximately 2, has been found on several occasions.

Takaku *et al* were the first to demonstrate the prolonged plasma clearance time of pegylated ADI compared to native ADI in mice. In their study, they showed that the immobilized enzyme exhibited decrease in the

Table 1: Persistence of native enzyme and Pegylated-enzyme conjugates in normal and tumour-bearing mice.

Enzyme	$t_{1/2}$ (days)	
	Normal mice	Tumour bearing mice
Native enzyme	2.20 ± 0.12	4.70 ± 0.30
Immobilized enzyme	4.60 ± 0.21	9.90 ± 0.23

Half-lives ($t_{1/2}$) of the enzymes were calculated from logarithmic plots of enzyme activity in the blood against time, and are shown as means ± S.D. of results from groups of three or four mice.

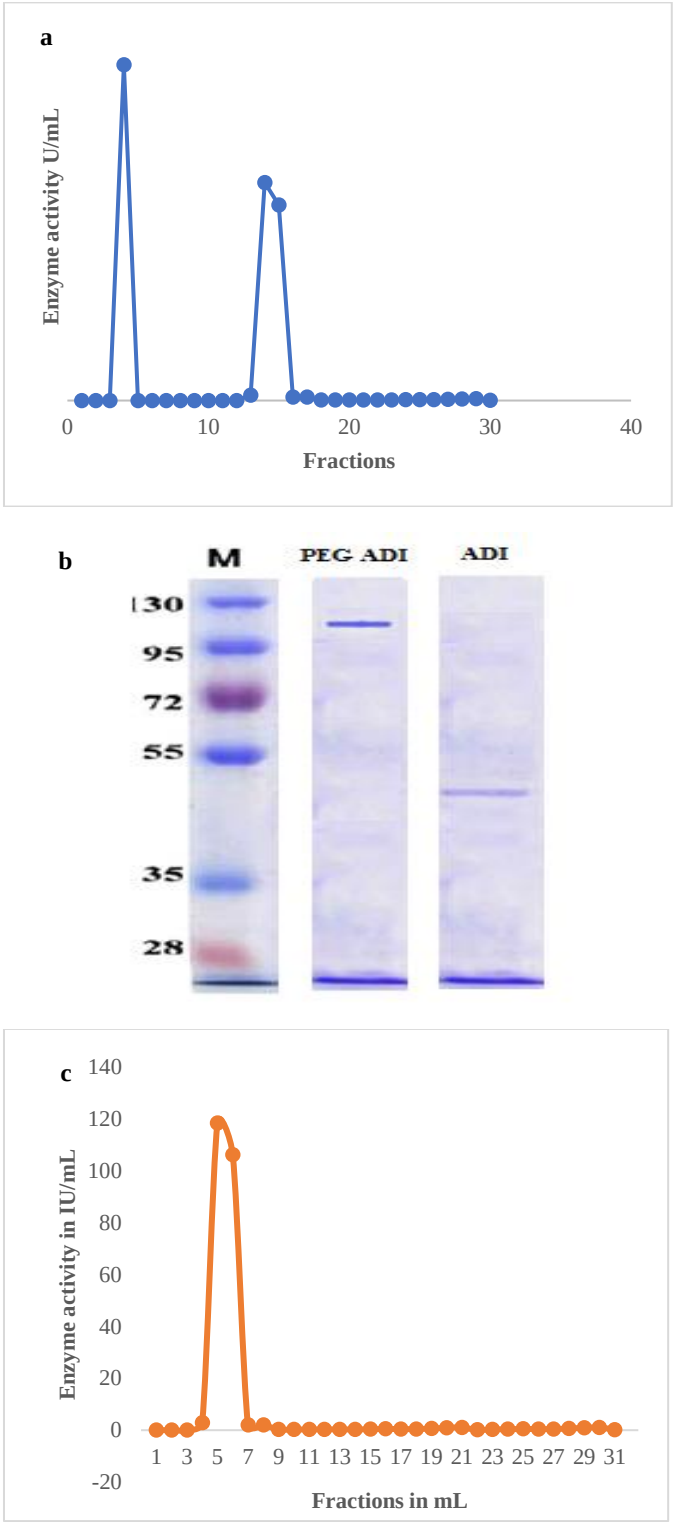

Figure 1 (a): Elution profile of PEG ADI using anion exchange chromatography (b): Molecular weight analysis by SDS PAGE. (c): Elution profile of PEG ADI in the gel chromatography.

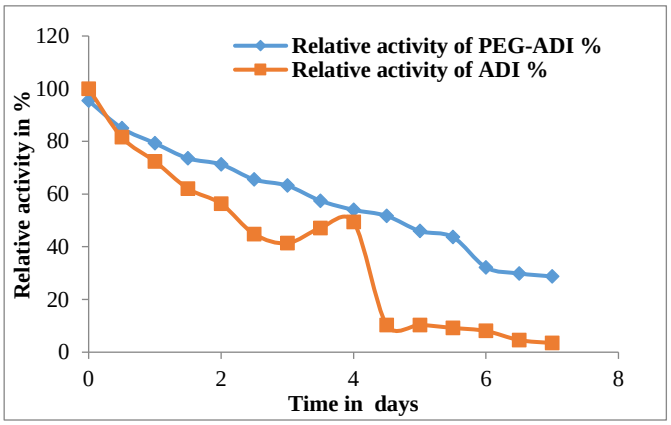

Figure 2: Determination of plama half life of the enzymes in normal mice.

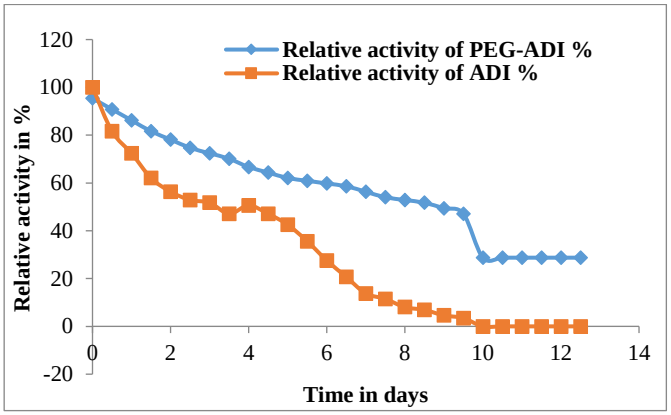

Figure 3: Determination of plama half life of the enzymes in tumour induced mice.

immunogenicity when compared to the free enzyme.[13, 14] Furthermore, pegylated ADI also inhibited the growth of human melanoma and hepatocellular carcinoma *in vivo* more efficiently than native ADI due to the longer circulation half-life.[15, 16]

In the study by El-Sayed *et al* on ADI isolated from thermophilic *Aspergillus fumigatus* KJ434941, there was an overall three-fold increase in the plasma half-life of the enzyme upon pegylation.[17]

The kinetic data of K_m and V_{max} values of PEGylated ADI was found to be 2.94 ± 0.13 mM and 129.87 ± 1.24 µmol/min, respectively. The K_m value of the ADI 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 ADI was found to have high substrate affinity when compared to immobilized ADI. The Michaelis-Menten constants (K_m) of ADI for various arginine concentrations were low (K_m = 1.51 ± 0.23 mM and V_{max} = 120.48 ± 3.24 µmol/min, respectively) and comparatively small amounts would be required to deplete total plasma arginine. However, it is expected that enzyme activity would have to be maintained for long periods to ensure depletion of the larger amino acid pool present in the circulation. The use of the conjugated ADI with its initially greater uptake and longer half-life would facilitate removal of this pool without the necessity of administering very high doses at frequent intervals with the risks that such schedules may involve.

Arginine analyses on blood samples from tumour bearing mice treated with native enzyme showed only a transient lowering in concentration 1-2 h after administration of the enzyme whereas with the PEG ADI-treated animals arginine concentrations were still decreased after 13 h (Figures 2 and 3). The incomplete removal of arginine (about 50%) was probably a reflection of the high K_m of the enzyme.

Conclusion

Covalent attachment of enzyme to PEG provides a straightforward and rapid method of enzyme immobilization. L-Arginine deiminase of *Vibrio sp.* remained effective even after pegylation and showed enhanced plasma persistence in normal as well as tumour bearing mice. Hence it can be a promising candidate in treatment of l-arginine auxotrophic cancers. Further clinical studies must be carried out to develop the enzyme in the form of chemotherapeutic agent.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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References

1. Kumar GT, Prakash NJ, Laxmi N, Krishna TD. Biotechnology based drug delivery by PEGylation method. IJRAP 2011; (1):95-102
2. Pasut G, Veronese FM. State of the art in PEGylation: The great versatility achieved after forty years of research. J Cont Rel. 2012; 61:461-472.
3. Knop K, Hoogenboom R, Fischer D. Poly (ethylene glycol) in drug delivery pros and cons as well as potential alternatives. Angew Chem Int Ed Engl. 2010; 49:6288-6308.
4. Teien A, Odegard OR and Christensen TB. Heparin coupled to albumin, dextran and ficoli influence on blood coagulation and platelets and in vivo duration. Thromb Res. 1975; 7:273-284.
5. Vegarud G and Christensen TB. Glycosylation of proteins: A new method of enzyme stabilization. Biotech Bioeng.1975; 17: 1391-1397.
6. Mishra P, Nayak B, Dey RK. PEGylation in anti-cancer therapy: An overview. Asian J Pharm Sci. 2015; 12:337-348.
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, Harker L. Effects of polyethelene glycol-conjugated recombinant human megakaryocyte growth and development factor on platelet counts after chemotherapy for lung cancer. New Engl J Med.1997; 336:404-409.
9. Unissa R, Sudhakar M, Kumar S, Reddy A. Evaluation of in vitro anti proliferative activity of l-arginine deiminase from novel marine bacterial isolate. BMRJ. 2016; 3(5):1-10.
10. Unissa R, Chauhan AK, Malleshwari A, Vinitha N, Haarika K, Aneesha CS, Navya D, Sree T, Mounika S, Begum MI. Evidence Based Comparative Studies on Free and Immobilized Therapeutic Protein. Biosci Biotech Res Asia. 2017; 14(1):343-348.
11. Lowry OH, Rosebrough NN, Farr AL, Randall RY. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193:265-275.
12. Oginsky EL. Isolation and determination of arginine and citrulline. Meth Enzymol. 1957; 3:639-643.
13. Takaku H, Misawa S, Hayashi H, Miyazaki K. Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from *Mycoplasma arginine*. Jap J Cancer Res. 993; 84(11):1195-1200.
14. Holtsberg FW, Ensor CM, Steiner MR, Bomalaski JS, Clark MA.Poly (ethylene glycol) (PEG) conjugated arginine deiminase: effects of PEG formulations on its pharmacological properties. J Cont Rel. 2002; 80(1-3):259-271.
15. Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA. Pegylated Arginine Deiminase (ADI-SS PEG20,000 mw) Inhibits Human Melanomas and Hepatocellular Carcinomas *in Vitro* and *in Vivo*. Cancer Res. 2002; 62(19):5443-5450.
16. Dillon BJ, Prieto VG, Curley SA, Ensor MC, Holtsberg FW, Bomalaski JS, Clark MA. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation. Cancer 2004; 100(4):26-33.
17. El-Sayed AS, Hassan MN, Nada HM. Purification, Immobilization and Biochemical Characterization of L-Arginine Deiminase from Thermophilic *Aspergillus fumigatus KJ434941*: Anticancer Activity in vitro. Biotechnol Prog. 2015; 31(2):396-405.