International Journal of Pharmacology and Pharmaceutical Technology

Manuscript 1010

Study on Pegylation of Therapuetic Enzyme Uricase And Its Physio-Chemical Properties For Improving Its Pharmaceutical Characteristics

Pooja Nanda

Study on Pegylation of Therapuetic Enzyme Uricase And Its Physio-Chemical Properties For Improving Its Pharmaceutical Characteristics

Pooja Nanda & P.E.Jagdeesh Babu

Department of Chemical Engineering, National Institute of Technology Karnataka, Srinivasnagar, Surathkal, Karnataka- 575 025, India

Abstract **-** Uricase (urate oxidase EC 1.7.3.3, UC) catalyses the oxidation of uric acid, a final product of purine catabolism to allantoin. In the present work, Uricase is bioconjugated using PEG (Polyethylene glycol) as a chemical tool for linking for obtaining conjugates with better and desirable pharmaceutical properties. Uricase from *Bacillus fastidisous* (Uc) was modified through PEGylation considering various concentrations of linear polyethyleneglycol-8KDa (PEG-8K). The Uc-PEG 8K conjugates, formed by conjugating Uc and PEG-8K in the ratio of 1:15 retained 87.5% of the initial uricolytic activity, and were highly stable at pH 6.0, which is quiet close to physiological pH and at a temperature of 16°C.

Keywords **-** *Uricase, Gout, PEGylation, Physiochemical Stability*.

I. INTRODUCTION

Uricase (EC 1.7.3.3, UC) is a therapeutic enzyme belonging to the class of the oxidoreductases, which catalyses the oxidation of uric acid, producing allantoin and acting in the purine degradation pathway. Uricase is useful for enzymatic determination of uric acid in biological fluids for clinical analysis [1]. Uricase can be also used as a protein drug to reduce toxic urate accumulation [2]. Immobilized uricase can be used as a uric acid biosensor [3]. Uricase is also used as an additive in commercial formulations of hair coloring agents [4].

Uricase is produced as a truncated, 10 amino acids long, inactive protein fragment in humans and apes, due to a nonsense codon inserted into its gene during the early primate evolution [5]. Due to its absence humans, the plasma concentration of uric acid is rather high [6], and an abnormal raise of this metabolite can promote renal failure and contribute to the development of disease known as gout. The normal serum urate level of uric acid is between 0.13 and 0.46 mM (2.18– 7.7 mg dl^{-1}). If the blood serum concentration of uric acid go beyond these particular levels due to tumor lysis syndrome and dietary reasons then, uric acid tends to precipitate and develop into inflammatory and painful gouty arthritis. The treatment of hyperuricemia has been performed with drugs that induce forced diuresis, urinary alcalinization and ones which decrease amount of uric acid synthesized by inhibiting xanthine oxidase enzyme. However, these treatments can sometimes prove to be inefficient, leading to serious clinical complications [7], [8]. Administration of uricase has proved to be a good alternative to treat gout, but uricase from microorganisms and animals in its native form is highly antigenic, and the chronicle treatment with uricase results in allergic reactions and anaphylactic shock [5]. Uricase administered externally, gets inactivated by proteolysis and hence exhibits a very short plasma half life. This problem can be overcome by conjugation of uricase with PEG (Polyethylene glycol).

Recently studies performed showed that PEGylation is a well succeeded methodology, used to widen the therapeutic and biotechnological uses of proteins. [9], [10], [11], [12], [13]. PEGylation increases the overall size and molecular weight of the protein-PEG conjugate and hence increases the plasma half life by avoiding ultrafiltration through kidneys. It brings about charge modification and epitope shielding and thus diminishes immunogenicity [14]. The administration of the PEGylated conjugate facilitates longer dosage intervals and reduces risk of adverse immunological reactions. Uricases from *Candida utilis* [15], [16], [17], [18], [19], [20], *Arthrobacter protoformiae* [21], *Bacillus fastidiosus* [7], [22] and from mammals [16], [23] have been PEGylated. Presently the PEGylated forms of uricase enzyme commercially available are PEGsitacase (Uricase-PEG 20) [EnzymeRx], KRYSTEXXA™ (Pegloticase),

Puricase® [Savient Pharmaceuticals], PEG_{40}-Uricase [Mountain View Pharmaceuticals].

There are only a few reports of conjugation of Uricase from *Bacillus fastidiosus* to PEG molecules. Uricase from *Bacillus fastidiosus* has been conjugated to linear PEG-5 kDa, branched PEG-10 kDa, Poly (N-acryloylmorpholine) -6 KDa by Schiavon *et al* in 2000 [7] and with monomethoxy-polyethylene glycol modified with NHS ester- 5 kDa by Chun *et al* in 2010 [22]. The present work is a new report on conjugation of Uricase from *Bacillus fastidiosus* with a linear molecule of Polyethylene glycol 8 kDa. Uricase and PEG were conjugated in different concentration ratios and their residual activities were found after conjugation, the stability at various pH and temperature conditions was also evaluated.

II. MATERIALS AND METHODS

A. Materials:

Uricase (Uc) from Bacillus *fastidiosus* (Molecular weight: 35 kDa and Enzymatic activity: 9 U/mg) and linear Polyethylene glycol (PEG-8K) were obtained from Sigma Aldrich Chemical Company (Germany). SDS-PAGE Kit and Reagents were bought from Chromous Biotech (India). Protein Marker (14 kDa-110 kDa) was also obtained from Chromous Biotech (India).

B. Methods:

a) Preparation of Uc-PEG 8K conjugates

Commercially obtained pure unmodified Uricase from *Bacillus fastidiosus* (1 mg/mL) was allowed to react with linear PEG-8K in the concentration ratios of 1:15, 1:20, 1:25 and 1:30 of Uc to PEG-8K, in 100mM sodium borate buffer solution having pH 9.0, as the reaction medium in a final volume of 1 mL. These components of the mixtures were allowed to react at 30°C with slight agitation for 2 hours.

b) SDS-PAGE analysis for the confimation of PEGylation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970) [24] for the determination of molecular weights of the conjugates formed. Staining and detection of protein bands was done using Coomassie blue stain and later destained.

c) Uricase enzyme assay and protein measurement

The enzymatic assay for uricase was carried out by the method described by Mahler *et al* (1955) [25]. To 3 mL of 20 mM boric acid buffer of pH 9.0, 75 µL of 3.57 mM uric acid solution and 20 µL of enzyme solution were added at 25°C. For the blank, 20 µL of buffer was added, instead of the enzyme solution to equate the reaction mixture volumes. The blank and the test solutions were incubated at 25°C for ten minutes, after which the decrease in the uric acid concentration was measured with the aid of a UV-Visible spectrophotometer at 293nm. The difference in absorbance of the test and blank is equivalent to the decrease in uric acid concentration during the enzyme reaction. One unit of enzyme activity was defined as the amount of uricase required to convert 1 µmol of uric acid into allantoin per minute at 25°C and at pH 9.0, considering milli molar extinction co-efficient of uric acid at 293nm as 12.6 $mM^{-1}cm^{-1}$. The activity of the enzyme before and after bioconjugation was determined using this method.

d) Effect of pH on the stability of unmodified uricase and the bioconjugate

The stability of the unmodified uricase and the bioconjugates was tested at different pH values by maintaining them in the following buffers solutions for an hour: for pH 6-7 sodium phosphate buffer, for pH 8-9, Borate buffer and for pH 10 Tris-HCl buffer. The enzymatic activities of the conjugates were determined after one hour of incubation. The pH at which the unmodified uricase showed the highest activity, was considered to possess 100% residual activity, and the activities at other pH values were evaluated with respect to the highest value of residual activity.

e) Effect of temperature on stability of unmodified uricase and the bioconjugate

Unmodified uricase and the bioconjugates were incubated at different temperatures which are as follows 4°C, 16°C, 32°C, 80°C, 100°C for an hour in 100mM sodium borate buffer pH 9. At the end of this incubation, the enzymatic activities of unmodified uricase and the bioconjugates was determined as described in the uricase enzyme assay procedure.

III. RESULTS AND DISCUSSIONS

A. Modification of Uricase by Bioconjugation:

Aliquots of commercially obtained uricase were subjected to bioconjugation reaction with linear PEG-8K used, as described in the method above and the enzyme activities of the conjugates were calculated and compared to that of the unmodified uricase, by considering their activity relative to that of the unmodified uricase whose activity is considered as 100%) as shown in Table 1.

Uc-PEG 8K conjugates retained 87.5% of the initial uricolytic activity when Uc and PEG-8K were reacted in a concentration ratio of 1:15. In previous studies of bioconjugation of a recombinant intracellular uricase from *Bacillus fastidiosus* ATCC 29604, Chun *et al* [19]

used modified NHS ester of the polymer and found out that the complex of mPEG (5 KDa) and uricase showed a residual activity of 65%. In another study, by Schiavon *et al* [7] on bioconjugation of uricase from *Bacillus fastidiosus* with linear PEG (Mol Wt 5 kDa), PEG (Mol wt 10 kDa) and poly (N-acryloylmorpholine) (PAcM) (Mol Wt 6 kDa), the residual activities were 40%, 76% and 24% respectively. From the data obtained through our present experimental results, retention of high residual activity, as high as 87.5% even after conjugation is very significant, as possession of high uricolytic activity is desired for an enzyme drug to be suited for administration.

Table 1. Residual activities after conjugation of Uc with PEG-8K in different concentration ratios

Uc:PEG8K ratio	Residual Activity (%)	Residual Activity (U/mg)
Unmodified Uricase	100	9.000
1:15	87.8	7.902
1:20	18.17	1.635
1:25	37.858	3.407
1:30	63.684	5.731

B) Visualization of Bioconjugates on SDS-PAGE gel and Molecular weight Determination

In the present work, results obtained from SDS-PAGE indicated that there is an increase in molecular weight of the bioconjugates formed, in comparison to the unmodified uricase enzyme, which is evident by visualizing the position of the protein bands formed on SDS-PAGE gels as shown in Fig 1.

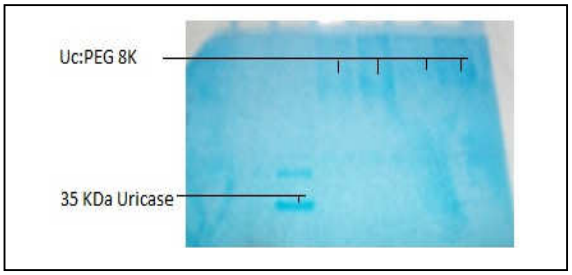

Figure 1. SDS-PAGE of Uricase after bioconjugation with PEG-8K. Lane 1: protein marker, Lane 2: unmodified uricase (35 KDa), Lane 3, 4, 5, 6: Bioconjugated complexes in various ratios mentioned earlier.

C) Stability of native and modified uricase at various pH

In the present work, aliquots of bioconjugates formed in the ratio of 1:15 of Uc:PEG 8K was choosen for stability testing based on the possession of maximum residual activities among all the other bioconjugates formed. The choosen bioconjugates were subjected to further studies of stability in various buffers of different pH values, in order to find an appropriate buffer which can be used for storage and maintenance of the bioconjugates for a prolonged time. Uc-PEG 8K bioconjugate was stable at pH 6.0 as indicated in Fig 2.

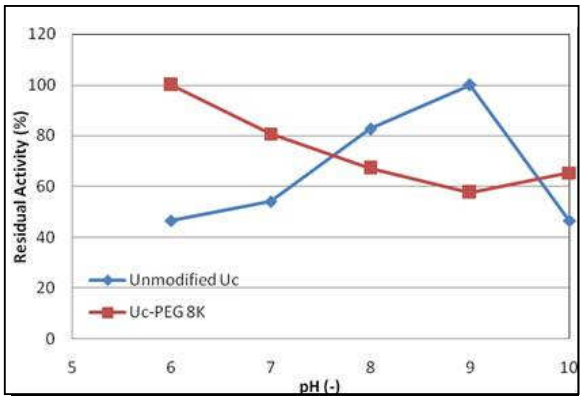

Figure 2. Stability of Unmodified uricase and Uc:PEG-8K at various pH values

In previous studies, Chun *et al* [19] observed that, the unmodified *Bacillus fastidiosus* ATCC 29604 uricase, had an optimum pH slightly below 9.2, which was not altered by modification with NHS ester of mPEG 5K or mPEG 350.

D. Stability of native and modified uricase at various temperatures

In the present work, aliquots of bioconjugates formed in the ratio of 1:15 of Uc:PEG-8K was choosen for stability testing based on the possession of maximum residual activities among all the other bioconjugates formed. In this trial, aliquots of bioconjugates formed, Uc:PEG-8K (in the concentration ratio of 1:15) were subjected to further studies of stability at various temperatures ie: 4°C, 16°C, 32°C, 80°C, 100°C, to investigate the temperature at which the conjugates remain the most stable. Uc-PEG 8K conjugates were most stable at 16 °C as indicated in Fig 3. In previous studies, Chun *et al* [19] observed that the unmodified uricase had an optimum reaction temperature below 25°C, and modification with the NHS esters of mPEG 5K and mPEG 350 with uricase, did not bring about any change in the optimum reaction temperature. But at 40°C in sodium borate buffer of pH 9.2, the unmodified uricase showed a thermo-inactivation half life of about 40 hours. Modification with NHS ester of acetate had no effect on its thermostability, while modification by mPEG350 slightly enhanced its thermostability, and modification by mPEG5K increased its thermo-

inactivation half –life to over 85 hours at 40°C in sodium borate buffer at pH of 9.2.

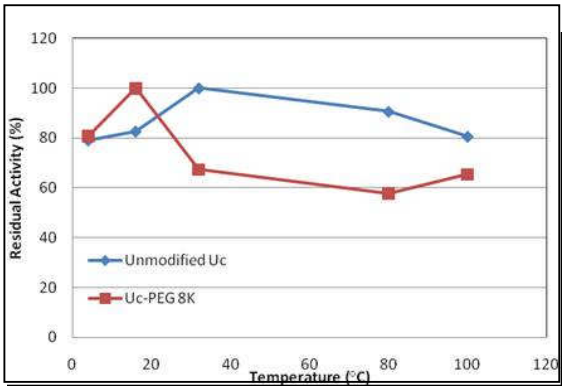

Figure 3. Stability of Unmodified uricase and Uc:PEG 8K at various temperature values

IV. CONCLUSION

The experimental results obtained by this work indicated an increase in the molecular weight of the conjugate in comparison to that of the unmodified uricase, indicating formation of Uc-PEG 8K conjugates, which were highly stable at pH 6.0, which is quiet close to the physiological pH, and at temperature of 16°C. This conjugate can be further characterized and tested for its pharmacodynamic and pharmacokinetic properties through clinical trials for its therapeutic usage.

ACKNOWLEDGMENT

The authors are thankful to the Ministry of Human Resource Development (MHRD), Government of India for financial assistance.

REFERENCES

[1] Gochman N and Schmitz MJ. Automated determination of uric acid, with use of a urease-peroxidase system. Clinical Chemistry. 1971; 17: 1154-1159.

[2] Colloc'h N, el Hajji M, Bachet B, L'Hermite G, Schiltz M, Prange T, Castro B & Mornon JP. Crystal structure of the protein drug urate oxidase-inhibitor complex at 2.05 Aº resolution. Nature Structural Biology. 1997; 4: 947-952.

[3] Fatma A. An Amperometric Biosensor for Uric Acid Determination Prepared From Uricase Immobilized in Polyaniline-PolypyrroleFilm. Sensors. 2008; 8: 5492-5500.

[4] Nakagawa S, Oda H. and Anazawa H. High cell density cultivation and high recombinant protein production of Eschericia coli strain expressing uricase. Bioscience Biotechnology and Biochemistry. 1995; 59: 2263–2267.

[5] Bomalaski JS, Holtsberg FW, Ensor CM, Clarck MA. Uricase formulated with polyethylene glycol (Uricase-PEG 20): biochemical rationale and preclinical studies. J. Rheumatol. 2002; 29: 1942–1948.

[6] Colloc'h N, Girard E, Dhaussy AC, Kahn R, Ascone I, Mezouar M, Fourme R. High pressure macromolecular crystallography: the 140-MPa crystal structure at 2.3 ´°A resolution of urate oxidase, a 135-kDa tetrameric assembly. Biochem. Biophys. Acta. 2006; 1764: 391–397.

[7] Schiavon O, Caliceti P, Ferruti P, Veronese FM. Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugate to linear or branched poly(ethylene glycol) and poly(N-acryloylmorpholine). Farmaco. 2000; 55: 264–269.

[8] Cannella AC, Mikuls TR. Understanding treatments for gout. Am. J. Manag. Care. 2000; 11: 451–458.

[9] Colonna C, Conti B, Perugini P, Pavanetto F, Modena T, Dorati R, Ladarola P, Genta I. Site-directed PEGylation as successful approach to improve the enzyme replacement in the case of prolidase. Int. J. Pharm. 2008; 358: 230–237.

[10] Song S, Liu D, Peng J, Sun Y, Li Z, Gu JR, Xu Y. Peptide ligand-mediated liposome distribution and targeting to EGFR expressing tumor in vivo. Int. J. Pharm. 2008; 363: 155–161.

[11] Veronese FM, Mero A. The impact of PEGylation on biological therapies. Bio. Drugs. 2008; 22: 315–329.

[12] Morille M, Passirani C, Letrou-Bonneval E, Benoit JP, Pitard B. Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting. Int. J. Pharm. 2009; 379: 293–300.

[13] Zhou J, Cai ZH, Li L, Kou C, Gao YF. Preparation and PEGylation of exendin-4 peptide secreted from yeast Pichia pastoris. Eur. J. Pharm. Biopharm. 2009; 72: 412–417.

[14] Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly(ethyleneglycol) protein conjugates. Adv. Drug Deliv. Rev. 2003; 55: 1261–1277.

[15] Nishimura H, Ashihara Y, Matsushima A, Inada Y. Modification of yeast uricase with polyethylene glycol: disappearance of binding ability towards antiuricase serum. Enzyme. 1979; 24: 261–264.

[16] Chen RHL, Abuchowski A, Van Es T, Palczuk NC, Davis FF. Properties of two urate oxidase modified by the covalent attachment of poly(ethylene glycol). Biochim. Biophys. Acta. 1981; 660: 293–298.

[17] Davis S, Park YK, Abuchowski A, Davis FF. Hypouricaemic effect of polyethylene glycol modified urate oxidase. Lancet. 1981; 8241: 281–283.

[18] Nishimura H, Matsushima A, Inada Y. Improved modification of yeast uricase with polyethylene glycol, accompanied with nonimmunoreacyivity towards anti-uricase serum and high enzymic activity. Enzyme. 1981; 26: 49–53.

[19] Caliceti P, Veronese FM, Jonak Z. Imunogenic and tolerogenic properties of monomethoxypoly(ethylene glicol) conjugate proteins. Farmaco. 1999; 54: 430–437.

[20] Caliceti P, Schiavon O, Veronese FM. Immunological properties of uricase conjugated to neutral soluble polymers. Bioconjug. Chem. 2001; 12: 515–522.

[21] Chua CC, Greenberg ML, Viau AT, Nucci M, Brenckman Jr WD, Hershfield MS. Use of polyethylene glycol-modified uricase (PEG-uricase) to treat hyperuricemia in a patient with non-Hodgkin lymphoma. Ann. Intern. Med. 1988; 109:114–117.

[22] Chun Z, Xiao LY, Juan F, Yonghua Y, Xiang L, Youquan B, Yanling X, Huidong Y and Fei L, Biosci.Biotechnol.Biochem. 2010; 74(6), 1298-1301.

[23] Sherman MR, Saifer MGP, Perez-Ruiz F. PEG-uricase in the management of treatment-resistant gout and hyperuricemia. Adv. Drug Deliv. Rev. 2008; 60: 59–68.

[24] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680–685.

[25] Mahler H, Hubscher G and Baum H. Studies on Uricase I. Preparation, Purification and Properties of a Cuproprotein. J. Biol. Chem. 1955; 216: 625-641.

◈◈◈