Research on PEG modification of uricase

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Abstract **Aim:** To modify uricase with PEG reagent in order to decrease uricase immunogenicity and increase its stability. **Methods:** The branched PEG of 40 kD was chosen to modify native uricase. The properties of the modified uricase including the stabilities to protease, pH and temperature, *in vivo* half-life time, as well as the immunogenicity were evaluated. The pharmacokinetic profiles of the midofied uricase were studied in mice. **Results:** It is demonstrated that the conjugation of PEG to lysine residues of *Candida utilis* uricase resulted in higher trypsin resistance, reduced immune response, and prolonged *in vivo* half-life. PEG modified uricase retained 80% of the enzymatic activity of native uricase. In addition, it was found that half-life in serum of the intravenously injected PEGylated uricase of up to 696 min was longer that that of native uricase of 45 min. Higher plasma drug concentrations were also reached with dosing of the PEGylated uricase to mice. Furthermore, the binding affinity was shown to be reduced for the PEG-uricase using ELISA assay, and it was one-eighth that of native uricase. Finally, it was indicated that the PEG uricase induced a delayed immunoresponse in mice following repeated administrations. **Conclusion:** These findings demonstrate that this chemically modified form of uricase may serve as a potentially effective drug to treat gout patients.

Key words uricase; PEGylation; immunogenicity; half-life
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聚乙二醇修饰尿酸酶的研究

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摘 要 **目的:**为了降低尿酸酶的免疫原性并提高其稳定性,利用 PEG 修饰剂对尿酸酶进行修饰,以期获得性质更优的治疗痛风的药物。**方法:**用相对分子质量为 40 kD,活化基团为羟基琥珀酰亚胺的 PEG 修饰剂对尿酸酶进行修饰,并比较修饰前后在酶解稳定性、pH 稳定性及温度稳定性方面的差异,以及 PEG 修饰对体内半衰期,免疫原性的影响。**结果:**发现 PEG 修饰后尿酸酶的酶解稳定性显著提高,PEG 化尿酸酶保留了原有尿酸酶 80% 的活性,体内半衰期从 45 min 延长至 696 min。PEG 化尿酸酶与抗体的结合能力为原型蛋白的 1/8。体内的免疫原性也明显降低。**结论:**化学修饰后的尿酸酶可望成为潜在的治疗痛风的有效药物。

关键词 尿酸酶;PEG 化修饰;免疫原性;半衰期

Gout is becoming more and more common because of the growing consumption of purine-rich foods such as meat and alcoholic beverages in consumers' regular diet. Because uric acid is execreted as the end product of purine metabolism, excessive uric acid usually precipitate and its low solubility in serum is responsible for the formation of crystal at bone joints, thereby resulting in gout. As a widely used drug, allo-

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purinol, a purine inhibitor of enzyme xanthine oxidase, inhibits the formation of uric acid *in vivo* but was associated with serious side effects in clinical use[1-2]. Uricase, catalyzing uric acid to a more soluble substance-allantoin, has been developed as an effective drug such as Eliket® to treat gout-suffering patients. Although uricase is an effective agent to decrease *in vivo* uric acid level in patients, its use is greatly limited because of high immunological reaction due to its foreign protein character[3].

To avoid or reduce foreign protein immunogenicity, various methods, including PEGylation, site-specific mutagenesis, co-expression with fusion proteins have been exploited[4]. In reality, PEG modification is the most popular and effective method in practical use[5-7].

In order to reduce the immunological response caused by this enzyme, the native uricase was chemically modified with PEG. When modified with PEG 5 kD, *Candida utilis* uricase containing 32 lysine residues retained 23% of the original activity with 57% modification rate[8]. While, according to another source of data[9], 71% modification rate brought out 11% activity retain. It was speculated that PEGylation with low molecular weight would greatly affect enzyme activity due to conformational change caused by high modification rate. In this study, branched PEG 40 kD was adopted to be conjugated to primary amino groups of uricase with an amide linkage.

Here, the aim was to examine the effect of PEGylation on chemical properties, pharmacokinetic profile and immunogenic properties of uricase. In detail, trypsin, pH and temperature stability of PEG-uricase was examined and *in vivo* pharmacokinetic behavior was studied following dosing of the PEG-uricas to mice. In addition, *in vitro* antibody binding ability and *in vivo* antigenic reaction were studied in this research.

1 Materials and methods

1.1 *Materials*

Branched PEG *N*-hydroxysuccinimide 40 kD (NHS-PEG, indicated as Figure 1) was purchased from Nektar (Huntsville, Al, USA). Uric acid, trypsin, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), 3, 5-dichloro-2-hydroxybenzene sulfonic acid, 4-amino-antipyrine, horseradish peroxidase conjugated goat antirabbit IgG, horseradish peroxidase conjugated goat antimouse IgG and *O*-Phenylenediamine were obtained from Sigma. The DEAE-Sepharose, Sephacryl S-200 and Q-Sepharose were supplied by Pharmacia Biotech. All other reagents were of analytical grade.

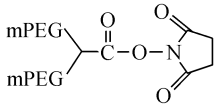


Figure 1 Structure of branched PEG *N*-hydroxysuccinimide

1.2 *Purification of uricase*

Escherichia coli containing plasmid pBV220 expressing *Candida utilis* uricase were cultured in LB medium at 37 °C until A_{600} reached 1 and induced at temperature 42 °C. Harvest *E. coli* were centrifuged at 8 000 r/min for 10 min. Next the precipitate was resuspended in Tris-Gly buffer (0.1 mol/L, pH 8.4) and processed by sonication. Then it was centrifuged at 10 000 r/min for 10 min. Supernatant was subjected to be precipitated by ammonium sulfate at concentration of 30%-60%. The precipitate was then dialyzed to remove ammonium sulfate and molecular cut off of the dialysis membrane was 3 kD. The crude extract was loaded on DEAE-Sepharose column and eluted with 20 mmol/L sodium borate buffer (pH 8.4) and 0-0.3 mol/L NaCl, and effective fractions were collected. To obtain further purified uricase, Sephacryl-S 200 column (1 cm × 100 cm) was used.

1.3 *Determination of uricase activity*

To detect uricase activity, at 25 °C, uric acid was dissolved in 0.1 mol/L, sodium borate buffer (pH 8.4) at the final concentration of 59.48 μmol/L. After the enzyme was added, absorbance at 293 nm was monitored at 30 s intervals for 5 min.

1.4 *Preparation and purification of PEG-uricase*

NHS-PEG was added to 5 mg/mL purified uricase solution (0.1 mol/L, pH 8.4 sodium borate buffer) with molar ratio of 4:1 (PEG: uricase). The reaction mixture was gently stirred for 1 h at 4 °C, and it was terminated by adding 10 mmol/L glycine.

Reaction mixture was diluted with 20 mmol/L sodium borate (pH 8.4) and rendered to Q-Sepharose column that was eluted with 20 mmol/L sodium borate (pH 8.4) with linear NaCl gradient. The eluate was analyzed by absorbance at 280 nm for protein content, iodine assay for polymer determination[10], and enzyme activity. Fractions containing the PEGylated

uricase were pooled and purified by exclusion chromatography.

1.5 *Electrophoresis*

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli on a slab gel containing 12% (*w/v*) polyacrylamide running gel and 4% (*w/v*) stacking gel. The protein bands were stained with commasim brilliant blue.

1.6 *Kinetic analysis*

Steady-state kinetic measurements were performed in 0.1 mol/L sodium borate (pH 8.4) at 25 °C, by varying the concentration of the substrate uric acid at 0.000 125%, 0.000 25%, 0.000 5%, 0.001% and 0.002% (*w/v*). The kinetic parameters K_{cat} and K_m were calculated by non-linear regression analysis of experimental steady-state data. Turnover numbers were calculated on the basis of one active site per 34 kDa subunit.

1.7 *Stability to proteolytic digestion*

Samples of native uricase and PEG-uricase (0.1 mg/mL) in 1 mL of 0.02 mol/L, pH 8.4 sodium borate were incubated at 37 °C with 1.8 mg of trypsin. Aliquots were taken at the intervals and assayed for residual enzymatic activity.

1.8 *Stability to pH*

Native uricase and the PEG-uricase (0.1 mg/mL) were dissolved in the following buffers: 0.1 mol/L sodium acetate (pH 3.6-5.2), 0.1 mol/L sodium phosphate (pH 5.6-7.2), 0.1 mol/L sodium borate (pH 7.6-8.4) and 0.1 mol/L glycine-NaOH (pH 8.8-10.4). After 24-h incubation, pH of the mixture was adjusted to 8.4 and enzyme activities were evaluated.

1.9 *Stability to temperature*

Thermal stability of native uricase and the PEG-uricase were evaluated on the basis of the residual enzyme activity of protein samples (0.2 mg/mL in 0.1 mol/L, pH 8.4 sodium borate) heated for 30 min at temperatures 4, 20, 30, 40, 50, 60, 70 and 80 °C, respectively, and cooled to room temperature.

1.10 *Half-life determination of PEG-uricase in vivo*

Half-life of uricase and the PEG-uricase were analyzed in Balb/C mice ($n=6$). 50 μg of the purified samples were injected into the tail vein. The blood samples were drawn at 10 min, 30 min, 1, 2, 4, 6, 8 and 24 h after injection. Blood samples were centrifuged for 5 min at 3 500 r/min, and serum was separated and diluted for later activity determination. One-compartmental pharmacokinetic modeling was performed.

1.11 *Antigenicity activity*

ELISA was performed to determine whether PEGylated uricase shields the protein from antibody binding. 96-well microplates were coated overnight with 100 μL of uricase or PEG-uricase. The wells were blocked with 10% FBS in PBS followed by incubation with rabbit anti-uricase serum. Binding ability was detected with HRP-labeled goat anti-rabbit IgG and OPD was utilized for colorimetric detection. The plates were measured at an absorbance of 492 nm.

The amount of the adsorbed protein on the well surface was estimated as follows[11]: To 96-well microplate coated with uricase or the PEG-uricase, 50 μL of uric acid solution (59.48 μmol/L) was added. Then 100 μL of detection mixture containing 0.2 mol/L sodium phosphate (pH 6), 1 mmol/L 3,5-dichloro-2-hydroxybenzene sulfonic acid, 0.25 mmol/L 4-aminoantipyrine, and 25 U/mL horseradish peroxidase was added, and microplate was placed in 37 °C condition. Absorbance at 510 nm was recorded.

1.12 *Immunogenicity evaluation*

Twenty male Balb/C mice were randomly divided into 2 groups and immunized on weeks 1, 2, 3 and 4 with 8 μg of native uricase or equimolar amounts of PEG-uricase, respectively. The immunizing solutions were prepared by dissolution of uricase or the PEG-uricase in 50 μL of PBS and 50 μL of Freund's adjuvant (complete adjuvant was used in the first immunization; incomplete adjuvant was used for boosting). The solutions were intraperitoneally injected, and blood for antibody determination were taken just before treatment on days 1 (predose) and after dose 8, 15, 22, and 29. Blood samples were centrifuged for 5 min at 3 500 r/min, and the separated serum was properly diluted in PBS. Anti-native uricase antibodies in serum were estimated by ELISA.

2 Results

2.1 *Preparation of PEG-uricase conjugates*

The purified uricase from Sephacryl S-200, with specific activity of 15 U/mg, exhibited purity of more than 95% when detected by SEC-HPLC (Figure 3) and then it was used to PEG modification. After the reaction, the PEGylated uricase was purified by ion-

exchange chromatography. The PEGylation solution, which contained PEG reagent, unreacted protein and PEG-uricase, was separated by Q-Sepharose chromatographer.

The PEGylated uricase eluted firstly, followed by unPEGylated uricase. Figure 2 tells that under this defined reaction condition, after purification, there is only mono-PEG modified conjugate in SDS-PAGE analysis. And according to protein and PEG content detection, the molar ratio of PEG: protein is between 2∶1 to 3∶1. it is concluded that every tetrameric uricase molecule is conjugated to 2-3 PEG molecules on average.

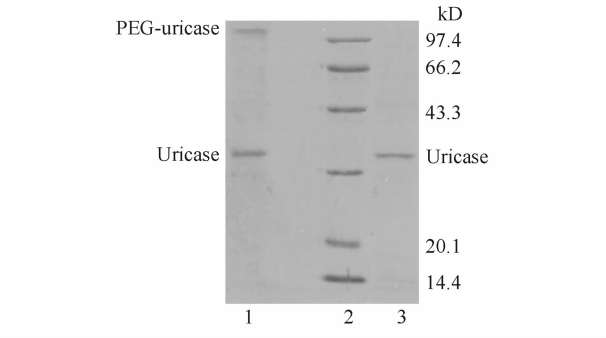


Figure 2 SDS-PAGE of uricase and PEG-uricase
1: Purified PEG-uricase; 2: Molecular mass marker; 3: Native uricase

Figure 3 shows the SEC-HPLC result of tetrameric uricase and PEG-uricase. It tells that the tetrameric uricase was eluted at 27.112 min, while the elution time of PEG-uricase was 21.964 min. PEG-uricase showed a purity of >95% in this detection.

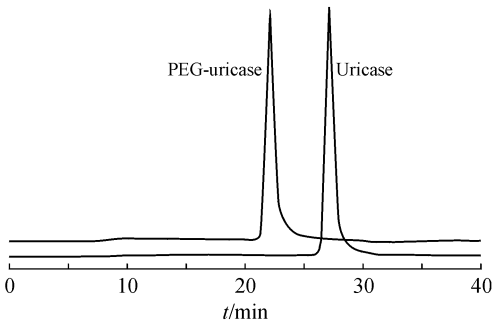


Figure 3 SEC-HPLC of PEG-uricase (t_R = 27.11 min) and native uricase (t_R = 21.96 min)

2.2 *Kinetic parameters*

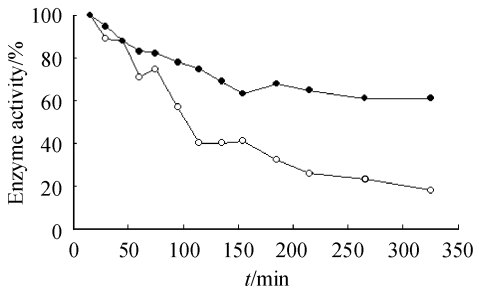
Table 1 shows kinetic parameters profiles of the PEG-uricase and uricase by measuring enzyme activity. After conjugation, maximum velocity of the PEG-uricase remained 80% of the initial value compared to native uricase and K_m value was diminished a little after the modification.

Table 1 Kinetic parameters of PEG-uricase and native uricase

Sample	V_{max}/(μmol/L · min)	K_m/(μmol/L)	K_{cat}/(1/s)
Uricase	9.3	128.4	2.4
PEG-uricase	7.45	113.0	1.9

2.3 *Stability of PEG-uricase to protease*

In Figure 4, the activity of the PEG-uricase under the proteolysis condition was determined. PEGylation often increases protease resistance, It was found that PEGylation provide considerable protection effect to protease, especially, after 325-min incubation. Native uricase retained 10% of the initial activity, while the activity of the PEG-uricase was 70% relative to that before digestion.

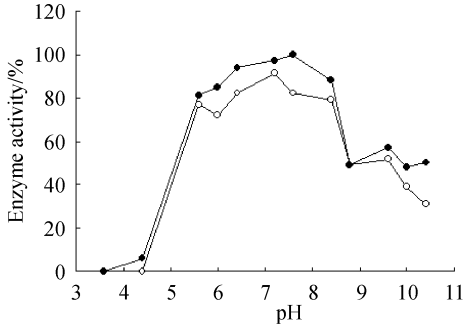


—○—Uricase; —●—PEG-uricase

Figure 4 Trypsin resistance of uricase and PEG-uricase

2.4 *Stability of PEG-uricase to various pH conditions*

The stability of the PEG-uricase to pH was indicated in Figure 5. PEG-uricase was more stable than that of native uricase at pH starting from 5.5 to 8.5.

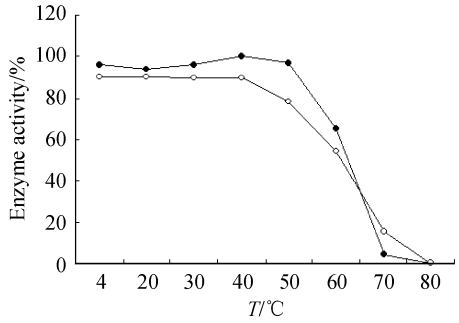


—○—Uricase; —●—PEG-uricase

Figure 5 pH-stability of PEG-uricase

2.5 *Stability of PEG-uricase to temperatures*

Thermostability studies demonstrated that after incubation in specific temperature, the PEG-uricase remained greater enzymatic activity relative to native uricase at temperature 20-60 °C. However, when temperature increased to 65 °C, the activity of PEG-uricase decreased more quickly than that of uricase (Figure 6).

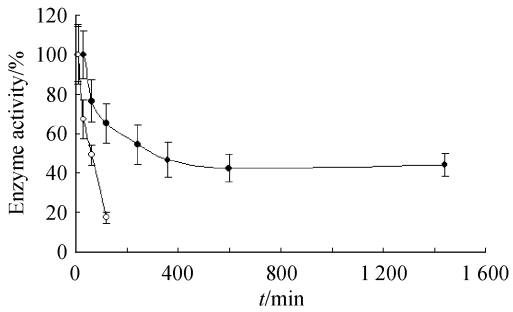


—●—PEG-Uricase; —○—Uricase

Figure 6 Thermostability of native uricase and PEG-uricase

2.6 *Prolonged serum drug half-life of PEGylated uricase*

Pharmacokinetic profiles of the PEG-uricase in mice were determined and compared to that of native uricase. The concentration-time curves of native uricase and the PEG-uricase were generated as serum enzyme activity (%) versus time after a single administration at dose of 50 μg per mouse. Figure 7 shows that native uricase was eliminated rapidly from the mice had a half-life of 45 min and PEGylated uricase has a half-life of 696 min. Pharmacokinetic parameters were listed in Table 2.



—●—PEG-Uricase; —○—Uricase

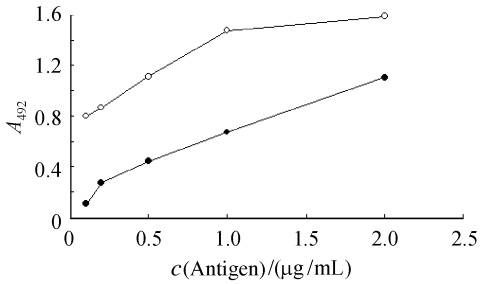
Figure 7 Plasma concentration-time profile of uricase and PEG-uricase

Table 2 Pharmacokinetic parameters of PEG-uricase and uricase in Balb/C mice (n = 6)

Parameter	Uricase	PEG-uricase
c/(U/mL)	0.858	1.147
AUC/(U · min/mL)	74	861.8
CL/(μL/min)	13.66	1.16
$t_{1/2}$(min)	45	696

2.7 *Anti-uricase antibody activity for PEG-uricase*

The ability of PEG to shield uricase from antibodies was tested *in vitro* with immunoassay (Figure 8). To examine the antigenic properties of the samples, different amounts of antigen were incubated with rabbit anti-uricase antiserum. Native uricase showed a standard antibody binding response at 0.2-5 μg/mL, and the PEG-uricase showed a standard antibody binding response at 0.1-2 μg/mL. Considerable amount of uricase or the PEG-uricase attached to microplates, the best shielding resulted in 8-fold reduction in antibody affinity to the PEGylated uricase at 0.1 μg/mL. It was indicated that antibody binding to the 40 kD conjugate was sufficiently reduced in comparison to native uricase. Its combination with the retained enzyme activity and the improvement in pharmacokinetic profile, making PEG-uricase an attractive candidate for a new derivative of uricase.



—○—Uricase; —●—PEG-Uricase

Figure 8 Determination of antigenicity of uricase and PEGylated Uricase.

2.8 *Reduced immunogenic activity*

Repeated administration of this therapeutic protein would elicit immune response no matter PEG-uricase or native uricase was used. In a month, uricase and the PEG-uricase were repeatedly administered to mice at weekly interval, and *in vivo* anti-uricase antibody formation was monitored. Native uricase was found to elicit rapid antibody levels whereas PEG-uricase stimulated a significantly lower immunoresponse. Figure 9 indicates that the PEG-uricase consistently elicited low antibody production relative to native uricase, strongly suggesting that PEG conjugates effectively lower *in vivo* immune reaction.

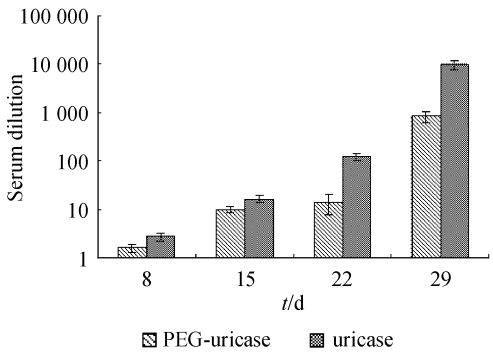


Figure 9 Immunological time course of native uricase and PEG-uricase

3 Discussion

PEG modification has long been regarded as an effective method to reduce immunogenic reaction and improve other properties of protein drugs. Modification of uricase with 40 kD banched PEG exerted little effect on enzyme activity, and PEG-uricase retained 80% activity of native uricase. It can be explained that higher hindrance effect prevents PEG to reach the active site of uricase, so its activity remains a lot. This property makes this conjugation a proper candidate for gout therapy for PEGylation probably leads to great activity decrease, while this PEG-uricase conjugate retained most enzyme activity.

In the proteolysis study, with the time progressing, differences in activity between uricase and PEG-uricase become greater. The target site of protease digestion is usually the peptide bond with lysine or arginie on the opposite of *N*-terminal, therefore, protease digestion is a proper standard to research PEGylation. This suggests that PEG provides efficient protection to uricase and will help prolong its *in vivo* half-life.

Modification of uricase, an intrinsic high immunogenicity protein, possesses obviously reduced binding activity with anti-uricase serum. The PEG moiety appears to be responsible for the shielding of antigen site from antibody. Assay of binding capacity of antiserum with samples, native uricase showed 3 to 8 fold higher affinity than PEG-uricase. Because of possible serious results arising from severe allergic response, reduced efficacy of therapeutic proteins and induce of autoimmunity to endogenous proteins caused by immune response, protein immunogenicity should be decreased to a proper level. Our study showed that antibody production was greatly reduced after administration of PEG-conjugated uricas instead of native uricase.

In conclusion, the data reported here are in favour of PEG conjugation for an improved use of uricase in treatment of gout. Especially, the prolonged half-life and the reduced immune response make it an attractive candidate in clinical utility.

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