JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 2909-2914

# Molecular Modification of Baboon Uricase to Reduce its Immunogenicity Employing Bovine Lactoferricin as Modifier

**Runsong Xiong, Sirajo Umar and Jinchun Chen***

College of Life Science and Technology, Beijing University of Chemical Technology, Beijing - 100 029, P. R. China.

(Received: 10 March 2013; accepted: 21 April 2013)

**Uricase is an important enzyme in purine degradation and receives considerable interests in therapy of hyperuricaemia. But as a heterogeneous protein, it may trigger immunoreactions when applied in human body. To reduce its immunogenicity, we designed three modified uricases, in which bovine lactoferricin was used as molecular modifier and embedded into baboon uricase by recombinant technology. In vitro immunological assay revealed decrease in uricase's immunogenicity corresponding to the number of modifiers in molecule and the maximum decrease was 48.67%. This study presents the possibility of using small peptide as modifier to reduce the immunogenicity of protein and provides a new clue for molecular modification.**

**Key words:** Baboon uricase, Bovine lactoferricin, Molecular modification, Immunogenicity.

Uricase or urate oxidase (UOX, EC 1.7.3.3) is an important enzyme in the metabolic pathway of purine, which decides the final degradation product of purine in body[1,2]. Since it was first discovered and prepared in 1957, uricase has been applied in the therapeutic purpose for the control of uric acid[3-6]. However, as a heterogeneous protein, it may arouse immunoreaction when applied in human body and its efficacy will diminish quickly when patients develop anti-uricase antibodies[7-9]. Moreover, serious allergic reactions including anaphylaxis have occurred sometimes[10]. Therefore, uricase with no or less immunogenicity is needed for therapy of hyperuricaemia.

* To whom all correspondence should be addressed.
TeleFax: +86-10-64439673;
E-mail: jingchunchen@hotmail.com

In chemical enzyme engineering cycle, employment of non-immunogenic macromolecules, such as PEG (polyethyleneglycol) and HSA (human serum albumin), as modifiers to erase protein's immunogenicity has been feasible via chemical cross link method[11]. But this process has some shortages, such as difficulty on control of the molecular size and pool yield of expected product. On the contrary, recombinant technology can generate bioactive products with uniform size and specific structures. This makes biological method more prospective than chemical method. To the best of our knowledge, there has been no report on the use of small peptide as modifiers to erase or reduce protein's immunogenicity.

In this study, bovine lactoferricin (LfcinB), a small bioactive peptide (MW: 2.86 kDa) was employed as molecular modifier by embedding it into target positions of baboon uricase (bUOX) molecule to reduce the latter's immunogenicity. Based on molecular structure of bUOX, three modified uricases were designed and then prepared by recombinant technology. Immunological assay

revealed that there was reduction of immunogenicity in modified uricase. The reduction corresponds to the number of modifiers incorporated in the uricase molecule.

## MATERIALS AND METHODS

**Design and amplification of rbUOX-LfcinB genes**

Immunogenicity is caused by the epitopes on protein surface[12]. So in theory protein immunogenicity can be erased if the epitopes are eliminated by introduction of non-immunogenic modifier. However, identification of epitopes, especially conformational epitopes on specific protein is a very difficult task till now[12]. This fact makes it more common for biological enzyme engineering to bind the modifier molecule to the N- and/or C-terminals of the target protein to erase its immunogenicity via recombinant technology before the protein's epitopes are identified. In this study, LfcinB was first embedded into bUOX molecule at N-terminal, and then at N-and C-terminals, respectively. Moreover, after comparing the sequences of uricases from three species, we found some clues for the third insertion site of LfcinB in bUOX molecule (Fig. 1A, B and C). Fig 1A showed the spans between active residues of uricases in three species. From these we found the spans were highly conserved in uricases. However, the spans varied between the residues R and S in different species, which indicated there might be some "redundant" residues or secondary structure in this part. Furthermore, we aligned these parts between bUOX and *Aspergillus flavus* uricase (afUOX) (Fig. 1B) using NCBI COBALT software and found aa 202-208 of bUOX corresponded to aa 191-205 of afUOX, which formed a small á-helix on the protein (Fig. 1C ). More important is that this á-helix did not contain any active or tetramer interface sites. Considering this, we determined it was the third insertion site of LfcinB in bUOX. Herefrom, all of the three modified rbUOX genes were identified (Fig. 2). In this part, pentaglynine ($G_5$) was used as linker between LfcinB and rbUOX, and pentaargine tag ($R_5$ tag) as alternative purification tag.

Thereafter, the three rbUOX-LfcinB genes were amplified (Supplementary Fig. S1; see supplementary Table S1 for sequence of primers) as the method of splicing by overlap extension (SOE) [13,14] and then inserted into corresponding sites of pET32a(+) vector (Novagen) (Fig. 2).

**Expression and preparation of rbUOX-LfcinB**

The constructs were respectively transfected into *E. coli* Origami B (DE3) (Novagen). After incubation for 2 h at 37°C, IPTG (isopropy-β-D-thiogalactoside) was added into the culture to induce the expression of target genes and meanwhile temperature was lowered to 25°C. After 8 h agitations, cells were harvested and lysed in reducing SDS sample buffer. And then the target proteins were purified from the supernatant of cell lysate under native condition by $Ni^{2+}$-NTA His-Bind Resin (Novagen) affinity chromatography according to the procedure of manufacture's manual No. TB273. Finally, the purified proteins were matured by cleavage of His-tag using recombinant enterokinase (rEK, Novagen).

**Characterization and enzyme activity assay of rbUOX-LfcinB**

MALDI-TOF-MS/MS was employed to analysis the prime structures of obtained rbUOX-LfcinB, which was carried out as described in[15]. The captured peptides were then compared with the peptides in a Mascot Search Database, which was composed of the three theoretical sequences of rbUOX-LfcinB.

Enzyme activity assay of the target proteins was conducted as reported by Koyama *et al*[16]. In this part, recombinant non-modified baboon uricase (rbUOX, prepared by our lab) was used as a control to estimate the enzyme activity reservation of rbUOX-LfcinB.

**Immunological detections of rbUOX-LfcinB**

Peripheral blood mononuclear cells (PBMC) separated from healthy adult as described in[17] was first sensitized in RPMI-1640 tissue culture (containing 1μg/mL of PWM (human pokeweed mitogen), 20 μg/mL of rbUOX, 10% (v/v) FCS (fetal calf serum), 50 μM thioglycol ) for 7 d at 37°C with 5% $CO_2$. Then cells were harvested by centrifugation at 2500 rpm for 25 min and suspended in RPMI-1640 (containing 10% FCS).

Thereafter, 96 wells plate was incubated by rbUOX and covered by HSA. And then the sensitized PBMC were added into the plate and incubated for 7 d at 37°C with 5% $CO_2$ to produce human anti-rbUOX antibody. After centrifugation at 2500 rpm for 25 min, the supernatant of cell culture containing human anti-rbUOX antibodies

was taken for immunogenicity assay.

Finally, an indirect ELISA was performed to quantify the immunogenicity of the matured rbUOX-LfcinB. The 96 wells plate was incubated by rbUOX and rbUOX-LfcinB proteins with equal amounts, respectively at 4°C overnight, and then covered by HSA. After being washed three times with 0.15 M PBS (containing 0.02% (w/v) $KH_2PO_4$, 0.29% (w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.8% (w/v) NaCl, 0.02% (w/v) KCl, and 0.05% (v/v) Tween-20, pH7.4), 100 µL of the supernatant with human anti-rbUOX antibody was added into the wells and kept at 37°C for 1.5 h. Later, the supernatant was removed and the wells were washed three times with PBS. Then goat anti-human IgG antibody (second antibody) conjugated previously horseradish peroxidase (HRP) was added into the wells and incubated at 37 °C for 1 h. Thereafter, 100 µL of TMB (10 mg of 3,3',5,5'-Tetramethylbenzidine (TMB) dissolved in 5 mL of ethanol, 95 mL of 0.15 M PBS, pH5.0, freshly prepared) was added into the wells and reacted with HRP in dark condition for 20 min. The reaction was stopped with 2 M $H_2SO_4$. The absorbance values of the mixture at 450 nm were then detected. In this part, HSA and rbUOX were utilized as negative and positive controls, respectively. Triplicates of each sample were examined. For qualitative assay on the immunogenicity of rbUOX-LfcinB, see supplementary materials and methods.

## RESULTS

### Expression and preparation of rbUOX-LfcinB

Fig. 3 showed the results of expression and purification of the three target proteins, which indicated that the target three proteins had been expressed and prepared successfully.

### Characterization and Enzyme Activity Assay of rbUOX-LfcinB

Basically, a fusion protein can be identified if more than 3 peptides derived from modifier and native protein respectively can be detected as well as protein score is great than the threshold value given by Mascot Search. Table 1 summarized the results of MALDI-TOF-MS/MS detection, which predicted LfcinB had been already embedded into the expected positions in rbUOX molecules. (see supplementary Fig. S2 and S3 for

**Table 1.** MALDI-TOF-MS/MS characterization of rbUOX-LfcinB

| Sample | Peptidehits | MW (kDa) | Score* | Thresholdvalue |
|---|---|---|---|---|
| rbUOX-LfcinB | 5 | 36.3 | 198 | 17 |
| rbUOX-LfcinB$_2$ | 7 | 39.5 | 143 | 17 |
| rbUOX-LfcinB$_3$ | 4 | 42.1 | 56 | 17 |

*Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits, which are significant when greater than 17, the threshold value provided by Mascot Search ($p<0.05$).

**Table 2.** Enzyme activity assay of rbUOX-LfcinB.

| Sample | Enzyme Activity(U/mL)* | | | Av. EA(U/mL) | Av. EA(U/mg) | EA Decrease/% |
|---|---|---|---|---|---|---|
| rbUOX | 1.723 | 1.815 | 1.819 | 1.786 | 17.86 | - |
| rbUOX-LfcinB | 1.423 | 1.475 | 1.417 | 1.438 | 14.38 | 19.5 |
| rbUOX-LfcinB$_2$ | 1.286 | 1.278 | 1.302 | 1.289 | 12.89 | 27.9 |
| rbUOX-LfcinB$_3$ | 1.187 | 1.193 | 1.170 | 1.183 | 11.83 | 33.8 |

* Triplicates of each sample were examined and one unit (U) was defined as the amount of enzyme necessary to transform 1 µmol of uric acid into allantoin in 1 min at 25°C. And the enzyme activity of prepared uricase was calculated by

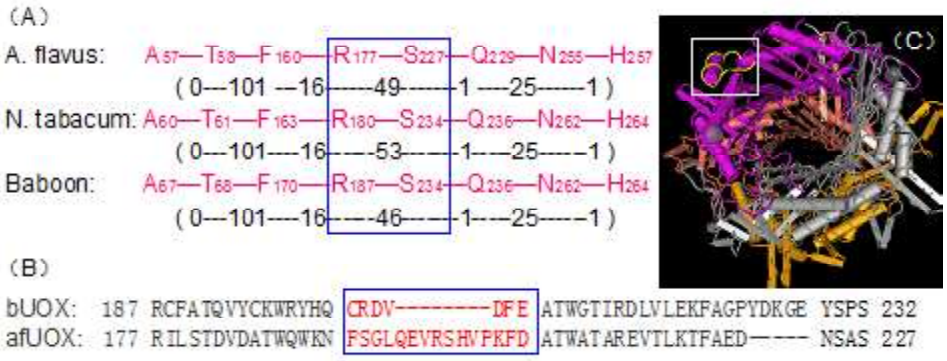
U/mg= ($^3$%A × Vt × n× c)/(12.6 × Ve)

where U is the enzyme activity of prepared uricase (U/mg); $^3$%A is the decrease per min of reaction mixture in absorbance at 293 nm; Vt is the volume of reaction mixture (mL); c is the concentration of uricase solution (mg/mL). n means the dilution times of uricase solution; 12.6 is the absorbance value per ìmole of uric acid at 293 nm; Ve is the volume of uricase solution added (mL)

**Table 3.** Immunogenicity assay of rbUOX-LfcinB.

| Sample | $OD_{450}$* | | | Av. $OD_{450}$ | P/N | Immune Reaction % | Decrease /% |
|---|---|---|---|---|---|---|---|
| HSA | 0.228 | 0.196 | 0.216 | 0.213 | - | - | - |
| rbUOX | 1.685 | 1.703 | 1.672 | 1.687 | 7.92 | 100 | 0 |
| rbUOX-LfcinB | 1.416 | 1.430 | 1.453 | 1.433 | 6.73 | 84.94 | 15.06 |
| rbUOX-$LfcinB_2$ | 1.275 | 1.306 | 1.312 | 1.298 | 6.09 | 76.94 | 23.06 |
| rbUOX-$LfcinB_3$ | 0.902 | 0.871 | 0.815 | 0.866 | 4.07 | 51.33 | 48.67 |

* Triplicates of each sample were examined.




(A) distribution of active residues in uricases from three species;
(B) sequence alignment of bUOX (baboon uricase) and afUOX (*Aspergillus flavus* uricase);
(C) secondary structure of aa 191-205 in afUOX (PDB ID: 3L8W).

**Fig. 1.** Identification of the third insertion site of LfcinB in rbUOX

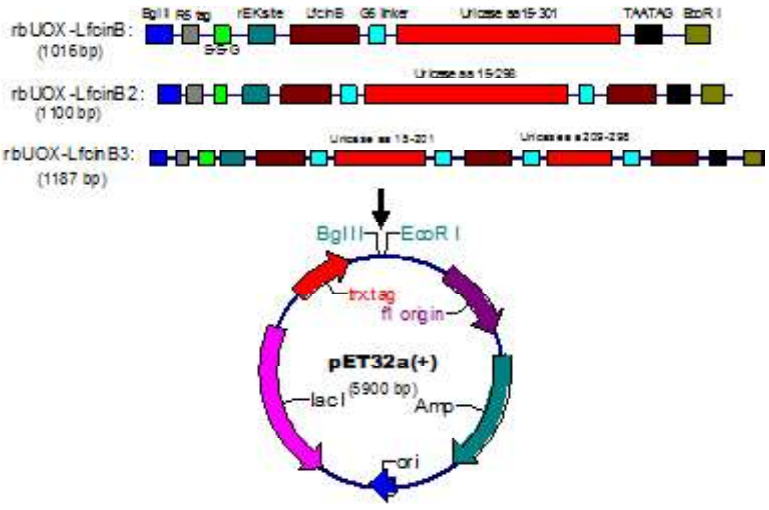



**Fig. 2.** Schematic diagram showing the design of modified rbUOX genes and the construction of the expression vectors pET32a(+)-rbUOX-LfcinB
Three modified rbUOX-LfcinB genes were respectively inserted to the corresponding sites of pET32a(+). $R_5$ tag: Arg-Arg-Arg-Arg-Arg; S-S-G: Ser-Ser-Gly; rEK site: recombinant enterokinase site, DDDDK; $G_5$ linker: Gly-Gly-Gly-Gly-Gly

more details of the results of MALDI-TOF-MS/MS detection on rbUOX-LfcinB). Table 2 exhibited the enzyme activity reservation of rbUOX after insertion of LfcinB, from which we can find the enzyme activity of rbUOX decreased in some extent after modified by LfcinB.

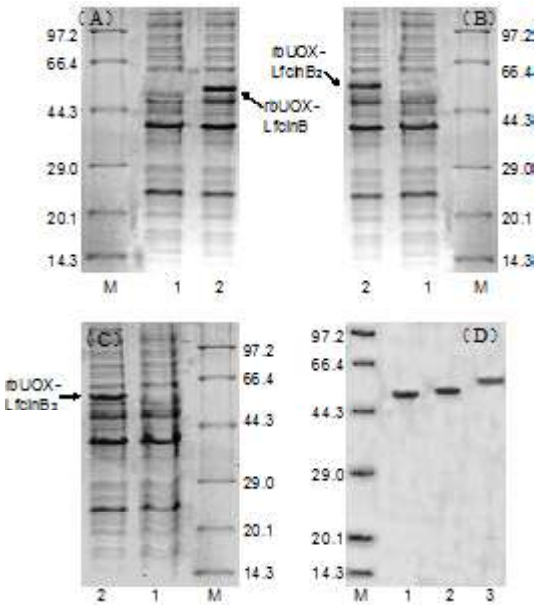
**Immunological detections on rbUOX-LfcinB**

Result of immunogenicity assay on rbUOX-LfcinB was showed in Table 3, which indicated that the immunogenicity of rbUOX was reduced successfully by introduction of LfcinB. And the immunogenicity decreases of modified rbUOX corresponded to the number of modifiers in molecule. The maximum decrease was 48.67%. (see supplementary Fig. S4 for the qualitative result of immunological assay on rbUOX-LfcinB). These results proved that LfcinB was beneficial to reduce the immunogenicity of rbUOX.

**Discussion and Conclusion**

For enzyme modification, the most important is to prevent the molecular structure from collapsing. When identified the third insertion site in bUOX molecule, we tried to avoid introducing the modifier to á and â structural motifs because these motifs in most cases comprise the core structures of proteins. From this point of view, the amino acid residues to form random coils in molecule, which in many cases do not contribute to the maintenance of the core structure of protein, seems to be preferred sites for introduction of modifier. Actually, there are two coil structures in baboon uricase molecule, which correspond to the residues of $I_{43}$ETSYTKADNSVIVA$_{57}$ and $F_{163}$LRDEVTTLK$_{172}$ of afUOX, respectively. However, $I_{43}$ETSYTKADNSVIVA$_{57}$ contained one active site and five tetramer interface sites. And $F_{163}$LRDEVTTLK$_{172}$ was a highly conserved sequence in uricase, which was too closed to the active sites $F_{160}$ and $R_{177}$ (supplementary Fig. S5). Thus, we considered both of these fragments were not suitable sites for the introduction of LfcinB.

The enzyme activity of rbUOX decreased in some extent after modified by LfcinB in this study. However, as a therapeutic protein, its biosafety such as immunogenicity should receive much attention when applied in human body besides its function. That's why in this study we keep eyes on reduction of the immunogenicity of rbUOX. As an attempt to reduce protein immunogenicity using small peptide as modifier, our result presented here proved it's helpful to reduce rbUOX immunogenicity by introduction of LfcinB and subsequently improved the safety of rbUOX in human body to some extent. Meanwhile, this study provided a novel strategy for molecular modification, especially for the selection of modifier in enzyme engineering.




(A), (B) and (C): expression of fusion proteins rbUOX-LfcinB (53.0 kDa), rbUOX-LfcinB$_2$ (56.1 kDa) and rbUOX-LfcinB$_3$ (59.3 kDa), respectively; M: molecular weight standard; 1: culture before induction; 2: culture after induction;
(D) purification of the target proteins by $Ni^{2+}$-NTA His-bind resin. M: molecular standard; 1: rbUOX-LfcinB; 2: rbUOX-LfcinB$_2$; 3: rbUOX-LfcinB

**Fig. 3.** Expression and purification of rbUOX-LfcinB fusions in *E.coli* Origami B (DE3)

**ACKNOWLEDGMENTS**

The authors would like to thank Xinwen Zhou for technical assistance to interpret mass-spectrograms.

**Abbreviations**

rbUOX, recombinant baboon uricase; PEG, polyethyleneglycol; HSA, human serum albumin; LfcinB, bovine lactoferricin; afUOX, *Aspergillus flavus* uricase; NCBI, National Center for Biotechnology Information; aa, amino acid; SOE, splicing by overlap extension; *E. coli*,

*Escherichia coli*; IPTG, isopropy-â-D-thiogalactoside; rEK, recombinant enterokinase; MALDI-TOF-MS/MS, matrix-assisted laser-desorption ionization-time-of-flight tandem MS; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; TMB, 3,3',5,5'-Tetramethylbenzidine; FCS, fetal calf serum.

## REFERENCES


1. Hediger M.A., Johnson R.J., Miyazaki H., et al. Molecular physiology of urate transport. *Physiology*, 2005; **20**: 125-33.
2. Richette P., Bardin T. Gout. *Lancet*, 2010; **375**: 318-28.
3. London M., Hudson P.M. Uricolytic activity of purified uricase in two human beings. *Science*, 1957; **125**: 937-8.
4. Fam A.G. Difficult gout and new approaches for control of hyperuricemia in the allopurinal-allergic patient. *Curr. Rheumatol. Rep.*, 2005; **3**: 29-35.
5. Rozenberg S., Roche B., Dorent R., Koeger A.C., Borget C., Wrona N., Bourgeois P. Urate-oxidase for the treatment of tophaceous gout in heart transplant recipients. A report of three cases. *Rev. Rhum. Engl. Ed.,* 1995; **62**: 392-4.
6. Bomalaski J.S., Clark M.A. Serum uric acid-lowering therapies: where are we heading in management of hyperuricemia and the potential role of uricase. *Curr. Rheumatol. Rep.,* 2004; **6**: 240-7.
7. Brogard J.M., Stahl A.: Stahl J. Enzymatic uricolysis and its use in therapy. In: *Uric Acid* (Kelley WN, Arnold WJ, Weiner IM, ed). New York: Springer-Verlag, 1978; pp 515-524.
8. Sibony G., North M.L., Bergerat J.P., Lang J.M., Oberling F. Hyperuricemia resistant to urate oxidase. Role of anti-serum urate oxidase precipitating antibodies (letter). *Presse. Med.,* 1984; **13**: 443.
9. Mourad G., Cristol J.P, Chong G., Andary M., Mion C. Role of precipitating anti-urate oxidase antibodies in urate oxidase-resistant hyperuricemia (letter). *Presse. Med.*, 1984; **13**: 2585.
10. Pui C.H, Relling M.V., Lascombes F., Harrison P.L., et al. Urate oxidase in prevention and treatment of hyperuricemia associated with lymphoid malignancies. *Leukemia,* 1997; **11**: 1813-16.
11. Yuan Q.S., Zhao J.: Molecular engineering of enzyme. In: *Enzymes and enzyme engineering* (Yuan QS, Zhao J, ed). Shanghai, China: East China University of Science and Technology Press, 2005; pp 342.
12. Huang J., Honda W. CED: a conformational epitope database. *BMC Immunology*, 2006; doi: 10.1186/1471-2172-7-7.
13. Horton R.M., Hunt H.D., Ho S.N., et al. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, 1989; **77**: 61-8.
14. Warrens A.N., Jones M.D., Lechler R.I. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. *Gene*, 1997; **186**: 29-35.
15. Xiong R.S., Chen J.J., Chen J.C. Secreted expression of human lysozyme in the yeast *Pichia pasteris* under the direction of the signal peptide from human serum albumin. *Biotech. Appl. Biochem*., 2008; **51**: 129-34.
16. Koyama Y., Ichikawa T., Nakano E. Cloning, sequence analysis and expression in *Escherichia coli* of the gene encoding the *Candida utilis* urate oxidase (uricase). *J. Biochem.*, 1996; **120**: 969-973.
17. Ge H.L., Zhang D.Q. Seperation and detection of immunocytes. In: *Protocols for immunology* (Ge HL, Zhang DQ ed). Beijing, China: Science Press, 2009; pp 1-2.