PROCESS FOR PRODUCTION OF RECOMBINANT BABOON URICASE IN ESCHERICHIA COLI ROSETTA (DE3)

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ABSTRACT

Hyperuricaemia is a serious purine metabolism disorder which may lead to gout, nephrolithiasis and other serious health problems. As a key enzyme responsible for the hydrolysis of uric acid to allantoin in the purine degradation pathway, uricase, especially recombinant uricase, shows great potential in the therapy of hyperuricaemia. In this paper, baboon uricase gene attached with Trx and hexahistidine tags was cloned and expressed in Escherichia coli Rosetta (DE3). The target protein formed a soluble structure in the cytoplasm at 25 °C, with a gradual enrichment in the induction time course, reaching its highest level at 6 h. After purification and maturation, the final yield of mature baboon uricase was 136.0 mg/L with enzyme activity of 17.93 U/mg. The $K_m$ of recombinant uricase was 6.15 μmol/L. And the optimum temperature and pH of the prepared uricase were 37 °C and 8.2, respectively. MALDI-TOF-MS/MS characterization confirmed the prepared protein was identical with the native baboon uricase. These results showed that the baboon uricase gene had been expressed effectively in E. coli and the obtained protein possessed promising enzymatic properties, which gives a new candidate for the therapy of hyperuricaemia.

Materials and Methods

Construction of pET-rbUOX plasmid

The target gene was amplified via splicing by overlap extension PCR (SOE) and then inserted into the corresponding sites of pET32a(+) expression vector (Novagen) (Fig. 1). Finally, the recombinant plasmid was transfected into E. coli Rosetta (DE3) (Novagen). (The primers utilized in this section are listed in the Online Supplementary Appendix (Table S1, www.diagnosisnet.com/bbeq).

Introduction

Uricase or urate oxidase (UOX, EC 1.7.3.3), the last enzyme in the metabolic pathway of purine nucleotides, determines the final breakdown product of purine nucleotides. Human beings and most primates, due to a non-sense mutation that occurred at some point in evolution in the uricase gene in their genome (5, 6, 12), cannot produce uricase, which subsequently brings about a higher physiological concentration of urate, close to its limit of solubility in people (6). Urate scavenges harmful radicals in our body. However, in conjunction with genetic or environmental (especially dietary) factors, urate may cause gout, nephrolithiasis, and vascular diseases (8). Compared to chemical drugs used for decrease of uric acid, uricase is considered to be a promising candidate for the treatment of gout or hyperuricaemia for its catalytic efficiency on uric acid. To date, several microorganism- and plant-derived uricases have been successfully obtained via recombinant technology (1, 2, 3, 4, 10, 11) and applied in therapeutic use for control of gout and hyperuricaemia-related diseases (7, 9). However, because of the evolutionary gap between the producers and human beings, the biosafety and biocompatibility of these uricases was a problem when used in human patients. From this point of view, among the animals possessing an active uricase gene, the baboon has a closest evolutionary relationship with human beings, so baboon uricase might have better biocompatibility than other native uricases. However, to the best of our knowledge, baboon uricase (bUOX) has not been produced till now. Therefore, we tried to clone and express the gene encoding baboon uricase (rbUOX). The induction conditions were optimized to avoid the aggregation of target protein in the cytoplasm. The amino acid composition and kinetic parameters were determined to characterize the prepared protein.
Expression of rbUOX in Rosetta (DE3)

The transformant was first grown in 100 mL LB with 100 μmol/L Ampicillin at 37 °C until the culture reached an A_{600} of 0.8–1.0 (approximately 3 h). Then, isopropylthio-β-D-galactoside (IPTG) was added into the culture to a final concentration of 1 mmol/L. Meanwhile, the temperature was reduced to 25 °C, 30 °C, and 37 °C, respectively. After induction for 8 h, samples were taken and analyzed by 10 % SDS-PAGE to ensure the influence of temperature on the expression of target protein. Moreover, the culture was induced at 25 °C for different hours (2 h, 4 h, 6 h, and 8 h, respectively) to determine the optimized induction time. To ensure the form of the expressed protein in the cytoplasm, the liquid culture was centrifuged at 10000× g for 2 min and then the supernatant was removed. The cell pellets were resuspended in 1× Ni^{2+}-NTA Bind Buffer (containing 300 mmol/L NaCl, 50 mmol/L sodium phosphate buffer, and 10 mmol/L imidazole, pH 8.0) with an equal volume to that of the liquid culture. Next, the cell pellets were lysed according to the manufacturer’s manual (No. TB273, Novagen); and then the cell lysate, supernatant and precipitate suspension of lysate were loaded on 10 % SDS-PAGE.

Purification of rbUOX fusion

The supernatant of the cell lysate was loaded on Ni^{2+}-NTA his Bind Resin column (novagen) and eluted under native conditions according to the procedure commended by the manufacturer (No. TB273). Thereafter, the purified protein was analyzed by 10 % SDS-PAGE. Quantity analysis of the target protein was performed as the method of bicinchoninic acid (BCA).

Characterization of expressed uricase

The separated protein was first matured by cleavage of N'-terminal fusion partners including Trx- and hexahistidine-tag using recombinant enterokinase (Novagen) for 16 h at 23 °C. Then enzyme activity detection was performed by the method of Koyama et al. (3). To determine the optimum temperature of the uricase, the enzyme activity was measured at different temperatures (25 °C, 28 °C, 30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C, and 45 °C). The enzyme activity of the uricase was also measured at different pH (4.0, 5.0, 6.0, 7.0, 7.2, 7.5, 7.8, 8.0, 8.2, 8.4, 8.6, 9.0, 10.0, 11.0, and 12.0) to determine its optimum pH. The reaction mixture contained 3 mL of uric acid solution (120 μmol/L) and 50 μL of uricase (10 μg/mL) dissolved in boric acid buffer. To determine the Km of the prepared enzyme, 3 mL of uric acid with different concentrations (5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, and 30 μmol/L) was allowed to react with 50 μL of uricase (10 μg/mL) for 1 min. The absorbance of the mixture at 293 nm was assayed. Finally, the Km of uricase was obtained from a Lineweaver–Burk double-reciprocal plot.

One unit (U) was defined as the amount of enzyme necessary to transform 1 μmol of uric acid into allantoin in 1 min. And the enzyme activity of prepared rbUOX was calculated by the following equation:

\[ U/mg = (\Delta A \times V_t \times n \times c) / (12.6 \times V_e) \]

where \( U \) is the enzyme activity of prepared uricase (U/mg); \( \Delta A \) is the decrease in absorbance at 293 nm per min of reaction; \( V_t \) is the volume of reaction mixture (mL); \( c \) is the concentration of rbUOX solution (mg/mL); \( n \) is the dilution factor of rbUOX.

TABLE 1

Production detection of rbUOX by BCA

<table>
<thead>
<tr>
<th>Samplea</th>
<th>OD562</th>
<th>AV. OD562</th>
<th>c/(mg/L)b</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbUOX in cell lysate</td>
<td>0.1771</td>
<td>0.1686</td>
<td>0.1859</td>
<td>0.1772</td>
</tr>
<tr>
<td>purified rbUOX</td>
<td>0.1486</td>
<td>0.1262</td>
<td>0.1702</td>
<td>0.1483</td>
</tr>
</tbody>
</table>

aThree batches of cell pellets were assayed;
bThe standard curve of BCA utilized in this section is shown in the Online Supplementary Appendix (Fig. S1, www.diagnosisnet.com/bbeq).

TABLE 2

Details about the peptides matched with native baboon uricase in MALDI-TOF-MS/MS determination

<table>
<thead>
<tr>
<th>Start – End</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Miss</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-18</td>
<td>1149.60</td>
<td>1148.60</td>
<td>1148.60</td>
<td>0.0086</td>
<td>1</td>
<td>KNDELEFVR</td>
</tr>
<tr>
<td>24-33</td>
<td>1254.61</td>
<td>1253.60</td>
<td>1253.69</td>
<td>-0.0866</td>
<td>1</td>
<td>DMVKVLHIQR</td>
</tr>
<tr>
<td>108-119</td>
<td>1517.81</td>
<td>1516.81</td>
<td>1516.80</td>
<td>0.0034</td>
<td>1</td>
<td>R.AQVYVEEIPWKRL</td>
</tr>
<tr>
<td>127-147</td>
<td>2459.20</td>
<td>2458.19</td>
<td>2458.20</td>
<td>-0.0079</td>
<td>0</td>
<td>HVHAFHTPTGTHFCVEQLR</td>
</tr>
<tr>
<td>162-175</td>
<td>1554.74</td>
<td>1553.73</td>
<td>1553.85</td>
<td>-0.1176</td>
<td>1</td>
<td>VLKTTQSGFEGFIK</td>
</tr>
<tr>
<td>199-203</td>
<td>706.29</td>
<td>705.28</td>
<td>705.30</td>
<td>-0.0190</td>
<td>0</td>
<td>YHQCR</td>
</tr>
<tr>
<td>238-249</td>
<td>1407.78</td>
<td>1406.77</td>
<td>1406.78</td>
<td>-0.0082</td>
<td>0</td>
<td>TLYDIQVLSLSR</td>
</tr>
</tbody>
</table>
solution; 12.6 is the absorbance value per μmol of uric acid at 293 nm; \( V_e \) is the volume of \( \text{rbUOX} \) solution added (mL).

The mature uricase was determined by means of MALDI-TOF-MS/MS. The target protein band was excised from the SDS-PAGE gel and destained with 15 mmol/L potassium ferricyanide and 50 mmol/L sodium thiosulfate (1:1, v/v) for 30 min at room temperature. After being washed twice with deionized water and shrunk by dehydration in acetonitrile, the sample was swollen in digestion buffer containing 20 mmol/L ammonium bicarbonate and 12.5 ng/μL trypsin (1:1, v/v) at 4 °C for 30 min. Then the gels were digested for more than 12 h at 37 °C. Peptides were extracted twice with 0.1 % trifluoroacetic acid and 50 % acetonitrile (1:1, v/v) and then subjected to a 4700 Proteomics Analyzer (Applied Biosystem 4700).

### Results and Discussion

#### Construction of pET-rbUOX plasmid

Fig. 2 indicates that the rbUOX gene was successfully amplified by SOE with a molecular weight of 973 bp (Lanes 1 and 2). After ligation to pCR 2.1 and sequencing, the baboon uricase gene was excised from pCR 2.1 by \( Bgl \) II and \( EcoR \) I and then inserted into the corresponding sites of the pET32a(+) vector.

**Fig. 2.** Analysis of the amplified gene in a 1 % agarose gel. Lanes 1 and 2: amplified rbUOX gene (974 bp); L: DNA ladder.

#### Expression and purification of the rbUOX in Rosetta (DE3)

Fig. 3A shows the rbUOX gene expressed under different temperatures. Since lower temperatures are beneficial for prevention of inclusion body formation in \( E. coli \), we fixed 25 °C as the final induction temperature. Fig. 3B indicates that 6 h were enough for the expression of the target gene in \( E. coli \) Rosetta (DE3) at 25 °C. Fig. 3C revealed the expressed protein located in the cytoplasm with a soluble form (Lane 2). These results indicated that a highly soluble fusion partner (Trx-tag) and lower induction temperature (25 °C) were beneficial for the target protein to form its correct structure in the cytoplasm. Fig. 3D shows the target fusion protein purified by nickel affinity chromatography (Lane 1). BCA detection revealed the production and the final yield of the expressed protein were 170.2 mg/L and 136.0 mg/L, respectively (Table 1). The recovery of the target protein was 79.91 %, which indicated the target protein could be separated effectively by nickel affinity chromatography.

**Fig. 3.** Expression and purification of rbUOX. (A) Expression of the target gene under different induction temperatures. Lane M: protein standard; Lane 1: cell culture before addition of IPTG; Lanes 2–4: cell cultures after addition of IPTG for 8 h at 25 °C, 30 °C and 37 °C, respectively. (B) Expression production of target protein at 25 °C after different induction times. Lane M: protein standard; Lanes 2–5: cell cultures after induction for 2 h, 4 h, 6 h and 8 h, respectively. (C) Identity of the expression form of the induced protein. Lane M: protein standard; Lane 1: precipitate of cell lysate; Lane 2: supernatant of cell lysate; Lane 3: cell pellets resuspended in 1× Ni-NTA Bind Buffer with an equal volume to that of the cell culture. (D) Purification of the expressed protein by nickel affinity chromatography. Lane M: protein standard; Lane 1 eluted fraction of the supernatant of cell lysate; Lane 2 supernatant of cell lysate. (10 % SDS-PAGE)

#### Characterization of expressed uricase

Fig. 4 showed the Biological activity assay of the matured rbUOX at different temperatures and pH, from which we can find the prepared uricase exhibits maximum activity at 37 °C (Fig. 4A) and the optimum pH is 8.2 (Fig. 4B). The enzyme activity of the obtained protein was 17.93 U/mg. The Lineweaver–Burk double-reciprocal plot revealed that the \( K_m \) of the prepared uricase was 6.15 μmol/L (Fig. S2 in the Online Supplementary Appendix, www.diagnosisnet.com/bbeq). As to MALDI-TOF-MS/MS determination of the obtained protein, Mascot search results predicted that the protein matched the native baboon uricase with a top score of 375, which was much greater than 75, the basic value for the probability of protein (\( p < 0.05 \)) (Table S2 in the Online Supplementary Appendix, www.diagnosisnet.com/bbeq).

Table 3 gives detailed the information about the peptides detected by MS/MS that matched the native baboon uricase. Fig. 6 presents the mass-spectrogram of the matched peptide, KNDELEFVR. These results confirmed the obtained protein
had an identical primary structure with that of native baboon uricase and possessed a molecular mass of 34 956 Da.

Conclusions
In this paper, we developed a simple but effective process for the production of recombinant baboon uricase in *E. coli*. The uricase gene was successfully expressed in the host cell. The target protein could be purified by affinity chromatography. The production and the final yield of uricase were satisfactory. The results from the characterization of the prepared protein indicated that the uricase possessed identical amino acids with the native baboon uricase and exhibited excellent enzymatic activity *in vitro*. We hope this study would give a guide for the mass production of uricase and supply a new candidate for the therapy of hyperuricaemia and related diseases.

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REFERENCES