

國立交通大學

生物科技學系

博士論文

尿酸氧化酵素之分子導向演化及其於晶片系統之應用

Molecular Evolution of Uricase and Its Applications

in Biochip System



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中華民國九十三年六月

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摘要

在臨床生物科技領域中，尿酸檢測是其中一個相當重要的工作。為了達到這個目標，獲得一個高活性與穩定性的尿酸氧化酵素，將其固定化在生物感測器上，以利用尿酸氧化酵素的活性檢測尿酸濃度是必要的程序。針對此一目標我們設計幾個實驗架構包括由自然界中，體外合成或藉由分子演化的技術等獲得一個新的尿酸氧化酵素，此外我們也發展出兩個測量酵素活性的方法，分別是96孔微量盤的呈色法及生物晶片的光學法。



尿酸氧化酵素基因是來自枯草桿菌 (*Bacillus subtilis*)CCRC14199的重組DNA，其基因序列包含1491 核苷酸約55 kDa蛋白質，及表現在麥芽糖結合蛋白(MBP)大約98 kDa之融合蛋白質(fusion protein)，並呈現高尿酸氧化酵素的活性(9.1 U/mg)。另外，比較枯草桿菌與*Bacillus sp.* TB-90之尿酸氧化酵素的氨基酸序列發現相似度為61%。

我們對應用分子演化策略以增進尿酸氧化酵素活性，也感到興趣。首先是經由一個修改的StEP突變的方法，以枯草桿菌尿酸氧化酵素當作模板，然後，利用改良式的呈色法，在96孔微量盤上使用尿酸(uric acid)、過氧化酵素(horseradish peroxidase)、4-aminoantipyrine 和3,5-dichloro-2-hydroxybenzene sulfonate 指示劑等產生呈色反應，檢

測突變株酵素活性。以篩選具有較原生態尿酸氧化酵素活性為高之變種尿酸氧化酵素。經由將StEP得到尿酸氧化酵素的突變基因庫轉殖進入大腸桿菌細胞，以上述方法篩選得到兩個具有活性的突變體尿酸氧化酵素基因。此突變體尿酸氧化酵素蛋白質比原生態枯草桿菌的尿酸氧化酵素的展現較高活性(13.1 U/mg)。經由基因序列分析發現，在真核與原核生物的尿酸氧化酵素中二個高度保留的區域(motifs)，也相同的在突變體尿酸氧化酵素基因中被發現。最後，利用改良式的呈色法檢驗比傳統檢驗更有效率，把分析所需時間從 4 天大大地減少至不到 20 小時。

我們也利用三個有機體中共有的密碼設計的寡核苷酸，經由將DNA序列以單一步驟集合並利用聚合酶連鎖反應合成與放大大人造尿酸氧化酵素基因，並將其轉殖進入麥芽糖結合蛋白表現系統，進行蛋白質之表現和純化。重組的基因進入大腸桿菌細胞，利用自動化DNA序列儀加以證實它的基因序列，並在大腸桿菌的表現系統中表現此麥芽糖結合蛋白-尿酸氧化酵素之融合蛋白質。利用蛋白酶因子Xa切割麥芽糖結合蛋白-尿酸氧化酵素，並經由澱粉親和性色層分析(amylose affinity chromatography)後，具有活性的純尿酸氧化酵素蛋白質可以在SDS-PAGE上產生一個單一條紋。

最後我們發展出一個以互補式金氧半導體(Complementary Metal Oxide Semiconductor, CMOS)陣列光感應器和共軛物酵素生物晶片為基礎的光學式共軛物生物晶片系統，以提供一種單步驟快速定量尿酸檢驗的程序。CMOS光感應器係依N⁺/P-well二極體構造設計，並且使用一個標準的 0.5 μm CMOS程序製造。聚合體酵素生物晶片是使用尿酸氧化酵素和過氧化酵素進行固定化，再滴入樣品反應物進行檢驗。這個酵素生

物晶片之最適化反應溫度在 20 °C 至 40 °C 之間，溫度越高顏色越濃，酸鹼度範圍在 6.0 至 10.0，最適酸鹼度在 8.5。純尿酸標準曲線線性濃度在 2.5 mg/dL 到 12.5 mg/dL。本酵素晶片並與 Beckman synchron method 方法做 20 個血清尿酸樣品臨床測量比較，結果顯示相關性極佳。



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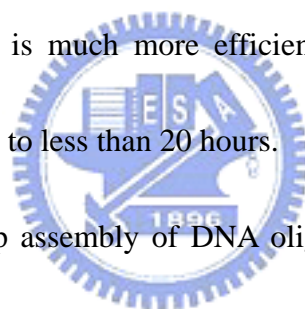
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Abstract

Facile and sensitive detection of uric acid in biological fluids is important in development of goal for biotechnology with many clinical applications. Ideally, detection requires a highly active and stable uricase enzyme for uric acid, and sensitive methods for uricase activity in a biosensor system. We report several achievements toward that goal including, assessment of novel uricase enzymes from nature, *in vitro* synthesis, and molecular evolution. In addition, we have developed two new assays, a 96 well plate modified colorimetric assay and an optical polymeric biochip assay for determination of uricase activity.

A uricase gene was cloned and sequenced from *Bacillus subtilis* strain CCRC 14199. The cloned uricase gene contained an open reading frame of 1491 nucleotides encoding a protein of about 55 kDa. A uricase-MBP fusion protein of about 98 kDa was expressed and purified. The enzyme exhibited high uricase activity (9.1 U/mg). *Bacillus subtilis* CCRC 14199 uricase is similar (61% amino acid identity) to the uricase from *Bacillus* sp. TB-90. We have applied a molecular directed evolution strategy and a new uricase assay to create new uricase functions. We used modified staggered extension process (StEP) mutagenesis to

generate mutant uricase enzymes using the thermophilic *B. subtilis* uricase gene as the template. Mutants were screened using a modified colorimetric assay we developed for uricase activity (a flexible 96-well microtiter plate assay using the uricase - uric acid - horseradish peroxidase - 4-aminoantipyrine - 3,5-dichloro-2-hydroxybenzene sulfonate colorimetric reaction). An *Escherichia coli* library of StEP-derived uricase mutant clones was screened two active and identical mutant uricase genes were obtained. Two motifs conserved in eukaryotic and prokaryotic uricases are highly conserved in the mutant uricase. The mutant uricase protein was found to exhibit high uricase activity (13.1 U/mg). The modified colorimetric method is much more efficient than conventional ones and greatly reduces assay time from 4 days to less than 20 hours.



We also present single-step assembly of DNA oligonucleotides by PCR to synthesize a *Bacillus* spp. uricase gene *in vitro*. The uricase gene was designed using preferred codons common to all the three organisms. The synthetic gene was cloned in *Escherichia coli* and its sequence was verified by DNA sequencing. The synthetic gene was cloned into the pMAL-c2 vector for expression in *E. coli* as a fusion protein with the maltose-binding protein (MBP). Uricase was purified as a MBP fusion, cleaved from the fusion protein with protease factor Xa, and purified from the flow-through following amylose affinity chromatography. The resulting uricase was fully active.

Finally, an optical polymeric biochip system for rapidly measuring uric acid in a one-step

procedure was developed based on the CMOS (Complementary Metal Oxide Semiconductor) photo array sensor and polymeric enzyme biochip. The CMOS sensor was designed with N⁺/P-well structure and manufactured using a standard 0.5 μm CMOS process. The polymeric enzyme biochip was immobilized with uricase-peroxidase and used to fill the reacting medium with the sample. The CMOS sensor response was stronger at a higher temperature range of 20-40 °C, with optimal pH at 8.5. The calibration curve of purified uric acid was linear in the concentration range from 2.5 mg/dL to 12.5 mg/dL. The results obtained for serum uric acid with this method correlated quite closely with those obtained using the Beckman Synchron method.



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Table of Contents

Abstract (Chinese).....	i
Abstract (English).....	iv
Acknowledgements.....	vii
Table of Contents.....	viii
List of Figures.....	xiii
List of Tables.....	xv
Chapter 1 Introduction.....	1
1.1 Introduction to uricase.....	1
1.2 Biosensor for biomolecular technology.....	10
1.2.1 Stable interfaces in biosensor.....	11
1.2.2 Transducers.....	13
1.3 CMOS technologys.....	18
1.4 References.....	24
Chapter 2 Experimental.....	33
2.1 Experimental of Materials.....	33
2.1.1 Strains and vectors.....	33
2.1.2 Reagents.....	33
2.1.3 Buffers and solution.....	34
2.1.4 Equipment.....	37
2.2 Experimental of Methods.....	39
2.2.1 Culture of bacterial strain.....	39
2.2.2 Isolation of genomic DNA.....	39
2.2.3 Polymerase chain reaction.....	40
2.2.4 Construction of recombinant DNA.....	41

2.2.5 Preparation of competent cells for electroporation.....	41
2.2.6 DNA sequence.....	42
2.2.7 Staggered-extension process (StEP).....	44
2.2.8 Modified colorimetric assay for screening of mutant gene.....	44
2.2.9 Purification of uricase.....	45
2.2.10 Quantitative assay of protein.....	46
2.2.11 Electrophoresis.....	46
2.2.12 Coomassie blue staining.....	47
2.2.13 Uricase activity.....	48
2.2.14 Electrophoresis.....	49
2.2.15 Coomassie blue staining.....	50
2.2.16 Uricase activity.....	50
Chapter 3 Cloning and Expression in <i>Escherichia coli</i> of the Gene Encoding <i>Bacillus subtilis</i> Uricase.....	51
3.1 Abstract.....	51
3.2 Introduction.....	52
3.3 Experimental.....	55
3.3.1 Cultures and media.....	55
3.3.2 Isolation of genomic DNA for uricase.....	55
3.3.3 Cloning of the uricase gene.....	55
3.3.4 Expression and purification of the fusion uricase.....	56
3.3.5 DNA sequencing and computer analysis.....	57
3.3.6 Measurements of uricase activity.....	57
3.4 Results.....	58
3.4.1 Cloning of the Uricase gene.....	58
3.4.2 Expression and Purification of the uricase.....	59

3.4.3 Nucleotide and amino acid sequence of uricase.....	60
3.5 Discussion.....	62
3.6 References.....	66
Chapter 4 Detection of Serum Uric Acid Using the Optical Polymeric Enzyme Biochip System.....	70
4.1 Abstract.....	70
4.2 Introduction.....	71
4.3 Experimental.....	73
4.3.1 Optical detection system setup.....	73
4.3.2 DNA manipulations.....	78
4.3.3 Expression and purification of the fusion uricase.....	78
4.3.4 Measurements of uricase activity.....	79
4.3.5 Preparation of the uricase-peroxidase conjugate.....	79
4.3.6 Immobilization of uricase-peroxidase conjugated on the biochip.....	80
4.3.7 Measurement of serum uric acid with optical enzyme biochip system and Beckman Synchron analyzer.....	82
4.4 Results.....	83
4.4.1 Expression and purification of uricase.....	83
4.4.2 Measurements of uricase activity.....	83
4.4.3 Optimization of the uricase-peroxidase-immobilized biochip.....	85
4.4.4 Evaluation of the uricase-peroxidase-immobilized biochip for measuring purified uric acid.....	85
4.4.5 Application of the optical polymeric biochip detection system for measuring serum uric acid.....	86
4.5 Discussion.....	89

4.6	References.....	92
Chapter 5 Modified Colorimetric Assay for Uricase Activity and a Screen for Mutant <i>Bacillus subtilis</i> Uricase Genes Following StEP Mutagenesis.....		
		95
5.1	Abstract.....	95
5.2	Introduction.....	96
5.3	Experimental.....	97
5.3.1	Materials.....	97
5.3.2	Mutagenesis of wild-type thermophilic bacterium <i>Bacillus subtilis</i> uricase gene.....	97
5.3.3	Screening for uricase-producing microorganisms.....	98
5.3.4	DNA sequencing and computer analysis.....	100
5.3.5	Expression and purification of the mutant fusion uricase protein.....	100
5.3.6	Measurements of uricase activity.....	101
5.4	Results.....	102
5.4.1	Mutagenesis of wild-type thermophilic bacterium <i>Bacillus subtilis</i> uricase gene.....	102
5.4.2	Screening for uricase activity via a modified colorimetric assay.....	103
5.4.3	Analyzing the motif sequence of mutant uricase.....	106
5.4.4	Comparison of expression and purification of the wild-type and mutant fusion uricase.....	108
5.4.5	Comparison of activity of the wild-type and mutant fusion uricase.....	110
5.5	Discussion.....	114
5.6	References.....	116
Chapter 6 Expression and purification of a PCR synthesized gene <i>Bacillus</i> spp. Uricase....		
		119
6.1	Abstract.....	119

6.2 Introduction.....	120
6.3 Experimental	121
6.3.1 Synthesizing oligonucleotides.....	121
6.3.2 Synthesizing a gene <i>Bacillus</i> spp. uricase by extending overlap and PCR. 121	
6.3.3 Sequencing and computer analysis of DNA.....	125
6.3.4 Expression and purification of the <i>Bacillus</i> spp. uricase protein.....	125
6.3.5 Assay of uricase activity.....	126
6.4 Results.....	127
6.4.1 Design of the synthetic <i>Bacillus</i> spp. uricase gene.....	127
6.4.2 Synthesizing the <i>Bacillus</i> spp. uricase gene using PCR.....	127
6.4.3 Nucleotide and amino acid sequence of <i>Bacillus</i> spp. Uricase.....	134
6.4.4 Expression and purification of the <i>Bacillus</i> spp. Uricase.....	134
6.5 Discussion.....	137
6.6 References.....	139



List of Figures

Figure 1-1 Metabolic degradation of purines.....	2
Figure 1-2 DCHBS oxidation catalyzed by HRP in the presence of hydrogen peroxide, and phenoxyl radical reaction with 4-AAP, producing a red quinoneimine dye.....	5
Figure 1-3 Uricase catalyzes the reduction of dissolved oxygen to peroxide in the presence of uric acid.....	7
Figure 1-4 CMOS structures. (a) p-well, (b) n-well, and (c) twin well.....	20
Figure 1-5 Optically generated electron-hole pair formation in a semiconductor.....	22
Figure 3-1 Agarose electrophoresis gel results of cloning.....	58
Figure 3-2 SDS-PAGE analysis of the fusion uricase produced in the transformed <i>E. coli</i> , and the uricase protein purified with amylose affinity spin column.....	59
Figure 3-3 DNA sequence of the uricase gene.....	61
Figure 3-4 Comparison of deduced amino acid sequence of the uricase with other uricase...	63
Figure 3-5 Fusion uricase activity.....	65
Figure 4-1 Optical arrangement built with the CMOS photo array sensor and used for polymeric enzyme biochip detection.....	75
Figure 4-2 The pixel structure of the CMOS photo array sensor.....	76
Figure 4-3 (a) The layout of the 240 x 140 CMOS photo array sensor which occupies an area of 1.8 mm x 1.05 mm. (b) The close up microphotograph of the fabricated CMOS photo array sensor showing few pixels. Each pixel is 7.5 μm x 7.5 μm in area...	77
Figure 4-4 Schematic polymeric enzyme biochip assay method for detecting uric acid using immobilized uricase and peroxidase.....	81
Figure 4-5 (a) Agarose electrophoresis gel results of cloning. (b) SDS-PAGE analysis of the uricase fusion protein produced in <i>E. coli</i> , and purified on an amylose affinity spin column.....	84
Figure 4-6 Effect of pH and temperature on the absorbance of the uricase-peroxidase immobilized biochip.....	87

Figure 4-7 (a) Calibration curve of purified uric acid concentration and absorbance at 520 nm using the uricase-peroxidase immobilized biochip. (b) Comparison of serum uric acid results by Beckman Synchron method and polymeric enzyme biochip assay method.....	88
Figure 5-1 Flow chart for the detection of uricase activity by the conventional method and the modified colorimetric method.....	99
Figure 5-2 Agarose gel electrophoresis results of mutant uricase gene cloning.....	102
Figure 5-3 Depiction of the hydrogen peroxide based colorimetric assay.....	104
Figure 5-4 Screening potential uricase mutants in 96-well microtiter plates using a microplate reader.....	105
Figure 5-5 Motif sequence analysis of the mutant uricase.....	107
Figure 5-6 SDS-PAGE analysis of wild-type and mutant uricase-MBP fusion proteins.....	109
Figure 5-7 Comparison of the wild-type and mutant uricase activities.....	111
Figure 5-8 Thermal stability of wild-type and mutant uricase enzymes.....	112
Figure 5-9 pH stability of wild-type and mutant uricase enzymes.....	113
Figure 6-1 Protocol for assembling synthetic <i>Bacillus</i> spp. uricase-pMAL-c2.....	124
Figure 6-2 Agarose electrophoresis gel results of first PCR product (B).....	129
Figure 6-3 Agarose electrophoresis gel results of second PCR product (C).....	130
Figure 6-4 Agarose electrophoresis gel results of third PCR product (D).....	131
Figure 6-5 Agarose electrophoresis gel results of cloning (E).....	133
Figure 6-6 DNA sequence of the synthetic <i>Bacillus</i> spp. uricase gene.....	135
Figure 6-7 SDS-PAGE analysis of uricase fragment from the flow-through of a second amylose column.....	136

List of Tables

Table 2-1 Compositions of SDS-PAGE.....	49
Table 6-1 Schematic representation of sixteen long oligonucleotide.....	123

