國立交通大學

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博 士 論 文

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Molecular Evolution of Uricase and Its Applications in Biochip System

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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天然界中，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 核甘酸約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 ℃至 40 ℃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。

Molecular Evolution of Uricase and Its Applications in Biochip Systems

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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 determunation 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 ºC, 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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