探討α-L-arabinofuranosidase E223 單點突變造成的反應機制之改變

研究生：陳凱郁　　指導教授：李耀坤博士

國立交通大學應用化學所碩士班

摘　要

木腐黴菌（*Trichoderma koningii* G-39）阿拉伯呋喃糖苷酵素(ABF)，屬於醣類水解酵素第 54 家族，其催化機制為保留機制（retention）且速率決定步驟為去醣基化（dearabinosylation）。其中 E223 和 D299 分別扮演親核基和一般酸/鹼基團之角色。在本研究中我們利用溶劑同位素效應（isotope solvent effect）再次強化 D299 在催化反應中之角色。然而，有趣的是突變酵素（E223G）仍保有明顯的活性。*Aspergillus kawachii* ABF 是目前此家族中唯有三維立體結構，利用蛋白質序列相比對，發現兩者之相同度（identity）達 72%，且其中活性區內之重要胺基酸均高度保留。因此我們利用其 *Aspergillus kawachii* ABF 之三維立體結構為模板，進行 *T. koningii* ABF 結構模擬。發現 D191 的位置可取代 E223 成為另一個重要胺基酸殘基，其與受質異位性碳原子（anomeric carbon）距離約 4 Å，故當 E223 被突變成 G223 時，可能使 E223G 反應轉變為反轉機制（inverting），而本研究旨於探討此突變酵素之催化性質與機構。

本研究利用 *Pichia pastoris* 系統表現酵素，經由單菌落 PCR 確認基因轉殖結果，再由活性測試與蛋白質電泳方法，可以成功的篩選表現效率最佳之單菌落，其再進一步被誘導產生大量酵素。經 80%飽和度之銨鹽沉澱與陽離子交換樹脂管柱層析，可以得到均質度達 95%的酵素以利

動力學研究使用。

根據酵素動力學的研究E223G/D191N與E223G/D191G活性值（k_{cat}/K_m）降為E223G的 0.69% ~ 4.6%，顯示D191 在E223G中可能是重要胺基酸。另外，E223G/D299N活性值降為wild type的 0.12%，E223G的2.8%，這很有可能說明D299 不僅在wild type之中，同時也在E223G中是重要胺基酸。

E223G 和Wild type的pH activity profile顯示兩鐘形曲線分佈，這兩酵素催化過程都分別由兩重要胺基酸基團調控。對其他突變酵素之pH profile研究顯示，其中E223G/D191G之 pK_{a2}=5.56，並且沒有pK_{a1}。E223G/D299N之pK_{a1}=2.23 沒有pK_{a2}。這些證據都顯示D191 和D299 分別是E223G的一般鹼基團與一般酸基團。

由分子模擬得知，D299 和 E223 距離約為 6 Å，是預期中保留機制兩重要胺基酸残基的距離。D299 與 D191 距離為 7.5 Å，則是預期可進行反轉機制的距離，因此我們認為 E223G 之催化反應為構型反轉之機構，而 D191 與 D299 是其重要胺基酸殘基，分別扮演一般酸與一般鹼之重要角色。

Convert a retaining α-L-arabinofuranosidase to inverting enzyme by single point mutation on E223

Student：Kai-Yu Chen　　Advisor：Dr. Yaw-Kuen Li

Department of Applied Chemistry
National Chiao-Tung University

ABSTRACT

The α-L-arabinofuranosidase (ABF) from *Trichoderma koningii* G-39 is a retaining enzyme belonging to GH family 54. Our previous study showed that breakdown of arabinosyl-enzyme intermediate is the rate limiting step of the catalytic reaction. The essential groups are E223 (nucleophile) and D299 (general acid/base). In this study, the investigation of kinetic solvent isotope effect re-confirmed that the D299 functions as the general acid/base in the catalytic reaction.

Surprisingly, E223G mutant was found to remain significant activity, while E223Q was completely inactive. The structure of *T. koningii* ABF was obtained from the homology simulation by using the structure of *Aspergillus kawachii* ABF (family 54) as the template. The structure exhibited that the distance between D191 and anomeric carbon of substrate is about 4 Å and the space is suitable for accommodating a water molecule. D191 was, thus, proposed to be essential for the catalysis of E223G, and the mechanism of E223G might become an inverting process. For studying this hypothesis, a series of double mutants, such as E223G/D191N, E223G/D191G and E223G/D299N, were constructed and over-expressed in *Pichia pastoris* system. The colonies with high-level expression were selected through various

steps of validation including colony PCR to confirm gene insertion and activity assay or protein electrophoresis (SDS-PAGE) to evaluate the protein expression level. After 80% ammonium sulfate precipitation following by a cation-exchanged chromatographic separation, enzymes can be purified to reach 95% homogeneity and used for further study.

Kinetic study revealed that the relative activity (k_{cat}/K_m) of E223G/D191N, E223G/D191G and E223G/D299N are 0.69%~4.6% of that of E223G. A bell-shaped pH-profile of E223G showed the catalytic activity of this enzyme is mediated by two pK_as, 1.8 and 4.2. However, a sigmoidal pH-profile was observed for E223G/D191G (pK_{a2}= 5.56) and for E223G/D299N (pK_{a2}= 2.23), indicating that D191 and D299 are the general base and the general acid of E223G, respectively. These findings are consistent with the suggestions obtained from the simulated structure of *T. koningii* ABF.