

# Purification and Characterization of an Intracellular Catechol 1,2-dioxygenase from *Pseudomonas aeruginosa* TKU002

羅翊璋、顏裕鴻 王三郎

E-mail: 9417913@mail.dyu.edu.tw

## ABSTRACT

*Pseudomonas aeruginosa* TKU002 is the production bacillus for catechol 1,2-dioxygenase ( abridged as C12O ) screened by this laboratory, capable of accumulating intermediate product catechol, with decomposition into cis,cis-mucomic acid by the C12O produced by the bacillus. The preferable culture medium for TKU002 to yield C12O is 0.05% urea, 0.3% glycerin, 0.6% Sodium Benzoate. Cultured at 30 for 3 days, the bacilli are retrieved for ultrasound homogenization. The supernatant from centrifugation is purified into a singular C12O enzyme through separation of nucleic acid with streptomycin sulfur and purification steps by DEAE-Sephacryl CL-6B and Sephacryl S-100, with molecular weight about 22 kDa, iso-electric point less than pH 5. In the substrate specificity respect, C12O shows a higher activity to pyrogallol; as for the influence of metallic ions on the enzyme, Zn<sup>2+</sup> inhibits its activity, while Mn<sup>2+</sup> promotes it

Keywords : 鄰苯二酚 ; 苯甲酸鈉 ; 兒茶酚

## Table of Contents

目錄 封面內頁 簽名頁 授權書.....	iii	中文摘		
要.....	iv	英文摘要.....	v	誌
謝.....	vi	目錄.....	vii	
圖目錄.....	xi	表目錄.....		
xii 第一章 緒言.....	1	第二章 文獻回顧.....		
3 2.1加氧?.....	3	2.1.1加氧?的分類.....	4	2.2雙加
氧?催化斷裂芳香環的反應式.....	4	2.3偏鄰苯二酚雙加氧?.....	6	2.4影響酵素活
性因子.....	7	2.5鄰苯二酚代謝路徑.....	8	2.6雙羥內、外雙加
氧?的催化反應比較.....	9	2.7芳香族化合物降解中間產物之利用.....	17	2.7.1 cis-Dihydrodiol化合
物之生產.....	17	2.7.2兒茶酚 ( catechol ) 之生產.....	18	2.7.3順、順 - 己二烯二酸之生
產.....	19	2.8苯甲酸1,2-雙加氧?.....	20	2.8.1蛋白質結構與催化原
理.....	20	2.8.2 酵素特性.....	21	2.9相關酵素之分類比
較.....	22	2.10蛋白質鹽析及沉澱法.....	22	2.11離子交換
法.....	23	第三章 實驗材料與器材.....	24	3.1實驗材
料.....	24	3.1.1菌株.....	24	3.1.2培養基材
料.....	24	3.1.3基質材料.....	25	3.1.4藥品與耗
材.....	25	3.1.5膠體材料.....	25	3.2實驗器
材.....	26	3.3最適培養基.....	27	3.4菌株活化
及保存.....	29	3.5粗酵素液調製.....	28	3.6兒茶酚雙加
氧?活性之測量.....	29	3.6.1 Catechol 1,2-dioxygenase ( C12O ) 活性測定.....	29	3.7兒茶酚含量測
定.....	30	3.8菌株生長情形探討.....	30	3.9酵素純
化.....	30	3.9.1大量培養.....	30	3.9.2菌體破
碎.....	31	3.9.3去核酸.....	31	3.9.4透
析.....	31	3.9.5 DEAE Sepharose CL-6B 管柱層析法.....	32	3.9.6濃
縮.....	32	3.9.7 Sephacryl S-200管柱層析法.....	32	3.9.8 冷凍乾燥
濃縮.....	33	3.9.9 Sephacryl S-200管柱層析法(第二次).....	33	3.9.10等電
點.....	33	3.10電泳分析.....	35	3.11 TKU002所
產生Catechol 1,2-dioxygenase ( C12O )之生 化性質探討.....	38	3.11.1 C12O之基質特		
異性.....	38	3.11.2金屬離子分別對粗酵素及純化後酵素之影響.....	39	第四章 結果與討
論.....	41	4.1菌株生長曲線測量.....	41	4.2酵素純
化.....	42	4.2.1大量培養.....	42	4.2.2菌體破

碎.....	42 4.2.3去核酸.....	42 4.2.4透
析.....	43 4.2.5 DEAE Sepharose CL-6B 管柱層析.....	43 4.2.6
Sephacryl S-200管柱層析法.....	43 4.2.7 Sephacryl S-200管柱層析法(第二次).....	44 4.2.8 C12O之
等電點.....	44 4.3電泳分析.....	51 4.4 TKU002所產
生Catechol 1,2-dioxygenase ( C12O )之生 化性質探討.....	52 4.4.1 C12O之基質特異	52 4.4.2金屬離子對C12O活性之影響.....
性.....	52 第五章 結	論.....
論.....	54 參考文獻.....	55 圖目錄
圖2.1鄰苯二酚被雙加氧?分解途徑.....	5 圖2.2原兒茶酚 3,4-雙加氧?催化五倍子酚開環斷裂所推測 之反	應機構.....
11 圖2.3鐵( III ) 雙加氧?催化雙脛內斷裂的反應機構示意圖..	12 圖2.4鐵( II	)雙加氧?催化雙脛外斷裂的反應機構示意圖.....
14 圖2.5雙加氧?MhpB與水解?MhpC催化的反應.....	15 圖2.6 MhpB的	雙脛外斷裂反應產生內酯中間體的證明式....
16 圖3.1 C12O純化流程圖.....	34 圖4.1 TKU	002生長曲線及產生catechol 量.....
41 圖4.2 DEAE Sepharose CL-6B之C12O純化圖.....	46 圖4.3	Sephacryl S-100之C12O純化圖.....
47 圖4.4 Sephacryl S-100之C12O <sup>2nd</sup> 純化圖.....	48 圖4.5 C12O之	等電點層析圖.....
49 圖4.6 C12O 電泳分析圖.....	51 表目錄 表3.1 酵素C12O與C23O反應溶液組成分析.....	29 表3.2 SDS-PAGE配
方.....	36 表3.3電泳液、追蹤染劑、退染劑配方.....	37 表3.4蛋白質標準
品組成之分子.....	38 表4.1 菌株TKU 002之catechol 1,2-dioxygenase純化表.....	50 表4.2 TKU 002
、KS-1及AN-22產生之C12O之基質特異性. 53 表4.2金屬離子對純化後C12O活性之影響.....	53	

## REFERENCES

- 吳文傑。2004。液化澱粉芽孢桿菌V656所生產蛋白?之研究。大葉大學生物產業科技系碩士論文。彰化。
- 施秋榮。2002。偏鄰苯二酚雙加氧?的基理型抑制反應 - 3-胺基甲基兒茶酚的合成與反應機構探討。成功大學化學系碩士論文。台南。
- 游淑玲。2004。Pseudomonas aeruginosa TKU002所生產兒茶酚1,2-雙加氧?之純化及定性。大葉大學分子生物科技學系碩士論文。彰化。
- 賴章旭。2002。3-(N-甲基胺基)甲基兒茶酚及3-胺基甲基兒茶酚與偏鄰苯二酚雙加氧?的反應。國立成功大學化學系碩士論文。台南。
- Bertini, I., Briganti, F., Mangani, S., Nolting, H. F., Scozzafava, A., 1995. Biophysical Investigation of Bacterial Aromatic Extradiol Dioxygenase Involved in Biodegradation Processes. *Coord. Chem. Rev.* 144: 321-345.
- Dagley, S., Stopher, D. A., 1959. A New Mode of Fission of the Benzene Nucleus by Bacteria. *Biochem. J.* 73:16- 17.
- Hayaishi, O., Katagin, M., Rothberg, S., 1955. Mechanism of The Pyrocatechase Reaction. *J. Am. Chem. Soc.* 77: 5450-5451.
- Horiguchi, S., and Yamada, K., 1968. Studies on the utilization of hydrocarbons by microorganisms. *Agr. Biol. Chem.* 32:555-560.
- Kita, A., Kita, S., Fujisawa, I., Inaka, K., Ishida, T., Horiike, K., Nozaki, M., Miki, K., 1999. An Archetypical Extradiol-Cleaving Catecholic Dioxygenase: The Crystal Structure of Catechol 2,3-Dioxygenase( Metapyrocatechase ) from Pseudomonas Putida mt-2. *Structure.* 7:25-34.
- Loh, K. C., Chua, S. S., 2002. Ortho Pathway of benzoate degradation in Pseudomonas putida: induction of meta pathway at high substrate concentrations. *Enzyme Microb. Technol.* 30:620-626.
- Lam, W. W. Y., Bugg, T. D. H., 1994. Chemistry of Extradiol Aromatic Ring-Cleavage - Isolation of a Stable Dienol Ring Fission Intermediate and Stereochemistry of Its Enzymatic Hydrolytic Cleavage. *J. Chem. Soc. Commun.* 1163-1164.
- Nozaki, M., Kotani, S., Ono, K., Senoh, S., Metapyrocatechase, . 1970. Substrate Specificity and Mode of Ring Fission. *Biochem. Biophys. Acta.* 220:224-238.
- Nozaki, M., Kagamiyama, H., Hayaishi, O., Metapyrocatechase, 1963. Purification Crystallization and Some Properties. *Biochem. Z.* 338: 582- 590.
- Nakai, C., Hori, K., Kagamiyama, H., Nozaki, M., Nakazawa, T., Inouye, S., Ebina, Y., Nakazawa, A., 1983. Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of Pseudomonas putida mt-2. *J. Biol. Chem.* 258:2923- 2928.
- Orville, A. M., Lipscomb, J. D., 1993. Simultaneous binding of nitric oxide and isotopically labeled substrates or inhibitors by reduced protocatechuate 3,4-dioxygenase. *J. Biol. Chem.* 268:8596-8607.
- Que, L. Jr., Ho, R. Y. N., 1996. Dioxygen Activation by Enzymes with Mononuclear Non-Heme Iron Active Sites. *Chem. Rev.* 96: 2607-2624.
- Saeki, Y., Nozaki, M., Senoh, S., 1980. Cleavage of pyrogallol by non-heme iron-containing dioxygenases. *J. Biol. Chem.* 255: 8465-8471.
- Sanvoisin, J., Langley, G. J., Bugg, T. D. H., 1995. Mechanism of Extradiol Catechol Dioxygenases: Evidence for a Lactone Intermediate in the 2,3-Dihydroxyphenylpropionate 1,2-Dioxygenase Reaction. *J. Am. Chem. Soc.* 117:7836-7837.
- Wolgel, S. A., Dege, J. E., Perkins-Olson, P. E.; Jaurez-Garcia, C. H., Crawford, R. L., Munck, E., Lipscomb, J. D., 1993. Purification and characterization of protocatechuate 2,3-dioxygenase from Bacillus macerans. *J. Bacteriol.* 175:4414-4426.
- Zukowski, M. M., Gaffney, D. F., Speck, D., Kauffmann, M., Findeli, A.; Eisecup, A., Lecocq, J. P., 1983. Chromogenic Identification of Genetic Regulatory Signal in Bacillus subtilis Based on Expression of a Cloned Pseudomonas Gene. *Proc. Natl. Acad. Sci.* 80:1101-1105.