

Characterization of glutamate decarboxylase and industrial production of GABA

張文翰、簡宏堅

E-mail: 321469@mail.dyu.edu.tw

ABSTRACT

Glutamate Decarboxylase 3 (GAD3) can remove carboxyl group and catalyze glutamate into GABA (γ -aminobutyric acid). Glutamate was used to control nerve impulse once changed into GABA which will inhibit nerve impulse. Human brain lacking GAD can't produce GABA normally, and cause hypertension, epilepsy, manic-depressive psychosis, psychomania, insomnia, and end shaking. This shows the important role of GAD in neuron metabolism, and the essential need in the proteomics are of health care system. In this study, the characteristics of the GAD 3 enzyme were analyzed by pH, ion requirements, substrate inhibition, product inhibition , substrate specificity, structure analysis and activity site analysis. These were helpful to promote mass production of GABA in the fermentation tank, and hoping to help mental patients. On the other hand, the random mutation of GAD3 gene was performed by Error prone PCR. However, mutant analysis of GAD3 random mutation was used by the method of TNB with a small amount of crude enzyme expressed after induction . The clones of low activity with L 1, L 24, and high activity with H 20, H 38, H 1, H 26, H 39 and H 46 Were obtained by the method of TNB and sequenced to find out the mutation points. L 1 and L 24 have the same mutation of D 296 A. H 20 has two mutation points of G 297 A and S 491 P. H 38 has a single mutation points of G 297 A. H 1 has two mutation points of V 78 A and F 271 S. H 26 has only one mutation of F 271 S. H 39 has five mutation points of R 50 L, V 78 A, F 271 S, I 375 V and G 515 V. While H 46 has six mutation points of T 16 S, V 78 A, D 128 G, F 271 S, S 471 T and W 423 L. However D 296 and S 491 were found to be the active site suggested by the previous comparison. Using analysis of SDS-PAGE, purified protein of GAD 3 and rest of GAD 3 mutants were found the protein molecular weight of 56 kDa. *Y. lipolytica* culture encoding glutamate decarboxylase 3 is being actively cooperate with the Vedan in a small 1.2 L fermentation to add 20 μ M Pyridoxal phosphate and 20 μ M Ferrous fumarate as cofactors. HPLC analysis of the GAD 3 activating has been accurately calculated 5 mM MSG disappeared with the ratio of 61% in 24 hrs, however in the biosynthesis of GABA on the conversion rate of 85 % in 24 hrs. The industrial production of GABA in the 5 L fermenter evaluation was, constantly improved to the conversion rate of 100 % with 1.42 % solid content analyzed by HPLC. While IN the 1000 L fermentor control conditions were determined to be pH: 5.4 ~ 5.3 and 0.05 ~ 0.018 (%) of glucose concentration. Vedan company recently want to produce a 10 Kg of GABA which can enter in the animal and human trials for health food.

Keywords : glutamic acid decarboxylase enzyme、 glutamic acid、 amino butyric acid、 activity analysis、 industry production 、 system、 after

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	vi	誌謝	viii	目錄	ix	圖目錄	xii	表目錄	xviii	1. 前言及目的與研究動機	1	1.1 前言	1	1.2 目的	2	1.3 研究動機	4	2. 材料與方法	6	2.1 材料	6	2.1.1 菌種與質體	6	2.1.2 藥品	6	2.1.3 酵素	6	2.1.4 引子	7	2.1.5 培養液	8	2.1.6 其他緩衝液及試劑	10	2.2 實驗方法	16	2.2.1 絲狀真菌染色體DNA 的抽取	16	2.2.2 PCR	17	2.2.3 隨機突變之PCR方法	19	2.2.4 DNA 電泳分析	20	2.2.5 DNA片段的回收及純化	21	2.2.6 GAD 3 基因表現載體的構築	22	2.2.7 酵素剪切	22	2.2.8 DNA 黏接	22	2.2.9 Competent cell的製備	23	2.2.10 轉殖入Escherichia coli	23	2.2.11 轉殖入Yarrowia lipolytica	24	2.2.12 Plasmid DNA之抽取	24	2.2.13 快速質體抽取套組	25	2.2.14 隨機突變GAD 3之E. coli小量菌體活性篩選	26	2.2.15 DNA 定序	30	2.2.16 GAD 3 E. coli胞內表現及破菌處理	30	2.2.17 Yarrowia lipolytica基因表現及透析	31	2.2.18 Ni-NTA column純化GAD 3	32	2.2.19 SDS-PAGE	33	2.2.20 酵素反應在Bromocresol green顯色	34	2.2.21 GAD 3最適pH測定法	34	2.2.22 GAD 3最適催化溫度測定法	35	2.2.23 GAD 3離子需求	35	2.2.24 GAD 3基質選擇性	36	2.2.25 GAD 3活性測定	36	2.2.26 GAD 3 之生長曲線測定	36	2.2.27 SC - GAD 3之生長期間GAD 3表現量與活性測定	37	2.2.28 SC - GAD 3之酵素表現期間GAD 3表現量與活性測定	38	2.2.29 SC - GAD 3 培養於YPD with L - Glutamate、 65 mM pH 6.6 phosphate buffer液態培養基中之酵素表現週 期GAD 3表現量與活性測定。	39	2.2.30 GAD 3針對不同廠牌Monosodium L-Glutamate (味精 MSG) 活性比較	39	2.2.31 不同濃度味全MSG對酵素活性影響	40	2.2.32 GAD 3產物抑制性	40	2.2.33 SC-GAD 3 1.2L搖瓶培養與活性測定	41	2.2.34 SC-GAD 3 5L發酵槽培養與生產條件	42	2.2.35 受質與產物之螢光HPLC分析	43	3. 結果	44	3.1 絲狀真菌染色體DNA 的抽取	44	3.2 PCR產物	44	3.3 回收DNA	45	3.4 轉殖質體製備檢查	45	3.5 Random mutation GAD 3之E. coli小量菌體活性篩選	46	3.6 GAD 3定序基因分析	46	3.7 SC - GAD3 生長曲線	47	3.8 GAD 3少量基因表現	48	3.9 酵素反應溴甲酚綠顯色	48	3.10 GABA與OPA反應線性回歸標準曲線	48	3.11 SC - GAD3 生長期間GAD 3之表現量與活性測定	49	3.12 SC - GAD3 酵素表現期間GAD 3之表現量測定	49	3.13 GAD 3	
----	----	----	-----	-----	-----	------	----	------	----	----	------	----	----	-----	-----	-----	-------	---------------	---	--------	---	--------	---	----------	---	----------	---	--------	---	-------------	---	----------	---	----------	---	----------	---	-----------	---	----------------	----	----------	----	----------------------	----	-----------	----	------------------	----	----------------	----	-------------------	----	-----------------------	----	------------	----	--------------	----	-------------------------	----	----------------------------	----	-------------------------------	----	-----------------------	----	-----------------	----	----------------------------------	----	---------------	----	-------------------------------	----	-----------------------------------	----	-----------------------------	----	-----------------	----	---------------------------------	----	---------------------	----	-----------------------	----	------------------	----	-------------------	----	------------------	----	----------------------	----	-------------------------------------	----	---------------------------------------	----	--	----	--	----	-------------------------	----	-------------------	----	-------------------------------	----	------------------------------	----	-----------------------	----	-------	----	--------------------	----	-----------	----	-----------	----	--------------	----	---	----	-----------------	----	--------------------	----	-----------------	----	----------------	----	-------------------------	----	-----------------------------------	----	----------------------------------	----	------------	--

的純化與回收50 3.14 GAD 3最適酸鹼環境50 3.15 GAD 3最適催化溫度51 3.16 GAD 3離子需求51 3.17 GAD 3基質選擇性51 3.18 GAD 3與不同廠牌MSG反應比較52 3.19 不同濃度味全MSG對GAD 3的受質抑制測定53 3.20 GAD 3產物抑制作用測定53 3.21 Wild type以及突變GAD 3的活性分析54 3.22 SC - GAD 3 1.2L搖瓶培養與活性測定57 3.23 SC - GAD 3 5L發酵槽培養與生產條件58 3.24 受質與產物之螢光HPLC分析59 4. 結論61 參考文獻148 附錄152 圖目錄 圖 1 L-Glutamic acid structure 和 Chemical structure of Gamma - aminobutyric acid.而 GAD 3 酵素可將L - Glutamic Acid 上的羧基去除轉變成為GABA.68 圖 2 Monosodium L - Glutamate 的化學結構.69 圖 3 了解Glutamate和GABA與神經元之關係70 圖 4 實驗流程設計71 圖 5 將GAD 3基因轉殖入pQE 30的表現載體72 圖 6 *Aspergillus oryzae* 的染色體抽出後，酵素切BamH I 73 圖 7 GAD 3 基因利用PCR增幅後產物74 圖 8 酵素切 BamH I 和 Sal I 回收後之結果75 圖 9 將轉殖好的菌體抽質體 DNA76 圖 10 將抽出後的質體 DNA 使用酵素切 BamHI 和SalI後的 結果77 圖 11 GAD 3的 DNA 和胺基酸的序列78 圖 12 使用胺基酸分析軟體，分析 GAD 3 酵素之基礎79 圖 13 SDS - PAGE小量酵素電泳分析80 圖 14 SDS - PAGE分析GAD 3酵素81 圖 15 SDS - PAGE分析GAD 3酵素82 圖 16 SDS - PAGE分析GAD 3酵素83 圖 17 OPA法之GAD 3 活性分析方法84 圖 18 使用OPA法測定GAD 3 酵素或性之最適波長85 圖 19 GABA 之 Standard Curve86 圖 20 利用TNB法測定GAD 3酵素活性最適波長87 圖 21 初試TNB 法之活性分析測定88 圖 22 篩出活性較低之L 1 - D 296 A，其DNA 和胺基酸序列 及回收酵素之SDS - PAGE89 圖 23 篩出 活性較高之H 20 - G 297 A and S 491 P，其DNA 和胺基酸序列及回收酵素之SDS - PAGE90 圖 24 篩出活性較高之H 38 - G 297 A，其DNA和胺基酸序 列及回收酵素之SDS - PAGE91 圖 25 篩出活性較高之H 1 - V 78 A and F 271 S，其DNA 和 胺基酸序列及回收酵素之SDS - PAGE92 圖 26 篩出活性較高之H 26 - F 271 S，其 DNA 和胺基酸序列 及回收酵素之SDS - PAGE93 圖 27 篩出活性較高之H 39 - L 50L、V 78 A、F 271 S、I 375 V and G 515 V，其 DNA 和胺基酸序列及回收酵素 之 SDS - PAGE94 圖 28 篩出活性較高之H 46 - T 16 S、V 78 A、D128 G、F 271 S和W 423 L其 DNA 和胺基酸序列及回收 酵素之 SDS - PAGE95 圖 29 為酵素動力學分析前GABA Standard Curve96 圖 30 GAD 3和隨機突變株之酵素動力學分析98 圖 31 將各種GAD酵素作胺基酸分析比對99 圖 32 與文獻比對後得知活性區域，並了解GAD 3酵素之作用機制100 圖 33 模 擬GAD 3酵素之3D立體結構，標示出活性中心與 各突變株之位置101 圖 34 溴甲酚綠顯色法，L - Glutamate102 圖 35 溴 甲酚綠顯色法，L - Glutamate 與熱失活之酵素反應103 圖 36 溴甲酚綠顯色法，L - Glutamate 與酵素反應104 圖 37 溴甲 酚綠顯色法，L - Glutamate 與兩倍酵素反應105 圖 38 溴甲酚綠顯色法未與任何物質反應106 圖 39 各種溴甲酚綠顯色法進 行趨勢比較107 圖 40 測定GAD 3酵素最適pH108 圖 41 測定GAD 3酵素最適溫度109 圖 42 測定金屬離子對GAD 3酵素之影 響110 圖 43 使用GAD 3酵素與L - Tyronly再做 OPA化反應111 圖 44 使用GAD 3酵素與D - Tyronly再做 OPA化反應112 圖 45 使用GAD 3酵素與Phelalanine再做 OPA化反應113 圖 46 使用GAD 3酵素與Tryptophan再做 OPA化反應114 圖 47 使 用GAD 3酵素與Lysine再做 OPA化反應.115 圖 48 使用GAD 3酵素與para - Hydroxybenzoate再做OPA化反 應116 圖 49 使 用GAD 3酵素與Leucine再做 OPA化反應117 圖 50 使用GAD 3酵素與Glycine再做 OPA化反應118 圖 51 使用GAD 3酵素 與MSG再做 OPA化反應119 圖 52 比較各種MSG與GAD 3酵素之反應120 圖 53 味全 MSG 測酵素活性抑制121 圖 54 GAD 3 酵素測產物抑制122 圖 55 載體pYLSC 1之圖譜123 圖 56 PLP 和 B6 之輔?124 圖 57 增幅GAD 3之基因.125 圖 58 增幅GAD 3 之基因後並增加KpnR之酵素切位.126 圖 59 回收已增加KpnR之GAD 3基因127 圖 60 將轉殖好的GAD 3抽質體DNA128 圖 61 將抽好的質體DNA 酵素切Kpn I 和Sfi I129 圖 62 SC - GAD 3 DNA和胺基酸序列130 圖 63 SDS - PAGE分析 SC - GAD 3酵素分泌至胞外結果131 圖 64 SC - GAD 3與po1g之生長情形132 圖 65 SC - GAD 3與po1g之誘導情形133 圖 66 SC - GAD 3與po1g在生長期間偵測酵素分泌至胞外 情況134 圖 67 1.2 L培養及誘導流程設計135 圖 68 1.2 L培養及誘導三重複之 情形136 圖 69 1.2 L培養與誘導pH值之變化137 圖 70 連續添加受質對於pH之影響138 圖 71 HPLC分析與計算出消失率與轉 換率139 圖 72 5 L發酵槽之酵素活性與pH值測定140 圖 73 消泡劑對於GABA與酵素活性之影響141 圖 74 利用5 L發酵槽做 初步生產與模擬1,000L發酵槽之生 產情況142 圖 75 在發酵槽內葡萄糖之消耗情形143 圖 76 利用監控條件產製並利 用HPLC分析144 圖 77 產物溶液與固型物百分比測定 145 圖 78 A組加入 (1 : 1) 與 (1 : 2) 付型劑之HPLC 分析146 圖 79 B 組加入 (1 : 1) 與 (1 : 2) 付型劑之HPLC分析147 表目錄 表 1 突變 GAD 3 之測小量活性方法 27 表 2 突變GAD 3 之測小量 活性第一組結果27 表 3 突變GAD 3 之測小量活性第二組結果28 表 4 突變GAD 3 之測小量活性第三組結果29 表 5 突 變GAD 3 酵素動力學之分析結果 56 表 6 突變GAD 3 酵素動力學之分析統整後做差異56

REFERENCES

參考文獻 1. 方于芃. 改良式發酵對普洱茶機能性成分之影響. 國立中興大學食品科技學系碩士論文. 2004. 2. 簡崇倫. 來自麴菌一種新 新的麩胺酸脫羧基?.在大腸桿菌中執行分子基因選殖與酵素活性分析. 大葉大學分子生物科技學系專題. 2008. 3. 王志傑. 紅麴菌二級 代謝物之生產與應用之研究. 國立台灣大學微生物與生化學研究所博士論文. 2003. 4. 吳奕韻. 富含 -胺基丁酸乳酸發酵糙米奶之研 製. 國立嘉義大學食品科技學系碩士論文. 2003. 5. Andres M, LozanoandSuneil K. Kalia.帕金森新解答.2005.科學人雜誌. 42:46-53. 6. Bio-Rad Laboratories. Bio-Rad Protein Assay, Bulletin No. 600-0005, Bio- Rad Laboratories GmbH, Munich, Germany. 1994. 7. Cartherine P. S. T., Owen R. V. C., Michael ang Barry J. S. , Regulation of -aminobutyric acid synthesis in situ by glutamate availability .1999. *Physiologia Plantarum*. 106:363-369. 8. Das s. k. and Ray P. K. , Ontogeny of GABA pathway in human fetal brains.1996. *Biochem Biophys Res commmum*. 228:544-548. 9. Daniele Marra de Freitas Silva, Vany P.Ferraz and Angela Maria Ribeiro:Improved high-performance liquid chromatographic method for GABA and glutamate determination in regions of the rodent brain. *Journal of Neuroscience Methods*.177:289-293. 2009. 10. Guido

Capitani, Daniela De Biase, Caterina Aurizi, Heinz Gut, Francesco Bossa, and Markus G. Gruetter, Crystal structure and functional analysis of Escherichia coli glutamate decarboxylase, *The EMBO Journal*, 22(16): 4027-4037. 2003. 11. Gustavo Fenalti, Ruby H P Law, Ashley M Buckle, Christopher Langendorf, Kellie Tuck, Carlos J Rosado, Noel G Faux, Khalid Mahmood, Christiane S Hampe, J Paul Banga, Matthew Wilce, Jason Schmidberger, Jamie Rossjohn, Ossama El-Kabbani, Robert N Pike, A Ian Smith and Ian R Mackay. GABA production by glutamic acid decarboxylase is regulated by a dynamic catalytic loop. *NATURE STRUCTURAL & MOLECULAR BIOLOGY*. 14 (4):280–286. 2007. 12. Guido Capitani, Daniela De Biase, Heinz Gut, Shaheen Ahmed, Markus G. Gritter: Structural Model of Human GAD 65. Prediction and Interpretation of Biochemical and Immunogenic Features. *Structure, Function, and Bioinformatics*. 59:7-14. 2005. 13. Hayakawa K, Ueno. Y. Kawamura, S., Taniguchi R. Oda K. , Production of γ -aminobutyric acid by lactic acid bacteria. *Seibutsu Kogaku*. 75:239-244. 1997. 14. Il Lae Jung and In Gyu Kim: Polymines and Glutamate Decarboxylase-based Acid Resistance in Escherichia coli. *The Journal of Biological Chemistry*. 278: 22846-22852. 2003. 15. Jacobs, W.A. o-Phthaldehyde-sulfite deriviation of primary amines for liquid chromatography-electrochemistry. *J. Chromatogr*. 392:435-441. 1987. 16. Kohama K. , Isolation and identification of hyposensitive principle in red mold rice. *Chem Pharm Bull*. 25, 2484-2489. 1987. 17. Komatsuzaki N., Shima J and Hayakawa, K. , Production of γ -aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol*. 22: 497-504. 2005. 18. Konol , Himeno K., Changes in γ -aminobutyric acid content during beni-kiji making. *Biosci Biotechnol Biochem*. 64(3), 617-619. 2000. 19. Kazuhito Akama and Fumio Takaiwa. C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in plant cells. *Journal of Experimental Botany*. 58(10), 2699-2707. 2007. 20. Leventhal A. g., Wang Y., Pu. M., Zhou. Y., Ma, Y.. GABA and its agonists improved visual cortical function in senescent monkeys. *Science*. 300:812-815. 2003. 21. Laemmli U.K. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685. 1970. 22. W. Lowry Caudill, Gregory P. Houck and R. Mark Wightman, Determination of γ -Aminobutyric acid by liquid chromatography with electrochemical detection. *Journal of Chromatography*. 227:331-339. 1982. 23. Madzak C., Blanchin-Roland, S., Cordero Otero, R and Gaillardin, C., Functional analysis of upstream regulating regions from the *Yarrowia lipolytica* XPR2 promoter; *Microbiology* 145: 75 – 87. 1999. 24. Madzak C, Treton B, Blanchin-Roland S. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*; *J Mol Microbiol Biotechnol*. 2(2):207-16. 2000. 25. Narahara, H. , Kohi-mold and its product koji. *J Brew Soc Japan*. 889: 873-881. 1994. 26. Nishimura A., Morita M., Nishimura Y., and Sugino Y. A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. *Nucleic Acids Research*, 18: 61-69. 1990. 27. Okada, Y., Taniguchi, H., and Schimada, C.. High Concentration of GABA and high Glutamate Decarboxylase Activity in Rat Pancreatic Islets and Human Insulinoma. *Science*, 194(4265): 620-622. 1976. 28. Pritchard L., Corne D., Kella D., Rowland J., Winson M. A general model of error-prone PCR. *Journal of Theoretical Biology* 234: 497-509. 2005. 29. Romain Chevrot, Ran Rosen, Elise Haudecoeur, Ame ' lie Cirou, Barry J. Shelp, Eliora Ron, and Denis Faur., GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*, *PNAS*, 10319:7460 – 746. 2006. 30. Struzynska L. and Sulkowski G.. Relationships between glutamine, glutamate, and GABA in nerve endings under Pb-toxicity condition. *J. Inorg. Biochem*. 98: 951-958. 2004. 31. Santosh K. , Narayan S. p, The metabolism of 4-aminobutyrate (GABA) in fungi. *Mycol Res*. 101:403-409. 1997. 32. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, and Erlich HA. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 239: 487-491. 1988. 33. Sambrook J. and Russell D.W. *Molecular cloning: a laboratory manual*, third edition. Cold spring harbor laboratory press, New York. 2001. 34. Ueno Y. , Hayakawa K. , Taksahashi S. , Oda K. , Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci Biotech Biochem*. 614: 1168-1171. 1997. 35. Yokoyama S. , Hiramatsu J. I and Hayakawa K. b , Production of γ -aminobutyric acid from alcohol distillery lees by *Lactobacillus brevis* IFO 12005. *J Biosci Bioeng*. 93(1): 95-97. 2002. 36. Yuki Kato, Yoko Kato, Keiji Furukawa, Shodo Hara. Cloning and Nucleotide Sequence of the Glutamate Decarboxylase-encoding Gene *gadA* from *Aspergillus oryzae*. *JSBA, Biosci. Biotechnol. Biochem.*, 66(12): 2600-2605. 2002.