

Production and characterization of the protease and chitinase from *Aspergillus fumigatus* TKU003

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ABSTRACT

本研究係探討 *Aspergillus fumigatus* TKU003 生產蛋白質及幾丁質之較適生產條件，並進行所得蛋白質之純化與特性。最適培養條件為：於含有 2% 蝦蟹殼粉、0.1% K₂HPO₄、0.05% MgSO₄·7H₂O 之液態培養基 (pH 4)，於 37°C 振盪，培養六天後，可得最高的蛋白質活性 (1.15 U/ml)。 *Aspergillus fumigatus* TKU003 發酵液經 DEAE-sepharose CL-6B 等步驟分離純化後，可得分子量 124 kDa，pI 為 8.3 的一種的蛋白質。此蛋白質之 pH 穩定性、最適反應 pH、熱安定性、最適反應溫度分別為 7~9、7~10、30~50、40，PMSF 會完全抑制酵素活性，因此推測此酵素係屬一種絲氨酸型蛋白質。幾丁質最適培養條件為：1% 蝦蟹殼粉、0.1% K₂HPO₄、0.05% MgSO₄·7H₂O、0.1% ammonium sulfate、0.1% ferrous sulfate 之液態培養基 (pH 4)，於 37°C 振盪，培養四天後，可得最高的幾丁質活性 (1.09 U/ml)。發酵液經由 DEAE Sepharose CL-6B 及 Sephacryl S-200 進行酵素之純化後可得一種幾丁質，分子量 29 kDa，pI 為 6.5 的一種的幾丁質。此幾丁質之 pH 穩定性、最適反應 pH、熱安定性、最適反應溫度分別為 5~7、4~7、30~50、40，EDTA 會完全抑制酵素活性，因此推測此酵素係屬一種金屬型酵素，又以 Mg²⁺ 及 Fe²⁺ 離子能夠增加酵素活性，而 Mn²⁺、Hg²⁺ 及 Cu²⁺ 抑制酵素活性。

Keywords : *Aspergillus fumigatus* TKU003

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REFERENCES

- 參考文獻 1. 王三郎 (2000) 應用微生物學, 高立圖書出版社。 2. 王三郎 (2000) 生物技術, 高立圖書出版社。 3. 宋賢一 (2002) 生物化學實驗原理與方法, 藝軒圖書出版社。 4. 李佩玲 (2002) Pseudomonas aeruginosa M-1001所生產抗菌成分之研究, 私立大葉大學食品工程學系研究所碩士論文。 5. 莊榮輝 (2000) 酵素化學實驗, 國立台灣大學農業化學系。 6. 陳石根、周潤琦 (2002) ?學, 九州圖書有限公司。 7. 梁慈雯 (2000) Bacillus subtilis V656所生產微生物抑制物質之研究, 私立大葉大學食品工程研究所碩士論文。 8. 黃群玲 (2003) Aspergillus fumigatus Fresenius所生產幾丁聚醣?之研究, 私立大葉大學食品工程學系研究所碩士論文。 9. 楊政國 (1999) 利用枯草菌進行蝦蟹殼去蛋白之研究, 私立大葉大學食品工程研究所碩士論文。 10. 劉錦芳 (2003) LCF007細菌所生產蛋白質分解酵素純化之研究。 11. 蔡文城 (2000) 微生物學, 藝軒圖書出版社。 12. 賴威安 (2000) Bacillus sp.P-6中蛋白?生產與性質分析, 國立中興大學食品科學系碩士論文。 13. A. Dayanandana, J. Kanagaraj, Lesley Sounderraja, (2003) Application of an alkaline protease in leather processing: an ecofriendly approach, Journal of Cleaner Production 11: 533-536。 14. Adil Anwar and Mohammed Saleemuddin (1998) Alkaline protease: a review. Biore Technol, 64: 175-183 15. Anwar, A., Saleemuddin, M. (1997) Alkaline pH actiog digestive enzyme of the polyphagous insect pest Spilosoma obliqua: stability and potential as detergent additives. Biotechnol. Appl. Biochem., 25: 43-46 16. Akihiko Ano, Tsutomu Takayanagi (2003) Characterization of a class Chitinase from Vitis Viniferacv. Koshu. JOURNAL OF BIOSCIENCE AND BIOENGINEERING. Vol. 95, No. 6, 645-647 17. Boonyaras Sookkheo, Supachok Sinchaikul, Suree Phutrakul, and Shei-Tein Chen (2000) Purification and Characterization of the Highyl Thermostable Proteases form Bacillus stearothermophilus TLS33. Protein Expression and Purification 20, 145-151 18. Babe, L. M. and Schmide, B. (1998) Purification and biochemical analysis of WprA, a 52-kDa serine protease secreted by B. subtilis as an active complex with its 23-kDa propeptide. Biochim. Biophys. Actas. 1386: 211-219 19. Babiker M. A. A / Banat, Yuko Kameyama, takanori Yoshioka, Daizo Koga (1999) Purification and characterization of a 54 kDa chitinase from Bombyx mori. Insect Biochemistry and Molecular Biology 29: 537-547 20. Cristina E. Goto, Elisangela P. Barbosa, Lais C. L. Kistner, Fabiana G. Moreira, Rosane M. Peralta (1998) Production of amylase by Aspergillus fumigatus utilizing -methyl D-glycoside, a synthetic of maltose, as substrate. FEMS Microbiology Letters 167: 139-143 21. Correa, J. U. Elango I., and Polacheck E., (1982) Endo-chitinase, a mannan-associated enzyme from Saccharoment cerevisiae. JOURNAL Biol. Chem, 257: 1392-1397 22. Dara O. Donnell, Liping Wang, Jianfeng Xu, Darin Ridgway, Tingyue Gu, Murray Moo-Young (2001) Enhanced Heterologous protein production in Aspergillus niger Through pH control of extracellular protease activity. Biochemical Engineering Journal 8: 187-193 23. Francoise Bono, Pierr Savi, Anne Tuong, Mohamed Maftouf, Jean-Marie Pereillo, Joel Capdevielle, Jean-Claude Cuillemost, Jean-Pierre Maffread, Jean-Marc Herbert (1996) Purification and characterization of a novel protease from culture filtrates of a Streptomyces sp. FEMS Microbiology Letters 141: 213-220 24. Fan-Chiang Yang and I.-Hsing Lin (1998) Production of acid protease using thin stillage from a rice-spirit distillery by Aspergillus niger. Enzyme and Microbial Technology 23: 397-402 25. Fenton DM and Eveleigh DE. (1981) Purification and mode of action of a chitinase from Penicillium islandicum. J. Gen Microbiol., 126: 151-165 26. Hsing-Chen Chen, Mei-Fang Hsu and

Shann-Tzong Jiang (1997) Purification and characterization of an exo-N,N,-diacetylchitinohydrolase-like enzyme from *Cellulomonas flavigena* NTOU1. *Enz. Microb. Technol.* 20:191-197 27. Hideaki Yamaoka, Hidenori Hayashi, Shuichi Karita, Tetsuya Kimura, Kazuo Sakka, And Kunio Ohmiya (1999) Purification and Some Properties of a Chitinase from *Xanthomonas* sp. Strain AK. *JOURNAL OF BIOSCIENCE AND BIOENGINEERING* Vol.88, No.3, 328-330 28. Ichiro Kawachi, Takuya Fujieda, Minoru Uita, Yuko Ishii, Kenzo Yamagishi, Hiroaki Sato, Toru Funaguma And Akira Hara (2001) Purification and Properties of Extracellular Chitinase from the Parasitic Fungus *Isaria japonica*. *JOURNAL OF BIOSCIENCE AND BIOENGINEERING* Vol.92, No.6, 544-549 29. Ikasari, L and Mitchell, D. A., (1996) Leaching and characterization of *Rhizopus oligosporus* acid protease from solid-state fermentation. *Enzyme Microb. Technol.* 19:171-175 30. Kazuo Ohishi, Masaaki Yamagishi, Toshiya Ohta, Mitsuaki Suzuki, Hitoshi Izumida, Hiroshi Sano, Miyuki Nishijima, And Tan Miwa (1996) Purification and Properties of Two Chitinases from *Vibrio alginolyticus* H-8. *JOURNAL OF FERMENTATION AND BIOENGINEERING* Vol.82, No. 6, 588-600 31. Kaustav Aikat, Bimal Chandra Bhattacharyya (2001) Protease production in solid state fermentation with Liquid medium recycling in a stacked plate reactor and in a packed bed reactor by a local strain of *Rhizopus oryzae* *Process Biochemistry* 36:1059-1068 32. Liao, C.H. and Daniel E. McCallus (1998) Biochemical and genetic characterization of an Extracellular protease from *Pseudomonas fluorescens* *Microb.*, 64:914-921 33. Lilik Ikasari and David A. Mitchell (1996) Leaching and characterization of *Rhizopus oligosporus* acid protease from solid-state fermentation. *Enzyme and Microbial Technology* 19:171-175 34. L.A.I. De Azeredo, D.M.G Freire, R.A.M. Soares, S.G.F Leite, R.R.R. Coelho (2004) Production and partial Characterization of thermophilic protease from *Streptomyces* sp. isolated from Brazilian cerrado soil. *Enzyme and Microbial Technology* 34:354-358 35. Masaru Sakurada, Diego P. Morgavi, Kenji Komatani, Yoshifumi Tomita, Ryoji Onodera (1996) Production and characterization of cytosolic chitinase from *Piromyces communis* OTS1. *FEMS Microbiology Letters* 137:75-78 36. N.-J. Remi Shih And Karen A. MCDONALD (1997) Purification and Characterization of Chitinase from Transformed Callus Suspension Cultures of *Trichosanthes kirilowii* Maxim *JOURNAL OF FERMENTATION AND BIOENGINEERING* Vol.84, No.128-34 37. Ogino H., Watanabe F., Yamada M., Nakagawa S., Hirose T., Noguchi A., Yasuda M. and Ishikawa H. (1999) Purification and characterization of organic solvent stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PTS-01. *JOURNAL OF BIOSCIENCE AND BIOENGINEERING* 87:61-68 38. Peter W. Inglis, John F. Peberdy (1997) Production and purification of a chitinase from *Ewingella americana*, a recently described pathogen of the mushroom, *Agaricus bisporus*. *FEMS Microbiology Letters* 157:189-194 39. R.F. Souza, R.C. Gomes, R.R.R Coelho, C.S. Alviano, R.M.A. Soares (2003) Purification and characterization of an endochitinase produced by *Colletotrichum gloeosporioides*. *FEMS Microbiology Letters* 222:40-50 40. Rashbehari Tunga, Binita Shrivastava, Rintu Banerjee (2003) Purification and characterization of a protease from solid state fermentation of *Aspergillus parasiticus*. *Process Biochemistry* 38:1553-1558 41. San-Lang Wang, Ing-Lung Shih, Tze-Wun Liang, and Chi-Hau Wang (2002) Purification and Characterization of Two Antifungal Chitinases Extracellularly Produced by *Bacillus amyloliquefaciens* V656 in a Shrimp and Crab Shell Powder Medium. *J. Agric. Food Chem.*, 50:2241-2248 42. San-Lang Wang, Jen-Kuo Yang, Ing-Lung Shih, Yew-Min Tzeng (2000) Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme and Microbial Technology* 26 :406—413 43. Shu-Yi Wang, Anne-Laure Moyne, George Thottappilly, Shaw-Jye Wu, Robert D. Locy, Narendra K. Singh (2001) Purification and characterization of a *Bacillus cereus* exochitinase. *Enzyme and Microbial Technology* 28:492-498 44. Sun Chul Kang, Sanggyu Park, and Gyu Lee (1999) Purification and Characterization of a Novel Chitinase From the Entomopathogenic Fungus, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* 73:276-281 45. Sandro Germano, Ashok Pandey, Clarice A. Osaku, Saul N. Rocha, Carlos R. Soccol (2003) Characterization and Stability of protease from *Penicillium* sp. produced by solid-state fermentation. *Enzyme and Microbial Technology* 32:246-251 46. Shuzheng Zhang, Gouqing Xia, Chunsheng Jin, Shoujun Yang and Cheng Jin (2001) A novel having a unique mode of Action from *Aspergillus fumigatus* YJ-407. *Eur. J. Biochem.* 268:4079-4085 47. Thangamani Anthony, Krishnan Chandra RAJ, Ayyappan Rajendran, And Paramasamy Gunasekaran (2003) Inhibition Of Proteases during Fermentation Improves Xylanase Production by Alkali Tolerant *Aspergillus fumigatus* AR1. *JOURNAL OF BIOSCIENCE AND BIOENGINEERING* 96:394-396 48. Thomas Aalbak, Morten Reeslev, Bo Jensen, Susanne H. Eriksen (2002) Acid protease and formation of multiple forms of glucoamylase in batch and continuous cultures of *Aspergillus niger*. *Enzyme and Microbial Technology* 30:410-415 49. T. Anthony, K. Chandra Raj, A. Rajendran, P. Gunasekaran (2003) High molecular weight cellulose-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. *Enzyme and Microbial Technology* 32:647-654 50. Utz Reichard, Michel Monod, Reinhard Ruchel (1995) Molecular cloning and sequencing of the gene encoding an Extracellular aspartic proteinase from *Aspergillus fumigatus*. *FEMS Microbiology Letters* 130:69-74 51. Veronique Planchot, Paul Colonna (1995) Purification and Characterization of extracellular alpha-amylase from *Aspergillus fumigatus*. *Carbohydrate Research* 272:97-109 52. Wang San-Lang and Chio S.H. (1998) Deproteinization of shrimp and crab shell with the protease of *Pseudomonas aeruginosa* K-187. *Enzyme and Microbial Technology* 22:629-633 53. Yoshinari Yamamoto, Yukio Fukunaga, Hideki Aoyagi, And Hideo Tanaka (1995) Purification and Characterization of Chitinase Secreted by Cultured *Wasabia japonica* Cells. *JOURNAL OF FERMENTATION AND BIOENGINEERING* Vol.80, NO.2. 148-152