

液化澱粉芽孢桿菌V656所生產蛋白?之研究

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摘要

由台灣中部土壤分離出一株能以蝦蟹殼粉為主要營養源之蛋白?生產菌, *Bacillus amyloliquefaciens* V656。初步證實此菌可生產蛋白?, 此蛋白?之純化與性質即為本研究之目的。透過比較各種條件下生產之蛋白?的活性, 希望發現此蛋白?對於植物病原菌抑制效果及對植物生長是否有促進的影響, 並探討蛋白?較適生長條件、生化性質及抑制機制等等相關之研究。純化自 *B. amyloliquefaciens* V656液體培養基上清液之蛋白?具耐熱性, 其分子量為28kDa。最佳反應酸鹼值與溫度分別是 pH8 及 37 。

關鍵詞: 蛋白質; #37238; 酵素純化; 蝦蟹殼粉; 液化澱粉芽孢桿菌V656

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參考文獻

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Takami, Teruhiko Akiba, Koki Horikoshi. (1989) Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH-101. *Appl. Microbiol Biotechnol.* 30:120-124.

34. Jean-Pierre Chessa, Ioan Petrescu, Mostafa Bentahir, Jozef Van Beeumen, Charles Gerday. (2000) Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species. *Biochim. Biophys.* 1479:265-274.

35. Jen-Kuo Yang, Ing-Lung Shih, Yew-Min Tzeng, San-Lang Wang. (2000) Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microb. Technol.* 26:406-413.

36. Johannes P. T. W. van den Homberg, Peter J.I. van de Vondervoort, Laurence Fraissinet-Tachet, J. Visser (1997) *Aspergillus* as a host for heterologous protein production: the problem of protease. *Tibtech July.* 15:256-263.

37. Jose Escobar, Stanley Barnett. (1995) Synthesis of Acid Protease from *Mucor miehei*: Integration of Production and Recovery. *Process Biochem.* 8:695-700.

38. Kaustav Aikat, Bimal Chandra Bhattacharyya (2000) Protease extraction in solid state fermentation of wheat bran by a local strain of *Rhizopus oryzae* and growth studies by the soft gel technique. *Process Biochem.* 35:907-914.

39. 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 Biochem.* 36:1059-1068.

40. K. L. Kohlmann, S. S. Nielsen, and M. R. Ladisch. (1991) Purification and characterization of an extracellular protease produced by *Pseudomonas fluorescens* M3/6. *J. Dairy Sci.* 74:4125-4136.

41. Kono, I., Himeni, K. (2000) Changes in —aminobutyric acid content during beni-joji making. *Biosci. Biotechnol. Biochem.* 64:617-619.

42. Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680-685.

43. Lahl, W.J., Braun, S.D. 1994. Enzymatic production of protein hydrolysates for food use. *Food Technol.* 48: 68-71.

44. L. C. Wu, Y. D. Hang. (2000) Acid protease production from *Neosartorya fischeri*. *Lebensm-Wiss. µ -Technol.* 33:44-47.

45. Leslie T. Mathaba, Catherine H. Pope, Jason Lenzo, Maria Hartofilis, Helen Peake, Robert L. Moritz, Richard J. Simpson, Andreas Bubert, Philip J. Thompson, Geoffrey A. Stewart. (2002) Isolation and characterization of a 13.8-kDa bacteriolytic enzyme from house dust mite extracts: homology with prokaryotic proteins suggests that the enzyme could be bacterially derived. *FEMS Immunol. and Med. Microb.* 19:171-175.

46. Lilik Ikasari, Daivd A. Mitchell (1996) Leaching and characterization of *Rhizopus oligosporus* acid protease from solid-state fermentation. *Enzyme Microb. Technol.* 19:171-175.

47. Löffler, A. (1986) Proteolytic enzymes: sources and application. *Food Technol.* 40:63-70.

48. Maria de Fatima Silva Lopes, Ana Lucia Leitao, J.J. Figueiredo Marques, Manuel Jose Teixeira Carrondo, Maria Teresa Barreto Crespo (1999) Processing of extracellular lipase of *Lactobacillus plantarum*: involvement of a metalloprotease. *FEMS Microbiol. Lett.* 176:483-487.

49. Maria Papagianni, Murray Moo-Young (2002) Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochem.* 37:1271-1278.

50. Marie-Claude Jobin, Daniel Grenier (2003) Identification and characterization of four proteases produced by *Streptococcus Suis*. *FEMS Microbiol. Lett.* 220:113-119.

51. Marianna Turkiewicz, Ewa Gromek, Halina Kalinowska, Maria Aielin ' ska (1999) Biosynthesis and properties of an extracellular metalloprotease from the Antarctic marine bacterium *Sphingomonas paucimobilis*. *J. Biotechnol.* 70:53-60.

52. Martha L. Diaz-Torres, Roy R. B. Russell. (2001) HtrA protease and processing of extracellular proteins of *Streptococcus mutans*. *FEMS Microbiol. Lett.* 204:23-28.

53. M.J. Zapelena, I. Astiasaran, J. Bello (1999) Dry Fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture. *Meat Sci.* 52:403-409.

54. Nathalie Durand-Poussereau, Michel Fevre (1996) Characterization and protease deficient strain of *Penicillium roqueforti* generated by heterologous plasmid integration: potential us for protein production. *J. Biotechnol.* 51:97-105.

55. Nora Muller, Frank Bordusa. (2000) Assay of diverse protease activities on the basis of a small synthetic substrate. *Analytical Biochemistry.* 286:86-90.

56. Oh YS, Shih IL, Tzeng YM, Wang SL. (2000) Protease produced by *Pseudomonas aeruginosa* K-187 and its application in the deproteinization of shrimp and crab shell wastes. *Enzyme Microb. Technol.* 27: 3—10.

57. Peng Y, Huang Q, Zhang RH, Zhang YZ (2003) Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. *Comp. Biochem. Physiol. B,* 134: 45—52.

58. Pirkko Helisto, Gleb Aktuganov, Nailia Galimzianova, Alexander Melentjev, Timo Korpela (2001) Lytic enzyme complex of an antagonistic *Bacillus* sp. X-B: isolation and purification of components. *J. Chromatogr B.* 758:197-205.

59. Price, N. C., Steven, L. (1999) *Fundamentals of enzymology*, pp. 184-185. Oxford University Press Inc., New York.

60. Rao, M. B. Tankale, A. M., Chatge, M. S., Deshpande, V. V. (1998) Molecular and Biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62:597-635.

61. R. K. Malik, D. K. Mathur. (1984) Purification and characterization of a heat-stable protease from *Pseudomonas* sp. B-25. *J. Dairy Sci.* 67:522-530.

62. 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 Microb. Technol.* 32:246-251.

63. S. E. Jensen, L. Phillippe, J. Teng Tseng, G. W. Stenmke, J. N. Campbell (1979) Purification and characterization of exocellular proteases produced by a clinical isolate and a laboratory strain of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 26:77-86.

64. Shaoliang Li, Shigemi Norioka, Fumio Sakiyama. (1997) Purification, staphylolytic activity, and cleavage sites of —Lytic protease from *Achromobacter lyticus*. *J. Biochem.* 122:772-778.

65. Shin-ichi Miyoshi, Sumio Shinoda. (2000) Microbial metalloproteases and pathogenesis. *Microb. Infect.* 2:91-98.

66. Suresh Shastry, M. S. Prasad. (2002) Extracellular protease from *Pseudomonas* sp. (CL 1457) active against *Xanthomonas campestris*. *Process Biochem.* 37:611-621.

67. Thomas Aalbaek, Moten 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 Microb. Technol.* 30:410-415.

68. T. Kobayashi, Y. Hakamade, J. Hitomi, K. Koike, S. Ito (1996) Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Appl. Microbiol Biotechnol.* 45:63-71.

69. Toma, C. (1999) Purification and characterization of an *Aeromonas caviae* metalloprotease that is related to *Vibrio cholerae* hemagglutinin/ protease. *FEMS Microbiol. Lett.* 170:237-242.

70. T. R. Patel, D. M. Jackman, F. M. Bartlett. (1983) Heat-stable protease from *Pseudomonas fluorescens* T16: Purification by affinity column chromatography and characterization. *Appl. Environ. Microbiol.* 46:333-337.

71. Wang SL, Shih IL, Liang TW, Wang CH, (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. 72. Yang JK, Shih IL, Tzeng YM, Wang SL, (2000) Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microb. Technol.* 26: 406—413.