

納豆激? 酶c與功能研究及腸道定殖之分子檢測技術開發

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摘要

納豆激? 酶c 孝莖狷c之胞外酵素，具有強力溶解血栓活性，在酪蛋白分解活性測試中發現納豆菌SJ與F所測得的比活性相差了有52%之多，推估這兩株菌所含納豆激? 酶b蛋白質的結構上有所差異，而比較其胺基酸序列及蛋白質的二級、三級結構分析結果，推論第53個胺基酸由絲胺酸 (serine) 改為脯胺酸 (proline) 可能是結構上造成其活性最主要的原因。本實驗選殖 (clone) 定序了九種從納豆所篩選得到的納豆激? 酶c [穉]，同時也從基因庫中搜尋得到納豆菌之16S核糖體基因片段序列，來建立特殊的引子 (primer)，以建立探討納豆菌在小鼠腸胃道定殖效果的分子檢測技術。小鼠定殖 (colonization) 試驗結果顯示，利用加熱將非耐熱及非產生芽孢之雜菌去除掉，再配合16S rDNA及納豆激? 酶c可以成功的在飼食耐酸納豆菌 (SJ)、耐鹼納豆菌 (JD11) 以及納豆菌孢子 (SP) 之小鼠糞便檢體中增幅出其特定的16S rDNA及納豆激? 酶c並鑑定其菌種，推論前述這三株菌在飼食後可以通過胃酸、膽汁的分解而成功的定殖在小鼠腸道中，間接證實該三株菌所產生的納豆激? 酶c在腸道被吸收而被小鼠利用。

關鍵詞：納豆菌；納豆激? 酶c；定殖；結構與功能；分子檢測

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參考文獻

- 參考文獻 1. 江曉、董明盛和江漢湖。2002。一種食源性纖維素(納豆激酶)學性質的研究。中國釀造 117: 23-25。 2. 董明盛、江曉和劉誠。2001。納豆激酶穩定性的研究。食品與發酵工業 27: 13-15。 3. 鄒文雄與黃明經。1997。蛋白質結構之電腦預測。中國化學會55: 101-109。 4. 謝秋玲、郭勇和林劍。2001。納豆激酶的合成機制。暨南大學學報(自然科學版) 22: 110-114。 5. Addison, J. M., Burston, D. and Matthews, D. M. 1972. Evidence for active transport of the dipeptide glycylsarcosine by hamster jejunum in vitro. Clin. Sci. 43 (6): 907-911. 6. Aoyama, M., Toma, C., Yasud, M. and Iwanaga, M. 2000. Sequence of the gene encoding an alkaline serine proteinase of *Bacillus pumilus* TYO-67. Microbiol. Immunol. 44 (5): 389-393. 7. Chiang, C. J. H., Chen, C., Chao, Y. P. and Tzen, J. T. 2005. Efficient system of artificial oil bodies for functional expression and purification of recombinant nattokinase in *Escherichia coli*. J. Agric. Food Chem. 53 (12): 4799-4804. 8. Chang, C. T., Fan, M. H., Kuo, F. C. and Sung, H. Y. 2000. Potent fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1. J. Agric. Food Chem. 48 (8): 3210-3216. 9. Chowdhury, P. and Soulsby, M. 2002. Lipid peroxidation in rat brain is increased by simulated weightlessness and decreased by a soy-protein diet. Ann. Clin. Lab Sci. 32 (2): 188-192. 10. Fei, Y. J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F. H., Romero, M. F., Singh, S. K., Boron, W. F. and Hediger, M. A. 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368 (6471): 563-566. 11. Fujita, M., Nomura, K., Hong, K., Ito, Y., Asada, A. and Nishimuro, S. 1993. Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Biochem. Biophys. Res. Commun. 197 (3): 1340-1347. 12. Fujita, M., Hong, K., Ito, Y., Fujii, R., Kariya, K. and Nishimuro, S. 1995. Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. Biol. Pharm. Bull. 18 (10): 1387-1391. 13. Fujita, M., Hong, K., Ito, Y., Misawa, S., Takeuchi, N., Kariya, K. and Nishimuro, S. 1995. Transport of nattokinase across the rat intestinal tract. Biol. Pharm. Bull. 18 (9): 1194-1196. 14. Guex, N., Diemand, A. and Peitsch, M. C. Protein modelling for all. 1999. Trends Biochem. Sci. 24 (9): 364-367. 15. Guo, J., Sun, Y. and Su, Y. Preparation of natto and its function in health care. 2002. Zhong. Yao Cai. 25 (1): 61-64. 16. Isezaki, M., Hosoya, S., Takeuchi, M. and Sato, T. 2001. A putative ATP-binding cassette transporter YbdA involved in sporulation of *Bacillus subtilis*. FEMS Microbiol. Lett. 204 (2): 239-245. 17. Jacobs, M. F. 1995. Expression of the subtilisin carlsberg-encoding gene in *Bacillus licheniformis* and *Bacillus subtilis*. Gene 152 (1): 69-74. 18. Ko, J. H., Yan, J. P., Zhu, L. and Qi, Y. P. 2004. Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. Comp Biochem. Physiol C. Toxicol. Pharmacol. 137 (1): 65-74. 19. Liu, B. Y. and Song, H. Y. 2002. Molecular cloning and expression of Nattokinase gene in *Bacillus subtilis*. Sheng Wu Hua Xue. Yu Sheng Wu Wu Li Xue. Bao. (Shanghai) 34 (3): 338-340. 20. Liang, R., Fei, Y. J., Prasad, P. D., Ramamoorthy, S., Han, H., Yang-Feng, T. L., Hediger, M. A., Ganapathy, V. and Leibach, F. H. 1995. Human intestinal H⁺/peptide cotransporter. cloning, functional expression, and chromosomal localization. J. Biol. Chem. 270 (12): 6456-6463. 21. Lucchini, F., Kmet, V., Cesena, C., Coppi, L., Bottazzi, V. and Morelli, L. 1998. Specific detection of a probiotic *Lactobacillus* strain in faecal samples by using multiplex PCR. FEMS Microbiol. Lett. 158 (2): 273-278. 21. Markland, F. S. and Smith, E. L. 1967. Subtilisin BPN. VII. Isolation of cyanogen bromide peptides and the complete amino acid sequence. J. Biol. Chem. 242 (22): 5198-5211. 22. Messina, M. 1999. Soy, soy phytoestrogens (isoflavones), and breast cancer. Am. J. Clin. Nutr. 70 (4): 574-575. 23. Nakamura, T., Yamagata, Y. and Ichishima, E. 1992. Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (natto). Biosci. Biotechnol. Biochem. 56 (11): 1869-1871. 24. Pace, N. R., Olsen, G. J. and Woese, C. R. 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell 45 (3): 325-326. 25. Patterson, H. G. and Graves, S. 2000. DNAssist: the integrated editing and analysis of molecular biology sequences in windows. Bioinformatics. 16 (7): 652-653. 26. Peng, Y., Huang, Q., Zhang, R. H. and Zhang, Y. Z. 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 Biochem. Mol. Biol. 134 (1): 45-52. 27. Samanya, M. and Yamauchi, K. E. 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. natto. Comp Biochem. Physiol A Mol. Integr. Physiol 133 (1): 95-104. 28. Sato, T., Yamada, Y., Ohtani, Y., Mitsui, N., Murasawa, H. and Araki, S. 2001. Production of menaquinone (vitamin K2)-7 by *Bacillus subtilis*. J. Biosci. Bioeng. 91 (1): 16-20. 29. Sato, T., Yamada, Y., Ohtani, Y., Mitsui, N., Murasawa, H. and Araki, S. 2001. Efficient production of menaquinone (vitamin K2) by a menadione-resistant mutant of *Bacillus subtilis*. J. Ind. Microbiol. Biotechnol. 26 (3): 115-120. 30. Stackebrandt, E., Liesack, W. and Witt, D. 1992. Ribosomal RNA and rDNA sequence analyses. Gene 115 (1-2): 255-260. 31. Sumi, H., Hamada,

H., Nakanishi, K. and Hiratani, H. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* 84 (3): 139-143.

32. Sumi, H., Hamada, H., Tsushima, H., Mihara, H. and Muraki, H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia* 43 (10): 1110-1111.

33. Stahl, M. L. and Ferrari, E. 1984. Replacement of the *Bacillus subtilis* subtilisin structural gene with an *In vitro*-derived deletion mutation. *J.Bacteriol.* 158 (2): 411-418.

34. Suzuki, K., Koike, H., Matsui, H., Ono, Y., Hasumi, M., Nakazato, H., Okugi, H., Sekine, Y., Oki, K., Ito, K., Yamamoto, T., Fukabori, Y., Kurokawa, K. and Yamanaka, H. 2002. Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. *Int.J.Cancer* 99 (6): 846-852.

35. Suzuki, Y., Kondo, K., Ichise, H., Tsukamoto, Y., Urano, T. and Umemura, K. 2003. Dietary supplementation with fermented soy beans suppresses intimal thickening. *Nutrition* 19 (3): 261-264.

36. Tai, M. W. and Sweet, B. V. 2006. Nattokinase for prevention of thrombosis. *Am.J.Health Syst.Pharm.* 63 (12): 1121-1123.

37. Suzuki, Y., Kondo, K., Matsumoto, Y., Zhao, B. Q., Otsuguro, K., Maeda, T., Tsukamoto, Y., Urano, T. and Umemura, K. 2003. Dietary supplementation of fermented soybean, natto, suppresses intimal thickening and modulates the lysis of mural thrombi after endothelial injury in rat femoral artery. *Life Sci.* 73 (10): 1289-1298.

38. Takano, A., Hirata, A., Ogasawara, K., Sagara, N., Inomata, Y., Kawaji, T. and Tanihara, H. 2006. Posterior vitreous detachment induced by nattokinase (subtilisin NAT): a novel enzyme for pharmacologic vitreolysis. *Invest Ophthalmol. Vis. Sci.* 47 (5): 2075-2079.

39. Urano, T., Ihara, H., Umemura, K., Suzuki, Y., Oike, M., Akita, S., Tsukamoto, Y., Suzuki, I. and Takada, A. 2001. The profibrinolytic enzyme subtilisin NAT purified from *Bacillus subtilis* cleaves and inactivates plasminogen activator inhibitor type 1. *J.Biol.Chem.* 276 (27):24690-24696.

40. Vasantha, N., Thompson, L. D., Rhodes, C., Banner, C., Nagle, J. and Filpula, D. 1984. Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. *J.Bacteriol.* 159 (3): 811-819.

41. Van de Peer Y., Chapelle, S. and De, W. R. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Res.* 24 (17): 3381-3391.

42. Van de Peer Y., Jansen, J., De, R. P. and De, W. R. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* 25 (1): 111-116.

43. Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18 (22): 6531-6535.

44. Welsh, J., Rampino, N., McClelland, M. and Peruchio, M. 1995. Nucleic acid fingerprinting by PCR-based methods: applications to problems in aging and mutagenesis. *Mutat. Res.* 338 (1-6): 215-229.

45. Wattiau, P., Renard, M. E., Ledent, P., Debois, V., Blackman, G. and Agathos, S. N. 2001. A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of wastewater biotreatment. *Appl. Microbiol. Biotechnol.* 56(5-6) : 816-819.

46. Yamaguchi, M., Kakuda, H., Gao, Y. H. and Tsukamoto, Y. 2000. Prolonged intake of fermented soybean (natto) diets containing vitamin K2 (menaquinone-7) prevents bone loss in ovariectomized rats. *J.Bone Miner. Metab* 18 (2): 71-76.

47. Zheng, Z. L., Ye, M. Q., Zuo, Z. Y., Liu, Z. G., Tai, K. C. and Zou, G. L. 2006. Probing the importance of hydrogen bonds in the active site of the subtilisin nattokinase by site-directed mutagenesis and molecular dynamics simulation. *Biochem.J.* 395 (3): 509-515.

48. Zheng, Z. L., Zuo, Z. Y., Liu, Z. G., Tsai, K. C., Liu, A. F. and Zou, G. L. 2005. Construction of a 3D model of nattokinase, a novel fibrinolytic enzyme from *Bacillus natto*. A novel nucleophilic catalytic mechanism for nattokinase. *J. Mol. Graph. Model.* 23 (4): 373-380.