

納豆菌發酵產物之乳化及凝乳活性之探討

Phan Thi Lan Anh、施英隆

E-mail: 9606876@mail.dyu.edu.tw

摘要

納豆是黃豆接種納豆菌絲發酵後之日本傳統食品，納豆含有很多有價值及有營養之生理活性物質。另外，納豆亦含有很多生物性高分子，如聚麣胺酸及果聚糖，及酵素。最近意外發現納豆萃取物具有乳化效果及凝乳效果。本研究因此製備液態及固態納豆，並且探討其乳化及凝乳之性質，液態納豆及固態納豆皆具有乳化活性，但固態納豆之乳化活性較高；相對的，果聚糖及聚麣胺酸則完全無任何乳化活性，但是納豆之乳化活性則較市售乳化劑低很多。在凝乳活性方面，液態納豆及固態納豆皆具有良好之凝乳活性，但聚麣胺酸及果聚糖皆完全為凝乳活性，在凝乳發酵土，凝乳活性受操作條件及營養因子而影響；當納豆菌在含燕糖（50g/L），氧化納（10g/L），硫酸鎂（0.5g/L），磷酸二氫鈉（3g/L），及硫酸氫鈉（3g/L）之培養基中，培養條件為370C，pH6及175rpm培養一天時，可得最高之凝乳活性685.7 SU/ml或12,000 SU/g，其中凝乳活性及蛋白質水解活性之比率在液態實驗中為2,981，在固態實驗中為52,174。凝乳活性物質在pH 5至pH 6之間相當穩定，當加熱至70 時凝乳活性在五分鐘內活性完全消失，但在25 至40 則相當穩定。雖然納豆中凝乳酵素之凝乳活性較市售之凝乳？(如小牛之凝乳酵素及黑黴素之凝乳酵素) 之活性低，但是納豆菌是益生菌，以納豆菌生產之凝乳酵素應當仍有很高之商業應用價值。

關鍵詞：納豆菌；枯草枯菌；凝乳？；活化活性

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參考文獻

REFERENCES

- 1.Amir Toren, Shiri Navon-Venezia, Eliora Z. Ron and Eugene Rosenberg, (2001). Emulsifying activities of purified Alasan Proteins from *Acinetobacter radioresistens* KA53. *Applied and environmental microbiology*, vol 67: 1102-1106
- 2.Andren, A. (1998). Milk-clotting activity of various rennets and coagulants: Background and information regarding IDF standards.*Bulletin of the International Dairy Federation* 332: 9-14
- 3.Arima K., (1970). Milk-clotting Enzyme from *Mucor pusillus* var. Lindt. *Methods in enzymology* 19: 446-459
- 4.Ashiuchi, M., Nawa, C., Kamei, T., Song, J.J., Hong, S.P., Sung, M.H., et al. (2001) Physiological and biochemical characteristics of poly- γ -glutamate synthetase complex of *Bacillus subtilis*. *Eur J Biochem* 268: 5321-5328
- 5.Ashiuchi, M., Tani, K., Soda, K., Misono, H., (1998). Properties of glutamate racemase from *Bacillus subtilis* IFO 3336 producing poly- γ -glutamate. *J. Biochem* 123: 1156-1163
- 6.Ashiuchi, M., Tani, K., Soda, K., Misono, H., (1999). A poly- γ -glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly- γ -glutamate produced by Escherichia coli clone cells. *Biochem. Biophys. Res. Commun* 263: 6-12
- 7.Bashir H. Yousif. Donald J. McMahon Khalid M. Shammet (1996). Milk-clotting enzyme from Solanum dulcamara plant. *Int. Dairy Journal* 6: 637-644
- 8.Bohlin, L., Hegg, P.O., Ljusberg-Wahren, H. (1984). Viscoelastic properties of coagulating milk. *Journal of Dairy Science*, 67(4): 729-734
- 9.Bovarnick, M. (1942). The formation of extracellular D(-)-glutamic acid polypeptide by *Bacillus subtilis*. *J. Biol. Chem* 145: 415-424
- 10.Castellani O., (2006). Oil-in-water emulsion properties and interfacial characteristics of hen egg yolk phosphatidylserine. *Food Hydrocolloids* 20: 35-43
- 11.Cavalcanti M.T.H., Teixeira M.F.S., Lima Filho J.L., Porto A.L.F., (2004). Partial purification of new milk-clotting enzyme produced by *Nocardiopsis* sp. *Bioresource Technology* 93: 29-35
- 12.Cheng, C., Asada, Y., Aida, T., (1989). Production of γ -polyglutamic acid by *Bacillus subtilis* A35 under denitrifying conditions. *Agric. Biol. Chem* 53: 2369-2375
- 13.Daninippon Pharmaceutical Co, Ltd., (1972), Ice cream stabilizer, Japanese Patent 19735/72
- 14.Fujita M, Hong K, Ito Y, Misawa S, Takeuchi N, Kariya K, Nishimuro S. (1995). "Transport of nattokinase across the rat intestinal tract." *Biological & pharmaceutical bulletin*. 18 (9): 1194-1196
- 15.Fujita M. (1993). "Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan." *Biochemical and Biophysical Research Communications* 197 (3): 1340-1347
- 16.Goto, A., Kunioka, M., (1992). Biosynthesis and hydrolysis of poly (D,L-glutamic acid) from *Bacillus subtilis* IFO 3335. *Biosci. Biotechnol. Biochem* 56: 1031-1035
- 17.Han, Y. W. Microbial levan (1990). *Adv. Appl. Microbiol* 35: 171-194
- 18.Haq, A., N.B. Webb, J.K. Whitfield, A.J. Howell, and B.C. Barbour. (1973). Measurement of Sausage Emulsion Stability by Electrical Resistance. *J. Food Sci.*, 38: 1124- 1127
- 19.Hara, T., Aumayr, A., Fujio, Y., Ueda, S., (1982a). Elimination of plasmid-linked polyglutamate production by *Bacillus subtilis* (natto) with acridine orange. *Appl. Environ. Microbiol* 44: 1456-1459
- 20.Hara, T., Fujio, Y., Ueda, S., (1982b). Polyglutamate production by *Bacillus subtilis* (natto). *J. Appl. Biochem* 4: 112-120
- 21.Hara, T., Ueda, S., (1982). Regulation of polyglutamate production in *Bacillus subtilis* (natto); transformation of high PGA productivity. *Agric. Biol. Chem* 46: 2275-2281
- 22.Healy M.G., Devine C.M., Murphy R., (1996). Microbial production of biosurfactants. *Resources, Conservation and Recycling* 18: 41-57
- 23.Hodges SJ, Pilkington MJ, Shearer MJ, Bitensky L, Chayen J (1990). Age-related changes in the circulating levels of congeners of vitamin K2, menaquinone-7 and menaquinone-8. *Clin Sci (Lond)* 78(1): 63-6
- 24.Hosoi T. Recent progress in treatment of osteoporosis (1996).
- 25.Nippon Ronen Igakkai Zasshi, 33(4): 240-4 [Article in Japanese] I-Chen Lu, (2004). Studies of Liquid Natto on Cosmetics. Master's Thesis, Providence University, Taichung Taiwan. Applied Chemistry department.
- 26.IDF (1997). Bovine rennets. Determination of total milk-clotting activity. International IDF Standard 157A. International Dairy Federation, Brussels, Belgium.
- 27.Ikeda H, Doi Y (1990). Avitamin-K2-binding factor secreted from *Bacillus subtilis*. *Eur J. Biochem*; 192-219
- 28.Ing-Lung Shih, Yi-Tsong Van, (2001). The production of poly-(D,L-glutamic acid) from microorganisms and its various applications. *Bioresource Technology* 79: 207-225
- 29.Innocente N., (1998). Emulsifying properties of the total fraction and the hydrophobic fraction of Bovine milk Proteose-peptones. *Int. Dairy Journal* 8: 981-985
- 30.Kaneki, (2001). Japanese Fermented Soybean Food as the Major Determinant of the Large Geographic Difference in Circulating Levels of Vitamin K2: Possible Implications for Hip-Fracture Risk. *Applied nutritional investigation*, vol 17: 315-321
- 31.Kang, S. K., Park, D. J., Lee, J. D., Lee, T. H. (2000) Physiological effects of levanoligosaccharide on growth of intestinal microflora. *J. Korean Soc. Food Sci. Nutr* 29: 35-40.
- 32.Kawana K, Takahashi M, Hoshino H, Kushida K (2001). Circulating levels of vitamin K1, menaquinone-4, and menaquinone-7 in healthy elderly Japanese women and patients with vertebral fractures and patients with hip fractures. *Endocr Res* 27(3): 337-43
- 33.Kunno, A., Taguchi, T. and Yamaguchi (1988b). Bakery products and noodles containing polyglutamic acid US Patent 4,888,193.
- 34.Kunno, A., Taguchi, T. and Yamaguchi, T. (1988a). New use of polyglutamic acid for foods. EP 0284386 B1.
- 35.Makino, S., Uchida, I., Terakado, N., Sasakawa, C., Yoshikawa, M., (1989). Molecular characterization and protein analysis of the cal region, which is essential for encapsulation in *Bacillus anthracis*. *J. Bacteriol* 171: 722-730
- 36.Marx, S. P., Winkler, S., Hartmeier, w. (2000) Metabolization of β -(2,6-linked fructose-oligosaccharides by different bifidobacteria. *FEMS Microbiol. Lett* 182: 163-169
- 37.Masao Kunioka and Atsuo Goto. (1992). Biosynthesis of poly (D,L-glutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in *Bacillus subtilis* IFO3335. *Applied Microbiology and Biotechnology*, Volume 40, No. 1: 867-872
- 38.McMahon, D. J., & Brown, R. J. (1982). Evaluation of formigraph for comparing rennet solutions. *Journal of Dairy Science*, 65(8): 1639-1642
- 39.Muller, M., Seyfarth, W. (1997). Purification and substrate specificity of an extracellular fructan-hydrolase from *Lactobacillus paracasei* ssp. *Paracasei* P 4134, *New Phytol*. 136: 89-96
- 40.Myers, C. (1988). Functional attributes of protein isolates. In F. Franks (Ed.), *Characterisation of protein*. Clifton, NJ:

Humana press 491-549 38. Nagai, T., Koguchi, K., Ito, Y., (1997). Chemical analysis of poly- γ -glutamic acid produced by plasmid-free *Bacillus subtilis* (natto): evidence that plasmids are not involved in poly- γ -glutamic acid production. *J. Gen. Appl. Microbiol* 43: 139-143 39. O' Connor C B. (1993). Traditional cheesemaking manual. ILCA (International Livestock Centre for Africa), Addis Ababa, Ethiopia. ISBN 92-9053-273-4 40. Okamoto Y, Furuno T, Hamano T and Nakanishi M (1995). Confocal fluorescence microscopy for studying thapsigargin-induced bivalent-cation entry into B cells. *Biochem. J.* 305: 1011-1015 (Printed in Great Britain) 41. Okamura-Metsui et al., (2001). Characteristics of a Cheese-Like Food Produced by Fermentation of the Mushroom *Schizophyllum commune*. *Journal of Biosciences and Bioengineering*. Vol. 92, No. 1: 30-32 42. Olayide S. Lawal, (2004). Functionality of African locust bean (*Parkia biglobosa*) protein isolate: effects of pH, ionic strength and various protein concentrations. *Food Chemistry* 86: 345-355 43. Omizo H, Hashimoto T, Shiraki M, et al. Nation-wide survey of the hip fracture incidence in Japan. *Jpn Med J* 1995, 37: 7-27 Orimo H, Hosoda Y, Fujiwara S et al. Fracture incidence in Japan. *JBMM* 1991: 9-89 44. Pearce, Kinsella, (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. *J. Argir. Food Chem* 26: 716-723 45. Pierre Monsan, Sophie Bozonnet, Cecile Albenne, Gilles Joucla, Rene-Marc Willemot, Magali Remaud-Simeon(2001). Homopolysaccharides from lactic acid bacteria. *International Dairy Journal* 11: 675-685 46. Sakai K., Sonoda C., and Murase K. (2000) Bitterness relieving agent. Jp. Patent. WO0021390. 47. Sakano, T., Notsumoto, S., Nagaoka, T., Morimoto, A., Fujimoto, K., Masuda, S., Suzuki, Y. and Hirauchi, K (1988). Measurement of K-vitamins in food by high-performance liquid chromatography with fluorometric detection. *Vitamins*, 62: 393-398. [Article in Japanese] 48. Sofia V. Silvaa, Toomas Allmere, F. Xavier Malcataa, Anders Andrlén. (2003). Comparative studies on the gelling properties of cardosins extracted from *Cynara cardunculus* and chymosin on cow's skim milk. *International Dairy Journal* 13: 559-564 49. Sumi H. (2000). Determination and Properties of the Fibrinolysis Accelerating Substance (FAS) in Japanese Fermented Soybean "Natto". *Nippon Nogeikagaku Kaishi*(Japanese). 74 (11): 1259-1264 50. Sumi, H., Yanagisawa, Y., and Kozaki, Y (1998). Vitamin K in the vegetable cheese natto. *Nippon Nogeikagaku Kaishi*. 72, 128. 4 [Article in Japanese] 51. Suzuki, M., Chatterton, N. J. (1993) Science and technology of Fructans. Boca raton: CRC Press. 52. Thakur M. S., Karanth N. G., and Krishna Nand, (1990). Production of fungal by *Mucor miehei* using solid state fermentation. *Applied microbiology and biotechnology* 32: 409-413 53. Torres M.R., Mar?n F.R., Ramos A.J., Soriano E., (2002). Study of operating conditions in concentration of chicken blood plasma proteins by ultrafiltration. *Journal of Food Engineering* 54: 215-219 54. Tsai R., Cassens R. G., Briskey E. J., (1970). The emulsifying properties of purified muscle proteins. *Journal of Food Science* 37: 286-288 55. Van Kranenburg, R., Boels, I. C., Kleerebezem, M., de Vos, W. M. (1999). Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. *Curr. Opin. Biotechnol.* 10: 498-504 56. Wang J. C. and Kinsella J. E. (1976). Functional properties of novel proteins: Alfalfa leaf protein. *Journal of Food Science* 41(2): 286-292 57. Webb NB, Ivey JF, Craig HB, Jones VA, Monroe RJ (1970). The measurement of capacity by electrical resistance. *J. Food Sci.* 35: 501-504 58. Wei Q., Wolf-Hall C., and Chang K.C., (2001). Natto characteristics as affected by streaming time, bacillus strain, and fermentation time. *J. of food science* 66: 167-173 59. Yamaguchi M, Kakuda H, Gao YH, 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): 71-86 60. Yamaguchi M, Taguchi H, Gao YH, Igarashi A, Tsukamoto Y (1999). Effect of vitamin K2 (menaquinone-7) in fermented soybean (natto) on bone loss in ovariectomized rats. *J Bone Miner Metab*; 17(1): 23-29 61. Yamamoto Y, Takahashi Y., Kawano M., Iizuka M., Matsumoto T., Saeki S., Yamaguchi H. (1999) In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats. *J. Nutr. Biochem* 10: 13-18 62. Yamanaka S. (1991) New gamma-polyglutamic acid, production therefore and drinking agent containing the same, JP 3047087 63. Yokota S, Togo SH, Maebuchi M, Bun-ya M, Haraguchi CM, Kamiryo T. (2002). Peroxisomes of the nematode *Caenorhabditis elegans*: distribution and morphological characteristics. *Histochemistry & Cell Biology* 118: 329-336 64. Yokota T (2000) Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 96(5): 1723-1732 65. Yoshinori Mine, (1998). Emulsifying characterization of hen's egg yolk proteins in oil-in-water emulsions. *Food Hydrocolloids* 12: 409-415 66. Yu-Wu Shih, (2005). Optimization of nutritional conditions for nattokinase production by an isolated *Bacillus subtilis* from natto health food. Thesis for Master of Science Department of Bioengineering Tatung University, Reference from Website: [1] 華文生技網 - 納豆菌製品日本人長壽秘方 (*Bacillus natto* products-the longevity secret of Japanese) [http://www.cop.ufl.edu/safezone/prokai/pha5100/Eagents.htm](http://www.bioweb.com.tw/feature_content.asp?ISSID=690&chkey1=%E7%B4%8D%E8%B1%86%E8%8F%8C&chkey2=%E6%97%A5%E6%9C%AC&chkey3=&chkey4=&chkey5=[2] PHA, emulsifying agents <a href=)