

以化學法及微生物法生產r-聚麴胺酸之衍生物及其應用

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摘要

聚麴胺酸是經由微生物發酵生產的天然生物性材料，其具有水溶性、生物可分解與可食等特性，且聚麴胺酸本身及其分解物對人體與環境無毒害，目前聚麴胺酸已知可應用於食品、化妝品、醫藥材料、環境保護等領域，因此工業化大量生產聚麴胺酸此一對環境友善之生物性材料，對於環境及環境保護將有相當的貢獻。本研究以十升發酵槽培養Bacillus subtilis C1並探討pH、曝氣量、葉片轉速對菌株生長、聚麴胺酸產量的影響，當Bacillus subtilis C1培養於T1培養基中（Glycerin：17%、Citric acid：2.2%、NH4Cl：0.7%、K2HPO4：0.05%、MgSO4·7H2O：0.05%、CaCl2·2H2O：0.015%、FeCl3·6H2O：0.004%、MnSO4·4·6H2O：0.0104%），發現發酵槽條件為pH=6.00、曝氣量為5、葉片轉速為150rpm、37°C下培養84小時，聚麴胺酸最高產量為8.25g/L，約為搖瓶培養生產聚麴胺酸的1倍（平均產量4.36g/L），Bacillus subtilis C1所生產之產物經結構鑑定證實為聚麴胺酸與甘油之共聚物，其光譜圖中化學位移1.8-2.1 (m, ,2H)、2.3-2.4 (b, ,2H)、4.1-4.2 (b, ,1H)代表r-聚麴胺酸各波?之位移，而化學位移3.50-3.54 (dd,2H)、3.60-3.62 (dd,2H)、3.72-3.77 (m,1H)相當於甘油各波?之化學位移。此產物之分子量為2,929,844。本研究亦探討聚麴胺酸衍生物之吸水性應用，使用Bacillus licheniformis CCRC 12826菌株所生產聚麴胺酸，並利用化學方法偶合聚麴胺酸與環氧樹脂以交聯形成結構堅固之凝膠，並測試其吸水性，實驗發現2.5%聚麴胺酸溶液於pH值為5.33與150 μl環氧樹脂進行反應4天，所得之凝膠可達到吸水重為乾凝膠重的50~60倍，吸水性較其它條件製備之凝膠優越。不同吸水材料之吸水實驗中，發現Bacillus subtilis C1所生產之聚麴胺酸-甘油複合物於30分鐘吸水實驗中，其吸水重為乾凝膠重之26倍，然而較長時間之吸水，此材料即完全溶解。

關鍵詞：聚麴胺酸、交聯、水凝膠

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范宜琮 (2001) 「以苔蘚桿菌生產聚麴胺酸之研究」，大葉大學環境工程學系 碩士論文。吳珮貞 (2004) 「以醬油篩選菌探討聚麴胺酸之生合成」，大葉大學環境工程學系 碩士論文。沈名豪 (2004) 「生物合成聚離胺酸之研究」，大葉大學環境工程學系 碩士論文。Abe K, Ito Y, Ohmachi T, Asada Y (1997) Purification and properties of two isozymes of α -poly glutamyltranspeptidase from *Bacillus subtilis* TAM-4. Biosci. Biotechnol. Biochem. 61:1621-1625. Ashiuchi M, Tani K, Soda K, Misono H (1998) Properties of glutamate racemase from *Bacillus subtilis* IFO3336 producing poly- α -glutamate. J. Biochem. 123:1156-1163. Ashiuchi M, Soda K, Misono H (1999a) A poly- α -glutamate synthetic system of *Bacillus subtilis* IFO3336: gene cloning and biochemical analysis of poly- α -glutamate produced by *Escherichia coli* clone cells.

Biochem. Biophys. Res. Commun. 263:6-12. Ashiuchi M, Soda K, Misono H (1999b) Characterization of yrpC gene product of *Bacillus subtilis* IFO3336 as glutamate racemase isozyme. Biotechnol. Biochem. 63:792-798. Ashiuchi M, Kamei T, Baek DH, Shin SY, Sung MH, Soda K, Yagi T, Misono H (2001) Isolation of *Bacillus subtilis* (chungkookjang), a poly-D-glutamate producer with high genetic competence. Appl. Microbiol. Biotechnol. 57:764-769. Birrer GA, Gromwick AM, Gross RA (1994) Poly(D-glutamic acid) formation by *Bacillus licheniformis* ATCC 9945A: Physiological and biochemical studies. Int. J. Biol. Macromol. 16:265-275. Bovarnick M (1942) The formation of extracellular D(-)-glutamic acid polypeptide by *Bacillus subtilis*. J. Biol. Chem. 145:424. Box G. E. P. and K. B., Wilson (1951) Experimental attainment of optimum conditions, Journal of the Royal Statistical Society, 13:1-45. Cheng C, Asada Y, Aaida T (1989) Production of D-poly glutamic acid by *Bacillus subtilis* A35 under denitrifying conditions. Agric. Biol. Chem. 53:2369-2375. Choi, H. J., Kunioka, M., (1995) Preparation conditions and swelling equilibria of hydrogel prepared by γ -irradiation from microbial poly-D-glutamic acid, Radiat. Phys. Chem. 46, 175-179. Choi, H. J., Yang, R., Kunioka, M., (1995) Synthesis and characterization of pH-sensitive and biodegradable hydrogels prepared by γ -irradiation using microbial poly(D-glutamic acid) and poly(L-lysine). J. Appl. Polym. Sci 58, 807-814. Cromwick AM, Gross RA, (1995a) Effect of manganese(II) on *Bacillus licheniformis* ATCC 9945A physiology and D-poly(D-glutamic acid) formation. Int. J. Biol. Macromol. 16:265-275. Cromwick AM, Gross RA, (1995b) Investigation by NMR of metabolic routes to bacterial D-poly(D-glutamic acid) using ^{13}C labeled citrate and glutamate as media carbon source. Can. J. Microbiol. 41:902-909. Daninippon Pharmaceutical Co, Ltd., (1972) Ice cream stabilizer, JP Patent 19735/72. Fujii H (1963) On the formation of mucilage by *Bacillus natto* Part I. Chemical constitutions of mucilage in natto (1). Nippon Nogeikagaku Kaishi. 37:407-411. Goto A, Kunioka M (1992) Biosynthesis and hydrolysis of Poly(D-glutamic acid) from *Bacillus subtilis* IFO3335. Biosci. Biotechnol. Biochem. 56:1031-1035. Guex-Holzer S, Tomcsik J, (1956) The isolation and chemical nature of capsular and cell-wall haptens in a *Bacillus* species. J. Gen. Microbiol. 14:14-25. Hanby WE, Rydon HN (1946) The capsule substance of *Bacillus anthracis*. J. Biochem. 40:297-309. Hasebe, K., Inagaki, M., (1999) Preparation composition for external use containing gamma-polyglutamic acid and vegetable extract in combination. JP Patent 11240827. Ing-Lung Shih, Yi-Tsong Van (2001) The production of poly(D-glutamic acid) from microorganisms and its various applications. Bioresource Technology. 79:107-225. Ito Y, Tanada T, Ohmachi T, Asada Y (1996) Glutamic acid independent production of Poly(D-glutamic acid) by *Bacillus subtilis* TAM-4. Biosci. Biotechnol. Biochem. 60:1239-1242. Ivanovics G, Bruckner V (1937) Chemische und immunologische Studien über den Mechanismus der Milzbrandinfektion und Immunität; die chemische Struktur der Kapselsubstanz des Milzbrandbazillus und serologisch identischen spezifischen Substanz des *Bacillus mesentericus*. Z Immunitätsforsch. 91:304-318. Ivanovice G, Erdos L (1937) Ein Beitrag zum Wesen der Kapselsubstanz des Milzbrandbazillus. Z Immunitätsforsch. 90:5-19. Karasawa, M., Tanimoto, H., Toride, Y., (1998) The use of poly-gamma-glutamic acid for preparing an agent for increasing the phosphorus assimilation. European patent EP0838160. Kubota H, Matsunobu Y, Uotani K, Satoh A, Tanaka T, Taniguchi M (1993a) Production of poly(D-glutamic acid) by *Bacillus subtilis* F-2-01. Biotechnol. Biochem. 57:1212-1213. Kubota H, Nambu Y, Ento T (1993b) Convenient and quantitative esterification of poly(D-glutamic acid) produced by microorganisms. J. Polym. Sci. Part A Polym. Chem. 31:2877-2878. Kunioka M (1995) Biosynthesis of Poly(D-glutamic acid) from L-glutamine, citric acid and ammonium sulfate in *Bacillus subtilis* IFO3335. Appl. Microbiol. Biotechnol. 44:501-506. Kunioka M (1997) Biosynthesis and chemical reactions of poly(amino acid) from microorganisms. Appl. Microbiol. Biotechnol. 47:469-475. Kunno,A., Taguchi, t., Yamaguchi, T., (1988a) New use of polyglutamic acid for foods. EP 0284386. Kunno,A., Taguchi, t., Yamaguchi, T., (1988b) Bakery products and noodles containing polyglutamic acid. US Patent 4,888,193. Leonard CG, Housewright RD, Thorne CB (1958) Effect of metal ions on glutamyl polypeptide synthesis by *Bacillus subtilis*. J. Bacteriol. 76:499-503. Leonard CG, Housewright RD, Thorne CB (1958) Effect of metal ions on the optical specificity of glutamine synthetase and glutamyl transferase of *Bacillus licheniformis*. Biochem. Biophys. Acta. 62:432-434. Nagai T, Phan Tran LS, Inatsu Y, Itoh Y (2000) A new IS4 family insertuon sequence, IS4Bsul, responsible for genetic instability of poly-D-glutamic acid production in *Bacillus subtilis*. J. Bacteriol. 182:2387-2392. Ogawa Y, Yamaguchi F, Yuasa K, Tahara Y (1997) Efficient production of poly-D-glutamic acid by *Bacillus subtilis* (natto) in jar fermenters. Biosci. Biotechnol. Biochem. 61:1684-1687. Otani Y, Tabata Y, Ikada Y (1996) A new biological glue from gelatin and poly(L-glutamic acid). J. Biomed. Mater. Res:31, 157-166. Otani Y, Tabata Y, Ikada Y (1996) Rapidly curable biological glue composed of gelatin and poly(L-glutamic acid). Biomaterials. 17:1381-1391. Perez-Camero G, Congregado F, Bou JJ, Munoz-Cuerra S (1999) Biosynthesis and Ultrasonic degradation bacterial poly(D-glutamic acid). Biotechnol. Bioengin. 63:110-115. Sakai, K., Sonoda, C., Murase, K., (2000) Bitterness reliving agent. JP Patent WO0021390. Stadtman ER (1966) Allosteric regulation of enzyme activity. Adv Enzymol. 28:41-154. Shih,I. L., Van, Y. T., (2001) The production of poly(D-glutamic acid) form microorganisms and its various applications. Bioresource Technol. 79:207-225. Tanimoto H, Sato H, Kuraishi C, Kido K, Seguto K (1995) High absorption mineral-containing composition and foods. US patent 5447,732. Tanimoto H, Sato H, Karasawa M, Iwasaki K, Oshima A, Adachi S (2000) Feed composition containing poly-D-glutamic acid. JP. Patent WO96353399. Tanaka T, Fujita K, Uotani K, Taniguchi M (1997) Existence of an optically heterogeneous peptide unit in poly(D-glutamic acid) produced by *Bacillus subtilis*. J Ferment Bioeng. 84:361-364. Thorne CB, Gomez CG, Blind GR, Housewright RD (1953) Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis*. III. Factors affecting peptide production in synthetic liquid media. J. Bacteriol. 65:472-478. Thorne CB, Gomez CG, Noyes HE, Housewright RD (1954) Production of glutamyl polypeptide by *Bacillus subtilis*. J. Bacteriol. 68:307-315. Troy FA (1973) Chemistry and biosynthesis of the poly(D-glutamyl) capsule in *Bacillus licheniformis*. 1. Properties of the membrane-mediated biosynthetic reaction. J. Biol. Chem. 248:305-316. Yokoi H, Natsuda O, Hirose J, Takasaki Y (1995) Characteristic of a biopolymer flocculant produced by *Bacillus subtilis* PY-90. J. Ferment. Bioeng. 79:378-380. Yokoi H, Natsuda O, Hirose J, Hayashi S, Takasaki Y (1996) Flocculation properties of poly(D-glutamic acid) produced by *Bacillus subtilis*. J. Ferment. Bioeng. 82:84-87.