

# The studies on the chitosanase from Aspergillus fumigatus Fresenius

黃群玲、王三郎、顏裕鴻

E-mail: 9300436@mail.dyu.edu.tw

## ABSTRACT

This thesis is a study of the utilization of chitosan by microbes to produce chitosanase. The chitosan-producing microorganism was identified as a strain of Aspergillus fumigatus Fresenius. Maximum chitosanase activity (0.4 U/mL) was obtained when the strain was grown aerobically in a medium consisting of 1g shrimp and crab shell powder, 0.1 g ammonium sulfate、0.1 g ferrous sulfate、0.1 g K<sub>2</sub>HPO<sub>4</sub> and 0.05 g MgSO<sub>4</sub> · 7H<sub>2</sub>O in 100 mL medium (pH 4.0) at 37 °C for 5 days. The chitosanase was purified from the culture supernatant of Aspergillus fumigatus Fresenius by ammonium sulfate fractionation, DEAE Sepharose CL-6B column chromatography and Sephadryl S-200 gel filtration. The purified enzymes estimated by SDS-PAGE have a molecular weight of 45 kDa . The optimal temperature , optimal pH and pH stability for chitosanase was 70 °C , 4 °C , 4~6 respectively. The activity of chitosanase was activated by Mg<sup>2+</sup> and 0.5 M Urea, but strongly inhibited by SDS、Mn<sup>2+</sup> and Hg<sup>2+</sup>.

Keywords : chitosan ,shrimp and crab shell powder , chitosanase , Aspergillus fumigatus Fresenius

## Table of Contents

第一章 緒言 .....	1 第二章 文獻回顧 .....	2 2.1 簡介			
.....2 2.2 幾丁質與幾丁聚醣之化學構造與特性 .....	3 2.2.1 幾丁質之化學構造與特性 .....	3 2.2.2 幾丁聚醣之化學構造與特性 .....	4 2.3 幾丁質類物質之生產 .....		
.....8 2.3.1 幾丁質之製備 .....	8 2.3.2 幾丁聚醣之製備 .....				
.....9 2.4 N-乙醯幾丁寡醣及幾丁寡醣 .....	12 2.4.1 結構 .....	12 2.4.2 N-乙醯幾丁寡醣及幾丁寡醣之抑菌效果 .....	12 2.5 幾丁聚醣 .....		
.....12 2.5.1 微生物產生之幾丁聚醣? .....	13 2.5.2 其他可水解幾丁聚醣之酵素 .....	13 2.6 幾丁聚醣?的一般特性 .....	14 2.7 幾丁聚醣?扮演之生物角色 .....		
.....14 2.8 幾丁聚醣?之應用 .....	15 第三章 研究材料與方法 .....				
.....23 3.1 材料及藥品 .....	23 3.2 研究設備 .....				
.....24 3.3 實驗方法 .....	25 3.3.1 懸浮態幾丁聚醣製備方法 .....				
.....25 3.3.2 酵素活性測定方法 .....	25 3.3.2 菌株之篩選 .....				
.....26 3.3.4 酵素生產培養條件的探討 .....	26 3.3.4.1 碳源 .....				
.....27 3.3.4.2 基礎培養基起始之pH .....	27 3.3.4.3 溫度 .....				
.....27 3.3.4.4 溶氧量 .....	27 3.3.4.5 氮源 .....				
.....27 3.3.4.6 微量金屬 .....	28 3.3.4.7 培養時間 .....				
.....28 3.3.5 酵素純化方法 .....	28 3.3.5.1 大量培養 .....				
.....28 3.3.5.2 硫酸銨沈澱 .....	28 3.3.5.3 DEAE Sepharose CL-6B .....				
.....29 3.3.5.4 Sephadryl S-200 gel filtration .....	29 3.3.5.5 蛋白質濃度測定 .....				
.....29 3.3.5.6 電泳分析 ( SDS-PAGE ) .....	30 3.3.5.7 銀染法 ( silver stain ) .....				
) .....	30 3.3.6 酵素基本性質探討 .....	32 3.3.6.1 最適pH .....			
.....32 3.3.6.2 pH 穩定性 .....	32 3.3.6.3 最適反應溫度 .....				
.....33 3.3.6.4 金屬離子及試劑 .....	33 第四章 結果與討論 .....				
.....34 4.1 菌種鑑定 .....	34 4.2 Aspergillus fumigatus Fresenius 生產幾丁聚醣?最適培養條件之探討 .....	37 4.2.1 Aspergillus fumigatus Fresenius 酵素活性 與pH 變化之關係 .....	37 4.2.2 碳源 .....	37 4.2.3 SCSP濃度 .....	
.....38 4.2.4 基礎培養基起始之pH .....	38 4.2.5 溫度 .....				
.....38 4.2.6 溶氧量 .....	39 4.2.7 氮源 .....				
.....39 4.2.8 微量金屬 .....	39 4.3 酵素純化及酵素基本特性探討 .....				
.....48 4.3.1 酵素純化 .....	48 4.3.1.1 粗酵素液的製備 .....	48 4.3.1.2 DEAE Sepharose CL-6B .....	48 4.3.1.3 Sephadryl S-200 gel filtration .....	49 4.3.1.4 酵素之純化概要 .....	51 4.3.1.5 蛋白質濃度測

定	51 4.3.1.6電泳分析	51 4.3.2 純化酵素基本特性探討
	51 4.3.2.1最適pH之測定	51 4.3.2.2 pH穩定性之測定
	52 4.3.2.3最適反應溫度	52 4.3.2.4金屬離子及試劑
條件	52 4.4 Aspergillus sp.生產之幾丁聚醣?之比較	53 4.4.1 幾丁聚醣?之最適培養
	53 4.4.2 幾丁聚醣?純化及其基本特性	53 第五章 結論
	66 參考文獻	67

## REFERENCES

- 王三郎 (1996) 水產資源利用學，高立圖書出版社。
- 王三郎 (1999) 海洋未利用生物資源之回收再利用-幾丁質及幾丁聚醣，生物資源生物技術1 (1) :1-8
- 王三郎 (2002) 應用微生物學，高立圖書出版社。
- 周婉萍 (1993) *Bacillus coagulans* NTU-FC-I 幾丁質?之研究，國立台灣大學農業化學研究所碩士班論文，台北。
- 周聰麟 (2000) *Aspergillus clavatus* NTU-FC-7 之幾丁聚醣?純化與酵素特性之探討，國立台灣大學農業化學研究所碩士論文，台北。
- 陳美惠、莊淑惠、吳志律 (1999) 幾丁聚醣的物化特性，食品工業月刊31 (10) :1-6
- 張珍田 (2000) 幾丁質?及幾丁聚醣?之研究，生命科學簡訊14 (3) : 3-8。
- 張文智 (1996) 蝦蟹殼加工之廢棄物回收與再利用，私立大葉大學食品工程系研究所碩士論文，台灣彰化縣。
- 吳真誼 (1995) 以幾丁聚醣自葡萄柚汁脫酸、澄清及抗菌之研究，東海大學食品科學研究所，台灣台中縣。
- 傅如嶽 (1999) 豬胃幾丁聚醣?之純化和生化性質研究，國立台灣大學農業化學研究所碩士論文，台北。
- 劉瓊淑 (1994) 幾丁質，幾丁聚醣及其相關酵素之特性與應用，食品工業26 (1) :26-36
- Ak, O., Bakir, U. and Guray, T. (1998) Production, purification and characterization of chitosanase from *Penicillium spinulosum*. *Biochem. Arch.*, 14: 221-225
- Alfonso, C., Martinez, M. J. & Reyes, F.(1992) Purification and properties of two endo-chitosanase from *Mucor rouxii* implicated in its cell wall degradation. *FEMS Microbiol. Lett.*, 95:87-94
- Boucher I, Dupuy A, Vidal P, Neugebauer WA and Brzeninski R. (1992) Purification and characterization of a chitosanase from *Streptomyces N174*. *App. Microbiol. Biotech.*, 38: 188-193
- Brine, C. J., and Austin, P. R. (1981) Chitin variability with species and method of preparation. *Comp. Biochem. Physiol.*, 69: 283-286
- Conrath, U., Domard, A. & Kauss, H., (1989) Chitosan elicited synthesis of callose and of coumarin derivatives in parsley cell suspension cultures. *Plant Cell Rep.*, 815: 2-5
- Chih-Yu Cheng and Yaw-Kuen Li. (2000) An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. *Biotechnol. Appl. Biochem.*, 32: 197-203
- Chui-Liang Chiang, Chen-Tien Chang, Hsien-Yi Sung (2003) Purification and properties of chitosanase from a mutant of *Bacillus subtilis* IMR-NK 1. *Enzyme Microb. Technol.*, 32: 260-267
- Davis, B. & Eveleigh, D. E. (1984) Chitosanase : occurrence, production and immobilization. In : *Chitin, Chitosan, and Related Enzymes* , ed. J.P. Zikakis. Academic Press,FL,161-179
- Davis(unpublished)
- Dumas-Gaudot, E. Grenier, J. Furlan, V. & Asselin, A. (1992) Chitinase, chitosanase and -1-3 glucanase activities in *Allium* and *Pisum* roots colonized by *Glomus* species. *Plant Sci.*, 84: 17-24
- Fenton DM and Eveleigh DE. (1981) Purification and mode of action of a chitinationase from *Penicillium islandicum*. *J. Gen. Microbiol.*, 126: 151-165
- Grenier, J. & Asselin, A. (1990) Some pathogenesis related proteins are chitosanases with lytic activity against fungal spores. *Mol. Plant Microb. Interact.*, 340: 1-7
- Hedges, A. & Wolfe, R. S. (1974) Extracellular enzyme from *Myxobacter AL-1* that exhibits both -1,4 glucanase and chitosanase activities. *J. Bact.*, 120: 844-853
- Ho-Geun yoon, Hee-Yun Kim, Hye-Kyung Kim, Bum-Shik Hong, Dong-Hoon Shin, and Hong-Yon Cho. (2001) Thermostable Chitosanase from *Bacillus* sp. Strain CK4:Its Purification, Characterization, and Reaction Patterns. *Biosci. Biotechnol. Biochem.*, 65(4): 802-809
- Imoto, T. and Yagishita, K. (1971) A simple activity measurement of lysozyme. *Agric. Biol. Chem.* , 35: 1154.
- Izume M, Nagae S, Kawagishi H, Mitsutomi M and Ohtakara A. (1992) Action pattern of *Bacillus* sp. No 7-M chitionase on partially N-acetylated chitionsan. *Biosci. Biotechnol. Biochem.*, 56: 448-453
- Jae Kweon Park, Kumiko Shimono, Nobuhisa Ochiai, Kazutaka Shigeru, Masako Kurita, Yukari Ohta, Katsunori Tanaka, Hideyuki Matsuda, and Makoto Kawamukai (1999) Purification, Characterization, and Gene Analysis of a Chitosanase (Cho) from *Matsuebacter Chitosanotabidus* 3001. *J. Bacteriol.* 21: 6642-6649
- Jean-Yves Masson, Isabelle Boucher, Witold A. Neugebauer, Dindial Ramotar and Ryszard Brzezinski (1995) A new chitosanase gene from a *Nocardiooides* sp. is a third member of glycosyl hydrolase family 46. *Microbiol.*, 141: 2629-2635
- Kauss, H., Jeblick, W. & Domard, A. (1989) The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. *Planta*, 178: 85-92
- Kendra, D. F. and Hadwiger, L. A. (1984) Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. *Exper. Mycol.*, 8: 276-281
- Kurakake Masahiro, Yo-U Shou, Nakagawa Kiyomi, Sugihara Minako, Komaki Toshiaki (2000) Properties of Chitosanase from *Bacillus cereus* S1..*Cur. Microbiol.*, 40: 6-9
- Linthorst, H. J. M. (1991) Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.*, 10:123-150
- Monaghan, R. L.,Eveleigh, D. E., Tewari, R. P. & Resse, E. T. (1973) Chitosanase, a novel enzyme. *Nature New Biol.*,245: 78-80
- Nongporn Hutadilok, Takako Mochimasu, Hiroki Hisamori, Kenichiro Hayashi, Hisashi Tachibana, Tomoko Ishii, Shigehiro Hirano (1995) The effect of N-substitution on the hydrolysis of chitosan by an endo-chitosanase. *Carbohydr. Res.*,268: 143-149
- Ohtakara, A., Ogata, H., Taketomi, Y., and Mitsutomi, M., (1984) In *Chitin , Chitosan and Related Enzyme*, ed. by J. P. Zikakis, Academic Press, New York, 147-160
- Ohajima, S., Ando, A., Shinoyama, H., Fujii, T. (1984) Purification and characterization of an extracellular chitosanase produced by *Amycolatopsis* sp.CsO-2. *J. Ferment. Bioeng.*, 77: 617-620
- Pirkko Helisto, Gleb Aktuganov, Nailia Galimzianova, Alexander Melentjev and Timo Korpela. (2001) Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. *J. Chromato. B*, 758: 197-205
- Price, J.S. and Stork, R. (1975) Production, Purification, and Characterization of an Extracellular Chitosanase from *Streptomyces*. *J. Bacteriol.*, 124: 1574-1585
- Pelletier, A., Lemire, I., Sygusch, J., Chornet,

E., Overend, R.P. (1990) Chitin/chitosan transformation by thermo-mechanochemical treatment including characterization by enzymatic depolymerisation. Biotechnol. Bioeng., 36: 310-315 41.Sakai, K., Katsumi, R., Isobe, A., Nanjo, F. (1991) Purification and hydrolytic action of a chitosanase from Nocardia orientalis. Biochem. Biophys. Acta., 1079: 65-72 42.Seino H, Tsukud K and Shimasue Y. (1991) Properties and action pattern of a chitosanase from Bacillus sp. PI-7S. Agric. Biol. Chem.,55: 2421-2423 43.Shimosaka, M., Nagawa, M., Ohno, Y., Okazaki, M. (1993) Chitosanase from the plant pathogenic fungus, Fusarium solani f. sp. phaseoli purification and some properties. Biosci. Biotechnol. Biochem.,57: 231-235 44.Shimosaka M., Fukumori Y., X.-Y. Zhang, N.-J. He, Kodaira, Okazaki M. (2000) Molecular cloning and characterization of a chitosanase from the chitosanolytic bacterium Burkholderia gladioli strain CHB101. Appl. Microbiol. Biotechnol.,54: 354-360 45.Somashekar, D., Joseph, R. (1992) Partial purification and properties of a novel chitosanase secreted by Rhodotorula gracilis.Lett. Appl. Microbiol., 14: 1-4 46.Somashekar D and Joseph R. (1996) Chitosanase properties and application:a review. Biores. Technol.,55: 35-45 47.Soon-Young, K., Dong-Hwa, S., Ke-Ho L.(1998) Purification and Characteristics of two types of chitosanases from Aspergillus fumigatus KH-94. J. Microbiol. Biotechnol., 8(6): 568-574 48.Stanley, W. L.,Watters, G. G.,Chan, B. G.,Kelly,S. H. (1976) Immobilization of glucose isomerase on chitin with glutaraldehyde and by simple adsorption. Biotechnol. Bioeng.17:439 49.Tamo Fukamizo, Takeshi Ohkawa, Yasuo Ikeda, Sachio Goto(1994)Specificity of chitosanase from Bacillus pumilus. Biochimica et Bio Physica Acta, 1205: 183-188 50.Tsuisaka, Y., Tominaga, Y., Iwai, M. (1975) Purification and some properties of the lytic enzyme from Bacillus R-4 which acts on Rhizopus cell wall. Agric. Biol. Chem., 39: 145-152 51.Uchida, Y., Ohtakara, A. (1988) Chitosanase from Bacillus species. Meth. Enzymol., 161: 501-505 52.Uchida, Y., Tateishi, K., Shida, O., Kodowaki, K. (1992) Purification and enzymic properties of chitosanase from Bacillus licheniformis UTK and their application. In Adv. Chitin Chitosan, ed.C.J.Brine, P.A. Sandford & J.P.Zikakis. Elsevier, London, 282-291 53.Yamasaki, Y., Hayashi, I., Ohta, Y., Matsuda, H. (1993) Purification and mode of action of chitosanolytic enzyme from Enterbacter sp.G-1. Biosci. Biotechnol. Biochem., 57: 444-449 54.Yabuki, M., Uchiyama, A., Suzuki, A., Ando, A., Feji, T. (1988) Purification and properties of chitosanase from Bacillus circulan MH-K1.J. Gen. Appl. Microbiol., 34: 255-270 55.Yamasaki Y, Hayashi I, Ohta Y, Nakagawa T, Kawamukai M and Matusda H. (1993) Purification and mode of action of chitosanolytic enzyme from Enterobacter sp. G-1. Biosci. Biotechnol. Biochem.,57: 444-449 56.Yoshihara K, Hosokawa J, Kubo T, Nishiyama M and Koba Y. (1992) Purification and properties of a chitosanase from Pseudomonas sp. H-14. Biosci. Biotechnol. Biochem.,56: 972-973 57.Xiao-Yong Zhang, An-Lan Dai, Xue-Kun Zhang, Kouji Kuroiwa, Ritsuko Kodaira, Makoto Shimosaka, Mitsuo Okazaki (2000) Purification and Characterization of Chitosanase and Exo- -D-Glucosaminidase from a Koji Mold, Aspergillus oryzae IAM2660. Biosci. Biotechnol. Biochem., 64: 1896-1902