The Antitumor Materials Produced by Bacillus amyloliquefaciens V656 and Monascus purpureus BCRC31499 Enzymes and Their..

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ABSTRACT

The biological function of protease, chitinase and hydrolysates were investigated in this study. In the first part, the protease of M. purpureus BCRC31499 was produced under the optimized culture condition. In the first step, the protease was precipitated and dialyzed by using ammonium sulfate. The further purification and separation procedures of the protease were processed by the use of DEAE Sepharose CL-6B ionic exchange chromatography. Purification was 27-fold with the crude enzyme solution. The overall activity yield of the purified protease was 6%, with specific protease activities of 10 U/mg. The final amount of the protease obtained was 1.6 mg. The protease had a molecular weight of 40 kDa and a pI of 7.9. The optimal pH, optimum temperature, pH stability of the protease were pH 7-9, 40℃, pH 5-9, and 40°C, respectively. In addition to protease activity, amino acids and peptides from the hydrolysis of the SCSP proteins by proteases also exhibited activity of enhancing vegetable growth. The protease would be used to produce biofertilizer in the future. In the second part, we investigated the optimized hydrolysis condition for chitinous materials (water-soluble chitosan, chitin and colloidal chitin). The chitinous materials were hydrolyzed by B. amyloliquefaciens V656 crude enzyme solution. The optimized hydrolysis conditions were that 1% of water-soluble chitosan or 1% of chitin or 3% of colloidal chitin with 20% of crude enzyme solution, pH 5, at 40℃. The composition of the hydrolysates were analyzed by HPLC. It was found that the optimum temperature and reaction time for production of (GlcNAc)6 were 40°C and 12 hours. Longer reaction time lead to the generation of (GlcNAc)n with lower DP ’ s. In the third part, we investigated the antitumor actions of the hydrolysates produce by B. amyloliquefaciens V656 and M. purpureus BCRC31499 crude enzymes on the growth of colon carcinoma cell line, CT26. However, we found that the hydrolysates of crude enzyme solution produce by M. purpureus BCRC31499 had no significant effect on the growth of CT26 cell. But colon carcinoma cell was challenged with the hydrolysates of chitinous materials by B. amyloliquefaciens V656 crude enzyme solution for 1,2,3 days. The cell growth has been measured by MTT assay. The change of cell cycle distribution and induction of apoptosis caused by the hydrolysates of chitinous materials were examined by flow cytometry. Results indicated that when cells were treated with 500 ?g/mL of the hydrolysates, cell proliferation rate was significantly inhibited. The hydrolysates-treated cells indicated a block of S-phase and an elevated level of DNA fragmentation. Additionally, sub-G1 fraction (apoptotic cells) increased with increasing concentrations of the hydrolysates as analyzed by flow cytometry, using agarose gel electrophoresis to analyze the hydrolysates-treated cells, a similar result was found as that of flow cytometry. For the hydrolysates-induced apoptosis, loss of mitochondrial membrane potential was noted. These results suggested that the hydrolysates of chitinous materials by B. amyloliquefaciens V656 crude enzyme solution inhibited the growth of colon carcinoma cell line, CT26 through an accumulation of cell cycle at S-phase and an induction of apoptosis. We expect that these results will provide a new strategy for therapy of colon carcinoma in human beings.

Keywords : protease ; chitinase ; N-acetylchitooligosaccharides ; apoptosis ; CT26 cell

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REFERENCES

1.王三郎 編著。2005。應用微生物學（第四版）。高立圖書公司出版。台北。 2.江孟燦、陳敏俐。1999。膳食幾丁聚醣對大白鼠脂質代謝的影響。中國農業化學會誌 37:20-31。 3.張克亮、李宗洋、傅文榮。2000。HPLC Analysis of N-acetyl- chitooligosaccharides during the acid hydrolysis of chitin. 藥物食品分析 8:75-83。 4.陳榮輝。1995。水產甲殼廢棄物開發高經濟價值之幾丁質材料研究報告。科學發展月刊 27: 413-421。 5.陳詠文、李錦楓。1998。以幾丁聚醣處理水產養殖池水的效益。食品科學 25: 626-637。 6.蘇遠志。2001。紅麴製品介紹及國內研究現況。機能性發酵製品研討會:67-112。中國農業化學會。台北。 7.Aalbaek, T, Reeslev M, Jensen B, and Eriksen SH. 2002. Acid protease and formation of multiple froms of glucoamylase in batch and continuous cultures of Aspergillus niger. Enzyme Microb Technol 30:410-415. 8.Aiba S. 1994. Preparation of N-acetyl-chitooligosaccharides by hydrolysis of chitosan with chitinase follow by N-acetylation. Carbohydr Res 265: 323-328. 9.Anwar, A. and Saleemuddin, M. 1998. Alkaline protease: a review Biosource Technol. 64: 75-83. 10.Babe, L.M. and Schmidt, B. 1998. Purification and biochemical analysis of WprA, a 52-kDa serine protease secreted by Bacillus subtilis as an active complex with its 23-kDa propeptide. Biochim Biophys Acta 1386:211-219. 11.Bible, K.C., and Kaufmann, S.H. 1997. Cytotoxic synergy between flavopiridol and various antineoplastic agents: the importance of sequence of administration. Cancer Res 57: 3375-3380. 12.Budi, S. W., Tuinen, D., Arnould, C., Dumas - Gaudot, E., Gianinazzi-Pearson, V. and Gianinazzi, S. 2000. Hydrolytic enzyme activity of Paenibacillus sp. Strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-brone pathogenic fungi. Appl Soil Ecol 15:191-199. 13.Carroad, P.A. and Tom, R.A. 1978. Bioconversion of shellfish chitin wastes : process conception and selection of microorganism. J Food Sci 43:1158-1160. 14.Chang, W.T., Chen, C.S. and Wang, S.L. 2003. An antifungal chitinase produced by Bacillus cereus using shrimp and crab shell powder as a carbon source. Cur Microbio 47:102-108. 15.Chang, C. T., Liao, Y. M. and Li, S. J. 1999. Preparation of low molecular weight chitosan and chitooligosaccharides by the enzymatic hydrolysis of chitosan. Advances in Chitin Science Vol 3: 233-238. 16.Chen, H.C., Hsu, M.F., and Jiang, S.T. 1997. Purification and characterization of an exo-N, N ’ - diacetylchitobiohydrolase - like enzyme from Cellulomonas flavigena NTOU 1. Enzyme Microb Technol 20: 191-197. 17.Chen, R. H., Huang, J. and Chiang, Y. 1999. Effects of various methods and chitosans used on pH stability and thermal properties of hydrogel prepared. Advances in Chitin Science Vol. 33:255-260. 18.Chen, R. H. and Hua, H. D. 1996a. Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membranes. Carbohydr. Polym. 29:353-358. 19.Chen, R. H. and Hua, H. D. 1996b. Effect of N-acetylation on the acidic solution stability, thermal and mechanical properties of membranes prepared from different chain flexibility chitosans. J. Appl. Polym. Sci. 61:749-754. 20.Chen, S. H. and Chen, H. C. 1999. Effect of oral administration of Cellulomonas flavigena NTOU1-degraded chitin hydrolysate of physiological changes in rat. Food Sci Agric Chem 1: 186-193. 21.Chen, Y., and Lai, M.Z. 2001. c-Jun NH2-terminal kinase activation leads to a FADD-dependent but Fas ligand-independent cell death in Jurkat T cells. J Biol Chem 276: 8350-8357. 22.Chiang, M. T., Yao, H. T. and Chen, H. C. 2000. Effect of dietary

chitosan with different viscosity on plasma lipids and lipid peroxidation in rats fed on a diet enriched with cholesterol. Biosci Biotechnol Biochem. 64: 965-971. 23.Cosio, I.G., Fisher, R.A.and Carroad, P.A. 1982. Bioconversion of shellfish chitin waste: waste pretreatment, enzyme production, process design, and economic analysis. J Food Sci 47:901-905. 24.Dayanandan, A. 2003. Application of an alkaline protease in leather processing:an ecofriendly approach. J Cleaner Product 11:533-536. 25.Don, T. M., Hsu, S. C. and Chiu, W. Y. 2001. Structures and Thermal Properties of Chitosan-Modified Poly (methyl methacrylate). J Polym Sci Part A : Polym Chem 39: 1646-1655. 26.Dunn, C, Crowley, J.J., Moenne-Loccoz, Y., Dowling, D.N., Bruijn F.J. and O ' Gara, F. 1997. Biological control of Pythium ultinum by Stenotrophomonas maltophilia W 18 is mediated by an extracellular proteolytic activity. Microbiol 143:3921-3931. 27.Durand-Poussereau N, Fevre M. 1996. Characterization and a protease deficient strain of Penicillium roqurforti generated by heterologous plasmid intergration:potential us for protein production. J Biotechnol 51: 97-105. 28.Elliott, B.W. and Cohen, J.C. 1986. Isolation and characterization of a Lysine-specific protease from Pseudomonas aeruginosa. J Biochem 261: 259-265. 29.Escobar, J. and Barnett, S. 1995. Synthesis of acid protease from Mucor miehei: integration of production and recovery. Process Biochem 8:659-700. 30.Evan, G.I. and Vousden, K.H. 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411: 342-348. 31.Fei, X. F., Wang, B. X., Li, T.J., Tashiro, S., Minami, M., Xing, de. J. and Ikejima, T. 2003. Evodiamine a constituent of Evodiae Fructus induces anti-proliferating effects in tumor cells. Cancer Sci 94:92-98. 32. Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont M. A., Chevolleau, S., Gasc, N., Tulliez, J., and Terce, F. 2000. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest, and apoptosis in HT29 human colon cancer cells. Cancer Res. 60: 1426-1433. 33.Germano S, Pandey A, Osaku AA, Rocha SN, and Soccol CR. 2003. Characterization and stability of protease from Penicillium sp. produced by solid-state fermentation. Enzyme Microb Technol 32:246-251. 34.Gessesse, A., Hatti-Kaul, R., Gashe, B.A. and Mattiasson, B. 2003. Novel alkaline protease from alkaliphilic bacteria grown on chicken feather. Enzyme Microb Technol 32:519-524. 35.Golstein, P. 1997. Controlling cell death. Science 275: 1081-1082. 36.Goping, I.S., Gross, A., Lavoie, J.N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J., and Shore, G.C. 1998. Regulated targeting of BAX to mitochondria. J Cell Biol 143: 207-215. 37.Green, D.R., and Reed, J.C. 1998. Mitochondria and apoptosis. Science 281: 1309-1312. 38.Greenlee, R.T., Hill-Harmon, M.B., Murray, T. and Thun, M. 2001. Cancer statistics. CA Cancer J Clin 51: 15-36. 39.Hageman, J.H., Shankweiler, G.W., Wall. P.R. and Quinones, C. 1984. Single chemically defined sporulation and extracellular protease production. J Bacteriol 30:41-62. 40.Harman, G.E., Hayes, C.K., Lorito, M., Broadway, R.M., DiPietro, A., Peterbaues, C. and Tronsmo, A. 1993. Chitinolytic enzymes of Trichoderma harzianum: purification of chitobiosidase and endochitinase. Phytopathol 83:313-818. 41.Hetts, S.W. 1998. To die or not to die: an overview of apoptosis and its role in disease. JAMA 279: 300-307. 42.Holinger, E.P., Chittenden, T., and Lutz, R.J. 1999. Bak BH3 peptides antagonize Bcl-xL function and induce apoptosis through cytochrome c-independent activation of caspases. J Biol Chem 274: 13298-13304. 43.Hsieh, P.S. and Tai, Y.H. 2003. Aqueous extract of Monascus purpureus M9011 prevents and reverses fructose-induced hypertension in rats. J Agric Food Chem 51:3945-3950. 44.Hwang, D. F., Lin,M. Y. and Chung, C. Y. 1999. Evaluation of food safety for chitin and chitosan. In Advances in Chitin Science Vol. III Chen, R. H. and Chen, H. C. (Eds.), P:356-361, Rita Advertising CO., LTD, Taipei, Taiwan, R.O.C. 45.Ichishima E, Ojima M, Yamagata Y, Hanzawa S, and Nakamura T. 1995. Molecular and enzymatic properties of an aspartic proteinase from Rhizopous hangchow. Phytochem 38:27-30. 46.Ikasari L, and Mitchell DA. 1996. Leaching and characterization of Rhizopous oligosporus acid protease from solid-state fermentation. Enzyme Microb Technol 19:171-175. 47.Jackman, M.R. and Pines, J.N. 1997. Cyclins and the G2/M transition. Cancer Surv 29: 47-73. 48.Joshi, S., Kozlowski, M., Selvaraj, G., Iyer, V.N., Davies, R.W., Jung, H.Y., Kim, C.Y., Kim, K. and Shin, C.S. 2003. Color characteristics of Monascus pigments derived by fermentation with various amino acids. J Agric Food Chem 51: 1302-1306. 49.Kaufmann, S.H. 1989. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 49: 5870-5878. 50.Kim, H.J. 1997. Purification and characterization of an extracellular metalloprotease from Pseudomonas fluorescens. J Biochem 121: 82-88. 51.Knorr D. 1984. Use of chitinous polymers in food—A challenge for food research and development. Food Technol 38: 85-97. 52.Kohlmann, K.L. 1991. Purification and characterization of an extracellular protease produced by Pseudomonas fluorescens M3/6. J Dairy Sci 74: 4125-4136. 53.Krek,W., Xu, G., and Livingston, D.M. 1995. Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83: 1149-1158. 54.Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R.A. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384: 368-372. 55.Kumar, C.G. 1999. Novel alkaline serine proteases from alkalophilic Bacillus spp. Purification and some properties. Process Biochem 34: 441-449. 56.Larcher G, Bouchara JP, Annaix V, Symoens F, Chabasse F, and Tronchin G. 1992. Purification and characterization of a fibrinogenolytic serine proteinase from Aspergillus fumigatus culture filtrate. FEBS Lett 308:65-69. 57.Li, X. K., Motwani, M., Tong,W., Bornmann,W. and Schwartz, G.K. 2000. Huanglian A chinese herbal extract inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. Mol. Pharmacol 58: 1287-1293. 58.Liang, T.W., Wang, S.L., Lin, J.J. and Yen, Y.H. 2006. Purification and characterization of a protease extracellularly produced by Monascus purpureus CCRC31499 in a shrimp and crab shell powder medium. Enzyme Microb Technol 38: 74-80. 59.Li, H., Zhu, H., Xu, C.J., and Yuan, J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491-501. 60.Lin, F.P., Chen, H.C. and Lin, C.S. 1999. Site-directed mutagenesis of Asp313, Glu315, and Asp391 residues in chitinase of Aeromonas caviae. IUBMB Life 48:199-204. 61.Ling, Y.H., Jiang, J.D., Holland, J.F., and Perez-Soler, R. 2002. Arsenic trioxide produces polymerization of microtubules and mitotic arrest before apoptosis in human tumor cell lines. Mol Pharmacol 62:529-538. 62.Lonhienne, T., Mavromatis, K., Vorgias, C.E., Buchon, L., Gerday, C., Bouriotis, V., Lorita, M., Harman, G.E., Hayes, C.K., Broadway, R.M., Tronsmo, A., Woo, S.L. and DiPietro, A. 1993. Chitinolytic enzymes produced by Trichoderma harzianum:

antifungal activity of purified endochitinase and chitobiosidase. Phytopathol 83:302-307. 63.Ma, J.Y., Li, Y.G., Ye, Q., Li, J., Hua, Y.J., Ju, D.j., Zhang, D.C., Cooper, R. and Chang, M. 2000. Constitutents of red yeast rice, a traditional Chinese food and medicine. J Agric Food Chem 48: 5220-5225. 64.Malik, R.K. and Mathur, D.K. 1984. Purification and characterization of a heat-stable protease from Pseudomonas sp. B-25. J Dairy Sci 67: 522-530. 65.Mancini, M., Nicholson, D.W., Roy, S., Thornberry, N.A., Peterson, E.P., Casciola-Rosen, L.A., and Rosen, A. 1998. The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J Cell Biol 140: 1485-1495. 66.Markaryan A, Morozova I, Yu H, Kolattukudy PE. 1994. Purification and characterization of an elestinolytic metalloprotease from Aspergillus fumigatus and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. Infect Immunol 62: 2149-2157. 67.Martikova, L., Patakova-Juzlova, P., Kren, V., Kucerova, Z., Havlicek, V., Olsovsky, V., Hovorka, O., Rihova, B., Vesely, D. and Ulrechova, J. 1999. Biological activities of oligeketide pigments of Monascus purpureus. Food Addit Contam 16: 15-24. 68.Michalides, R.J. 1999. Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. J Clin Pathol 52: 555-568. 69.Miyata, H., Doki, Y., Yamamoto, H., Kishi, K., Takemoto, H., Fujiwara, Y., Yasuda, T., Yano, M., Inoue, M., Shiozaki, H., Weinstein, I. B. and Monden, M. 2001. Overexpression of CDC25B overrides radiation-induced G2-M arrest and results in increased apoptosis in esophageal cancer cells. Cancer Res. 61: 3188-3193. 70.Moracova, J. and Chaloupka, J. 1984. Repression of the synthesis of exocellular and intracellular proteinases in Bacillus megaterium. Folia Microbiol 29: 273-281. 71.Mori T, Okumura M, Matsuura M, Ueno K, Tokura S, Okamoto Y, Minami S, and Fujinaga T. 1997. Effect of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. Biomaterials 18: 947-951. 72.Muller, N. and Bordusa, F. 2000. Assay of diverse protease activities on the basis of a small synthetic substrate. Anal Biochem 286: 86-90. 73.Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., Riccardi, C. 1991. A rapid, and simple method for measuring thymocyte apoptosis by propidium iodine staining, and flow cytometry. J. Immunol. Methods 139: 271-279. 74.Nishimura K, Nishimura S, Seo H, Nishi N, Tokura S, and Azuma I. 1987. Effect of multiporous microspheres derived from chitin on the activation of mouse peritoneal macrophages. Vaccine 5: 136-140. 75.O ' Keefe-Owens, J.M. amd Chynoweth, D.P. 1996. Anaerobic composting of crab-picking wastes for byproduct recovery. Biores Technol 58: 265-272. 76.Ogasawara, M., Matsubara, T., and Suzuki, H. 2001. Inhibitory effects of evodiamine on in vitro invasion and experimental lung metastasis of murine colon cancer cells. Biol Pharm Bull 24: 917-920. 77.Ogasawara, M., Matsubara, T., and Suzuki, H. 2001. Screening of natural compounds for inhibitory activity on colon cancer cell migration. Biol. Pharm. Bull 24:720-723. 78.Oh, Y.S., Shih, I.L., Tzeng, Y.M. and Wang, S.L. 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. 79.Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619. 80.Pae HO, Seo WG, Kim NY, Oh GS, Kim GE, Kim YH, Kwak HJ, Yun YG, Jun CD, and Chung HT. 2001. Induction of granulocytic differentiation in acute promyelocytic leukemia cells (HL-60) by water-soluble chitosan oligomer. Leuk Res 25: 339-346. 81.Palmieri G, Bianco C, Cennamo G, Giardina P, Marino G, and Monti M. 2001. Purification, characterization, and functional role of a novel extracellular protease from Pleurotus ostreatus. Appl Environ Microbiol 67: 2754-2759. 82.Papa, S. 1996. Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications. Biochem Biophys Acta 1276: 87-105. 83.Pardee, A.B. 1989. G1 events and regulation of cell proliferation. Science 246: 603-608. 84.Patil, R.S., Ghormade, V. and Deshpande, M.V. 2000. Chitinolytic enzymes: an exploration. Enzyme Microb Technol 26: 473-483. 85.Piao, W., Yoo, J., Lee, D. K., Hwang H. J., and Kim, J. H. 2001. Induction of G2/M phase arrest and apoptosis by a new synthetic anti-cancer agent, DW2282, in promyelocytic leukemia (HL-60) cells. Biochem. Pharmaco. 62: 1439-1447. 86.Piazza G. A., Rahm, A. L., Krutzsch, M., Sperl, G., Paranka, N. S., Gross, P. H., Brendel, K., Burt, R. W., Alberts, D. S., Pamukcu, R., and Ahnen, D. J. 1995. Antineo-plastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. Cancer Res. 55: 3110-3116. 87.Qi LF, Xu ZR, Li Y, Jiang X, and Han XY. 2005. In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells. World J Gastroenterol 11: 5136-5141. 88.Rao, M.B., Tanksale, A.M., Chatge, M.S. and Deshpande, V.V. 1998. Molecular and Biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597-635. 89.Reed, J. C. 1997. Cytochrome c: can ' t live with it-can ' t live without it. Cell 91: 559-562. 90.Reed, J. C., Jurgensmeier, J. M. and Matsuyama, S. 1998. Bcl-2 family proteins and mitochondria. Biochim. Biophys. Acta 1366: 127-137. 91.Reichard U, Buttner S, Eiffert H, Staib F, and Ruchel R. 1990. Purification and characterization of an extracellular serine proteinase from Aspergillus fumigatus and its detection in tissue. J Med Microbiol 33:243-251. 92.Schwartz, G.K., Farsi, K., Maslak, P., Kelsen, D.P., and Spriggs, D. 1997. Potentiation of apoptosis by flavopiridol in mitomycin-C-treated gastric and breast cancer cells. Clin Cancer Res 3: 1467-1472. 93.Sellers, W.R., and Fisher, D.E. 1999. Apoptosis and cancer drug targeting. J Clin Invest 104: 1655-1661. 94.Shaikh, S.A. and Deshpande, M.V. 1993. Chitinolytic enzymes: their contribution to basic and applied research. World J. Microbiol Biotechnol 9: 468-475. 95.Shastry, S. and Prasad, M.S. 2002. Extracellular protease from Pseudomonas sp. (CL 1457) active against Xanthomonas campestris. Process Biochem 37: 611-621. 96.Shi, Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9: 459-470. 97.Shibata Y, Foster LA, Metzger WJ, and Quentin NM. 1997. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. Infect Immunol 65: 1734- 1741. 98.Shih, I.L., Chang, W.T., Chen, L.G., Yu, T.S. and Wang, S.L. 2003. Microbial reclamatiom of fish processing wastes for the production of fish sauce. Enzyme Microb Technol 33: 154-162. 99.Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H. and Tsujimoto, Y. 1996. Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. Oncogene 13: 21-29. 100.Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. S., Alnemri, E. S., Green, D. R. and Martin, S. J. 1999. Ordering the cytochrome c-initiated caspase cascade: hierarchical

activation of caspases-2, -3, -6, -7, -8 and – 10 in a caspase-9-dependent manner. J. Cell Biol. 144: 281-292. 101.Slee, E. A., Zhu, H., Chow, S. C., MacFarlane, M., Nicholson, D. W., and Cohen, G. M. 1996. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) inhibits apoptosis by blocking the processing of CPP32. Biochem. J. 315: 21-24. 102.Sookkheo, B. 2000. Purification and characterization of the highly thermostable protease from Bacillus stearothermophilus TLS33. Protein Expr Purifi 20: 143-151. 103.Sugiyama, K., Akiyama, T., Shimizu, M., Tamaoki, T., Courage, C., Gescher, A., and Akinaga, S. 1999. Decrease in susceptibility toward induction of apoptosis and alteration in G1 checkpoint function as determinants of resistance of human lung cancer cells against the antisignaling drug UCN-01 (7-hydroxystaurosporine). Cancer Res 59: 4406-4412. 104.Suh, N., Luyengi, L., Fong, H. H., Kinghorn, A. D. and Pezzuto, J. M. 1995. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. Anticancer Res. 15:233-239. 105.Sun, X. M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R. and Cohen, G. M. 1999. Distinct caspase cascades are initiated in receptor- mediated and chemical-induced apoptosis. J Biol Chem 274: 5053- 5060. 106.Suzuki S, Ogawa Y, Okura Y, Hashimoto K, and Suzuki M. 1982. Proceedings of the Second International Conference on Chitin and Chitosan. pp. 210-212. Sapporo, Japan. 107.Suzuki K, Mikami T. and Okawa Y, 1986. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. Carbohydr Res 151: 403-408 108.Suzuki K, Tokro A, and Okawa Y, 1985. Enhancing effects of N-acetyl-chito-oligosaccharides on the active oxygen-generating and microbicidal activities of peritoneal exudate cells in mice. Chem Pharm Bull 33: 886-888. 109.Suzuki K Tokoro A, and Okawa Y, 1986. Effect of N-acetylchitooligosaccharides on activation of phagocytes. Microbiol Immunol 30: 777-787. 110.Suzuki K, Okawa Y, and suzuki S, 1984. Protecting effect of chitin and chitosan on experimentally induced murine candidiasis. Microbiol Immunol 28: 903-912. 111.Suzuki S, Okawa Y, and Suzuki K, 1987. Candidacidal effect of peritoneal exudae cells in mice administered with chitin or chitosan: the role of seine protease on the mechanism of oxygen-independent candidacidal effect. Microbiol Immunol 31: 375-383. 112.Suzuki S, Watanabe T, Mikami T, Matsumoto T, and Suzuki M. 1992. Immuo-enhancing effects of N-acetyl-chitohexanose. In: Advance in Chitin and Chitosan, pp. 96-105. Brine CJ, Sandford PA, and Zikakis JP (ed.). Elsevier Applied Science, NY. 113.Takami, H., Nakamura, S., Aono, R. and Horikoshi, K. 1992. Degradation of human hair by a thermostable alkaline protease by alkalophilic Bacillus sp. AH-101. Biosci Biotechnol Biochem 56: 1667-1669. 114.Taylor, W.R. and Stark, G.R. 2001. Regulation of the G2/M transition by p53. Oncogene 20:1803-1815. 115.Thornberry, N.A., and Lazebnik, Y. 1998. Caspases: enemies within. Science 281: 1312-1316. 116.Tokoro A, Kobayashi M, Tatewaki N, Suzuki K, Okawa Y, Mikami T, Suzuki S, and Suzuki M. 1989. Protective effect of N-acetyl chitohexaose on Listeria monocytogenes infection in mice. Microbiol Immunol 33: 357-367. 117.Tokoro A, Tatewaki N, Suzuki K, Mikami T, Suzuki S, and Suzuki M. 1988. Growth-inhibitory effect of hexa-N-acetylchitohexanose against Meth A solid tumor. Chem Pharm Bull 36: 784-790. 118.Toma, C. 1999. Purification and characterization of an Aeromonas caviae metalloprotease that is related to the Vibrio cholerae henagglutinin/protease. FEMS Microbiol Lett 170: 237-242. 119.Tsai, G. J. and Hwang, S. P. 2001. Effects of shrimp chitosan on the intestinal epithelia tissue. Proceedings of 3rd Int. Symposium on Chitin Enzymology. 120.Tsai, G. J. and Su, W. H. 1999. Antibacterial activity of shrimp chitosan against Escherichia coli. J Food Prot 62: 239-243. 121.Tsai, G. J., Wu, Z.Y. and Su, W.H. 2000. Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. J. Food Prot 63: 747-752. 122.Tsujibo, H., Miyamoto, J., Kondo, N., Miyamoto, K., Baba, N., Inamori, Y. and Turkiewicz, M. 1999. Biosynthesis and properties of an Extracellular metalloprotease from the Antarctic marine bacterium Sphingomonas paucimobilis. J Biotechnol 70: 53-60. 123.Vantieghem, A., Xu, Y., Assefa, Z., Piette, J., Vandenheede, J.R., Merlevede,W.,DeWitte,P.A. and Agostinis, P. 2002. Phos- phorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. J Biol Chem 277: 37718-37731. 124.Wang, C.H., Shih, I.L. and Wang, S.L. 2002. Production of antifungal compounds from chitin by Bacillus subtilis. Enzyme Microb Technol 31: 337-344. 125.Wang, I.K., Lin-Shiau, S.Y., Chen, P.C. and Lin, J.K. 2000. Hypotriglyceridemic effect of anka (a fermented rice products of Monascus sp.) in rats. J Agric Food Chem 48: 3183-3189. 126.Wang, S.L. and Chang, W.T. 1997. Purirfication and characterization of two bifunctional chitinases/lysozymes extracellularly produced by Pseudomonas aeruginosa K-187 in a shrimp and crab powder medium. Appl Environ Microbiol 63: 380-386. 127.Wang, S.L., Chen, Y.S., Wang ,C.L., Yen, Y.H. and Chern, M.K. 2005. Purification and characterization of a serine protease extracellularly produced by Aspergillus fumigatus in a shrimp and crab shell powder medium. Enzyme Microb Technol 36:660-665. 128.Wang, S.L., Chio, S.H. and Chang, W.T. 1997. Production of chitinase from shellfish waste by Pseudomonas aeruginosa K-187. Proc Natl Sci Cncl 21: 71-78. 129.Wang, S.L., Hsiao, W.J. and Chang, W.T. 2002. Purification and characterization of two antimicrobial chitinases extracellularly produced by Monascus purpureus CCRC31499 in a shrimp and crab shell powder medium. J Agric Food Chem 50: 2249-2255. 130.Wang, S.L., Shih, I.L. and Liang, T.W. 2002. Purification and characterization of two antimicrobial chitinases extracellularly produced by Bacillus amyloliquefaciens V656 in a shrimp and crab shell powder medium. J Agric Food Chem 50:2241-2248. 131.Wang, S.L., Yen, Y.H., Hsiao, W.J., Chang, W.T. and Wang, C.L. 2002. Production of antifungal compounds by Monascus purpureus CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme Microb Technol 31: 337-344. 132.Wang, S. L., Shih, I. L., Wang, C. H., Chang W. T. and Liang, T.W. 2002. Production of antifungal compounds from chitin by Bacillus subtilis. Enzyme Microb Technol 31:321-328. 133.Wolf, B. B., and Green, D. R. 1999. Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem 274: 20049-20052. 134.Wu, L. C., Chen, Y. C., Ho, J. A. and Yang, C. S. 2003. Inhibitory effect of red koji extracts on mushroom tyrosinase. J Agric Food Chem 51: 4240-4246. 135.Wu, L. C. and Hang, Y. D. 2000. Acid protease production from Neosartorya fischeri. Lebensm-Wiss U-Rechnol 33:44-47. 136.Yang, J. K., Shih, I. L., Tzeng, Y. M. and Wang, S. L. 2000. Production and purification of protease from a Bacillus subtilis that can deproteinize crustacean wastes. Enzyme Microb Technol 26: 406-413. 137.Yasuda, M., Aoyama, M., Sakaguchi, M., Nakachi, K., and Kobarnoto, N. 1999. Purification and characterization of a

soybean-milk-coagulating enzyme from Bacillus pumilus TYO-67. Appl Microb Biotechnol 51: 474-479.