Optimization of α - chymotrypsin - catalyzed dipeptide derivative (N-Ac-Phe-Gly-NH2) synthesis

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ABSTRACT

The dipeptide derivative, N-Ac-Phe-Gly-NH2, has a heat-resistant character to prevent protein agglutination due to heat and makes inflammation or pathological changes for normal cells. This study focused on the immobilization of α-chymotrypsin and its catalysis to synthesize the dipeptide derivative under optimal conditions. Three sections are included, and they are: (1) optimal synthesis of N-Ac-Phe-Gly-NH2 catalyzed by α-chymotrypsin, (2) optimal immobilization of α-chymotrypsin on Fe3O4-chitosan nanoparticles by covalent-binding and (3) optimal synthesis of N-Ac-Phe-Gly-NH2 catalyzed by the immobilized enzyme in a mixing reactor furnished with a magnet. In the first section (Chapter 3), the dipeptide derivative, N-acetyl-phenylalanine- glycinamide (N-Ac-Phe-Gly-NH2), was synthesized from N-acetyl phenylalanine ethyl ester (N-Ac-Phe-OEt) and glycinamide hydrochloride (Gly-NH2・HCl) and catalyzed by α-chymotrypsin (a protease) in a biphasic system. Response surface methodology with a four-factor-five-level central composite rotatable design was employed to evaluate the effects of selected variables that included the incubation time, reaction temperature, enzyme activity, and pH level on the yield of the dipeptide derivative. The results indicated that pH significantly affected the yield of N-Ac-Phe-Gly-NH2. From a ridge max analysis, the optimum conditions for this synthesis included an incubation time of 30.9 min, a reaction temperature of 35.8 ° C, an enzyme activity of 159.2 U, and a pH of 8.98. The predicted and the actual (experimental) yields were 98.0% and 95.1%, respectively. The second section (Chapter 4) investigated the immobilization of α-chymotrypsin onto magnetic Fe3O4-chitosan nanoparticles by covalent binding. The RSM with a 3-factor-3-level Box-Behnken experimental design was employed to evaluate the effects of the manipulated variables, including the incubation time, reaction temperature and pH level, on the activity of the immobilized enzyme. The results indicated that the temperature significantly affected the enzyme activity. The binding sites of functional groups were identified by FTIR. By a ridge max analysis, the optimum condition for α-chymotrypsin immobilization was obtained to include a reaction temperature of 21.7 ° C, a pH level of 7.6, and an incubation time of 1.1 h. The predicted and the actual (experimental) enzyme activities were 353.51 and 346.76±46.51 U/g-support, respectively, under the optimum condition. The immobilized enzyme (Fe3O4-chitosan-chymotrypsin nanoparticles) has a good acid-resisting character and can reduce the operating time to separate the product from the enzyme by a magnetic field. Therefore, the immobilized enzyme could be used to produce N-Ac-Phe-Gly-NH2 in a large-scale. In the third section (Chapter 5), N-Ac-Phe-Gly-NH2 synthesis was catalyzed by the immobilized enzyme in a mixing reactor furnished with a magnet. The RSM with a 3-factor-3-level Box-Behnken experimental design was employed to evaluate the effects of the manipulated variables, including the incubation time, reaction temperature and pH level, on the N-Ac-Phe-Gly-NH2 yield. The results indicated that the reaction temperature and pH level significantly affected the yield. The yield increased with the increase of the reaction temperature or pH. By ridge max and canonical analyses, the optimal condition for N-Ac-Phe-Gly-NH2 synthesis was obtained to include an incubation time of 92.3 min, a reaction temperature of 36.2 ° C, and a pH level of 8.7. The predicted and the actual (experimental) yields under the optimal condition were 84.22% and 82.26±0.39%, respectively. In summary, this study first obtained an optimal condition for N-Ac-Phe-Gly-NH2 synthesis catalyzed by α-chymotrypsin using CCRD. Then, α-chymotrypsin was immobilized onto Fe3O4-chitosan nanoparticles by covalent binding. The optimal condition for enzyme immobilization was obtained by a Box-Behnken experimental design. The immobilized enzyme has a good acid-resisting character and its catalytic capability is about the same as the free-enzyme. Moreover, the immobilized enzyme can be reused up to more than ten times and therefore could be used to produce N-Ac-Phe-Gly-NH2 in a large-scale. The last part of this study investigated the optimal condition for N-Ac-Phe-Gly-NH2 synthesis in a mixing reactor (with a rotational speed of 150 rpm) furnished with a magnet by a Box-Behnken design. Although the yields obtained in Chapter 5 was 10%-15% lower than those obtained in Chapter 3, the immobilized enzyme can be used up to more than ten times. Furthermore, the immobilized enzyme can be easily separated from the product by a magnetic field and therefore can reduce the operating cost substantially.

Keywords : N-Ac-Phe-Gly-NH2、α-Chymotrypsin、Fe3O4-chitosan nanoparticles、Enzyme immobilization、Mixing reactor with a magnet、Optimization

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REFERENCES

1. 史宗淮。1995。微粉製成技術簡介。化工。42:28 – 35。 2. 江晃榮，2003。生物反應器在特化及食品產業之應用。化工資訊與商情，第六期。 3. 沈明來。2005。實驗設計學，第三版。第41 – 42頁。九州圖書文物公司。台北。 4. 何國慶，丁立孝。2007。食品酵素學。第8頁。五南圖書出版公司。台北。 5. 洪佃玠，2003，利用幾丁聚醣固定脂肪分解酵素之研究。碩士論文。國立清華大學化學工程學系。新竹，台灣。 6. 洪哲穎，陳國誠。1992。回應曲面實驗設計法在微生物酵素生產之應用。化工。39:3 – 12。 7. 黃世宏。2003。氧化鐵磁性奈米粒子在酵素固定化及分離上之應用。第7 – 8頁。碩士論文。成功大學化學工程學系。台南，台灣。 8. 陳美瑩。1995。固相胜?合成新方法之研究。碩士論文。大葉大學食品工程研究所。彰化，台灣。 9. 陳盛樂，2004。真菌Aspergillus terreus固定化技術之探討－包埋法。碩士論文。朝陽科技大學應用化學系。台中，台灣。 10. 陳淑玲。2004。固相胜?合成DOTA-Try3-Octreotide。第3頁。碩士論文。中原大學化學系。中壢，台灣。 11. 陳國誠，1990，生物固定化技術與產業應用。第125 – 164頁。茂昌圖書，台北。 12. 陳國誠，2000，生物固定化技術與產業應用。第121 – 155頁。茂昌圖書。台北。 13. 陳順宇，鄭碧娥。1993，Statistica手冊(II)工業統計。第五章，第1頁。華泰書局。台北。 14. 陳順宇，鄭碧娥。2002，實驗設計。第十二章，第1-33頁。華泰書局。台北。 15. 張碩芬。2006。以

聚麩胺酸為擔體探討脂解酵素AY共價固定化之研究。碩士論文。大葉大學生物產業科技系。彰化。台灣。 16. 葉怡成。2005。實驗計劃法—製程與產品最佳化。第六章，第1-21頁。五南圖書。台北。 17. 賴滋漢，賴業超。1994。食品科技辭典。第1078頁。富林出版社。台中。台灣。 18. 薛蓮，孟琴，呂德偉。2002。用吸附法固定化培養紫草細胞。植物生理與分子生物學學報。28(5)。369-374。 19.Abdul Rahman, M. B., Tajudin, S. M., Hussein, M. Z., Abdul Rahman, R.-N. Z.-R., Salleh, A. B. and Basri, M. 2005. Application of natural kaolin as support for the immobilization of lipase from Candida rugosa as biocatalyst for effective esterification. Appl. Clay Sci. 29: 111 – 116. 20.Abdul Rahman, M.B., Zaidan, U.H., Basri, M., Hussein, M.Z., Abdul Rahman, R. N. Z. R. and Salleh, A. B. 2008. Enzymatic synthesis of methyl adipate ester using lipase from Candida rugosa immobilised on Mg, Zn and Ni of layered double hydroxides (LDHs). J. Mol. Catal. B-Enzym. 50: 33 – 39. 21.Abdus Salam, S. M., Kagawa, K. I. and Kawashiro, K. 2006. α-Chymotrypsin-catalyzed peptide synthesis in frozen aqueous solution using N-protected amino acid carbamoylmethyl esters as acyl donors. Tetrahedron: Asymmetry 17: 22 – 29. 22.Ataie, A. and Heshmati-Manesh, S. 2001. Synthesis of ultra-fine particles of strontium hexaferrite by a modified co-precipitation method. J. Eur. Ceram. Soc. 21: 1951-1955. 23.Bacheva, A. V., Belyaeva, A. V., Lysogorskaya, E. N., Okesnoit, E. S., Lozinsky, V. I. and Filippova, I. Y. 2005. Biocatalytic properties of native and immobilized subtilisin 72 in aqueous-organic and low water media. J. Mol. Catal. B-Enzym. 32: 253 – 260. 24.Bai, Y. -X., Li, Y. -F. and Wang, M. -T. 2006. Study on synthesis of a hydrophilic bead carrier containing epoxy groups and its properties for glucoamylase immobilization. Enzyme and Microb. Tech. 39: 540 – 547. 25.Balca?o, V. M., Paiva, A. L. and Malcata, F. X. 1996. Bioreactors with immobilized lipases: State of the art. Enzyme and Microb. Tech. 18: 392 – 416. 26.Barros, R. J., Wehtje, E. and Adlercreutz, P. 2001. Modeling the performance of immobilized α-chymotrypsin catalyzed peptide synthesis in acetonitrile medium. J. Mol. Catal. B-Enzym. 11: 841 – 850. 27.Bayramo?lu, G. and Arica, M. Y. 2008. Preparation of poly(glycidylmethacrylate -methylmethacrylate) magnetic beads: Application in lipase immobilization. J. Mol. Catal. B-Enzym. 55: 76 – 83. 28.Berson, S. A. and Yalow, R. S. 1973. Peptide Hormones. p. 202. American Elsevier, New York. 29.Biro, E., Nemeth, A. Sz., Sisak, C., Feczko, T. and Gyenis, J. 2008. Preparation of chitosan particles suitable for enzyme immobilization. J. Biochem. Bioph. Meth. 70: 1240 – 1246. 30.Bodalo, A., Bastida, J., Maximo, M.F., Montiel, M.C., Gomez, M., and Murcia, M.D. 2008. Production of ricinoleic acid estolide with free and immobilized lipase from Candida rugosa. Biochem. Eng. J. 39: 450 – 456. 31.Bommarius, A. S. and Riebel, B. R. 2003. Biocatalysis. p. 30 – 571. Wiley-VCH, Atlanta, GA. 32.Boonsongrit, Y., Mueller, B. W. and Mitrevej, A. 2008. Characterization of drug-chitosan interaction by 1H NMR, FTIR and isothermal titration calorimetry. Eur. J. Pharm. Biopharm. 69: 388-395. 33.Box, G. E. P. and Wilson, K. B. 1951. On the experimental attainment optimum conditions. J. R. Stat. Soc. 13: 1 – 45. 34.Brandsch, M., Brandsch, C., Prasad, P. D., Ganapathy, V., Hopfer, U. and Leibach, F. H. 1995. Identification of a renal cell line that constitutively expresses the kidney-specific high-affinity H+/ peptide co-transporter. FASEB J. 9: 1489 – 1496. 35.Calvet, S., Clapes, P., Torres, J. L., Valencia, G., Feixas, J. and Adlercreutz, P. 1993. Enzymatic synthesis of X-Phe-Leu-NH2 in low water content system: Influence of the N-α protecting group and the reaction medium composition. Biochim. Biophys. Acta 1164: 189 – 196. 36.Cao, L. 2005. Immobilised enzymes: science or art? Curr. Opin. Chem. Biol. 9: 217 – 226. 37.Chandrasekaran, G. and Nimy Sebastian, P. 1998. Magnetic study of Znx Mg1-zFe2O4 mixed ferrites. Mater. Lett. 37: 17-20. 38.Costodes, V. C. T. and Lewis, A. E. 2006. Reactive crystallization of nickel hydroxycarbonate in a fluidized-bed reactor: fines production and column design. Chem. Eng. Sci. 61: 1377 – 1385. 39.Cumbal, L., Greenleaf, J., Leun, D. and SenGupta, A. K. 2003. Polymer supported inorganic nanoparticles: characterization and environmental applications. React. Funct. Polym. 54: 167 – 180. 40.Curtius, T. 1881. Ueber die einwirkung von chlorbenzoyl auf glycocollsiber. J. Prakt. Chem. 24: 239 – 240. 41.Denkba?, E. B., Kilicay, E., Birlikseven, C. and Ozturk, E. 2002. Magnetic chitosan microspheres: preparation and characterization. React. Funct. Polym. 50: 225-232. 42.D’Souza, S. F. 1999. Immobilized enzymes in bioprocess. Curr. Sci. 77: 69 – 79. 43.Duke, S. D. and Abbas, H. K. 1995. Natural products with potential use as herbicides. ACS Symp. Ser. 582: 348 – 362. 44.Faber, K. 1992. Biotransfermations in Organic Chemistry. p. 2-4. Springer-Verlag, Germany. 45.Fazzi, R., Galimberti, S., Pacini, S., Testi, R., Azzara`, A., Orciuolo, E., Trombi, L., Metelli, M. R. and Petrini, M. 2002. Bone and bone marrow interactions: Hematological activity of osteoblastic growth peptide (OGP)-derived carboxy-terminal pentapeptide. II. Action on human megakaryocytopoieses: focus on essential thrombocythemia. Leuk. Res. 26, 839 – 848. 46.Feliciano, A. S., Dias, A. I. and Prazeres, D. M. F. 2000. The effect of stirring and seeding on the Ac-Phe-Leu-NH2 synthesis and crystallization in a reversed micellar system. Enzyme Microb. Technol. 27: 264 – 269. 47.Fomuso, L. B. and Akoh, C. C. 2002. Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. Food Research. Int. 35: 15 – 21. 48.Fuganti, C. and Grasselli, P. 1986. Immobilized penicillinacylase: Application to the synthesis of the dipeptide aspartame. Tetrahedron Lett. 27: 3191-3194. 49.Ganapathy, M. E., Brandsch, M., Prasad, P. D., Ganapathy, V. and Leibach, F. H. 1995. Differential recognition of β-lactam antibiotics by intestinal and renal peptide transporters, PEPT1 and PEPT2. J. Biol. Chem. 270: 25672 – 25677. 50.Ganapathy, M. E., Prasad, P. D., Mackenzie, B., Ganapathy, V. and Leibach, F. H. 1997. Interaction of anionic cephalosporins with the intestinal and renal peptide transporters PEPT1 and PEPT2. Biochim. Biophys. Acta 1324: 296 – 308. 51.Ganapathy, M. E., Huang, W., Wang, H., Ganapathy, V. and Leibach, F. H. 1998. Valacyclovir: A substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. Biochem. Biophys. Res. Commun. 246: 470 – 475. 52.Garrett, R. H. and Grisham, C. M. 2005. Chap.14.7: Mechanisms of Enzyme Action. p. 459. Biochemistry (3nd). Thomson Books/Cole, California. USA. 53.Gerisch, S., Jakubke, H. D. and Kreuzfeld, H. J. 1995. Enzymatic peptide synthesis in frozen aqueous systems: use of N α-unprotected unusual acyl donors. Tetrahedron: Asymmetry 12: 3039 – 3045. 54.Ghanem, A. and Ghaly, A. 2004. Immobilization of glucose oxidase in chitosan gel beads. J. Appl. Polym. Sci. 91: 861 – 866. 55.Grant, G. A., 1992. Synthetic Peptide. A User’s Guide. Freeman, New York. 56.Guinn, R. M., Margot, A. O., Taylor, J. R., Schumacher, M., Clark, D. S. and Blanch, H. W. 2004. Synthesis and characterization of polyamides containing unnatural amino-acids. Biopolymers 35: 503 – 512. 57.Guo, Z. and Sun, Y. 2004. Characteristics of immobilized lipase on hydrophobic superparamagnetic microspheres to catalyze esterification. Biotechnol. Prog. 20: 500-506. 58.Hansler, M. and Jakubke, H. D. 1996. Non-conventional protease catalysis in frozen aqueous solutions. J. Pept. Sci. 2: 279 – 289. 59.Hess, G. P. 1971. Chymotrypsin, chemical properties and catalysis, in The Enzymes, 3rd ed., vol. 3, Boyer, P. D. (Ed.), p. 213-248. Academic Press. New York. 60.Hong, J., Gong, P., Xu, D., Dong, L. and Yao, S. 2007. Stabilization of α-chymotrypsin by covalent immobilization on amine-functionalized superparamagentic nanogel. J. Biotechnol. 128: 597 – 605. 61.Hong, J., Gong, P., Yu, J. -H., Xu, D. -M., Sun, H. -W. and Yao, S. 2006. Conjugation of α-chymotrypsin on a polymeric hydrophilic nanolayer covering magnetic nanoparticles. J. Mol. Catal. B-Enzym. 42: 99 – 105. 62.Hong, J., Xu, D., Gong, P., Ma, H., Dong, L. and Yao, S. 2007. Conjugation of enzyme on superparamagnetic nanogels covered with carboxyl groups. J. Chromatogr. B 850: 499 – 506. 63.Hong, J., Xu, D., Gong, P., Sun, H., Dong, L. and Yao, S. 2007. Covalent binding of α-chymotrypsin on the magnetic nanogels covered by amino groups. J. Mol. Catal. B-Enzym. 45: 84 – 90. 64.Hong, J., Xu, D., Gong, P., Yu, J., Ma, H. and Yao, S. 2008. Covalent-bonded immobilization of enzyme on hydrophilic polymer covering magnetic nanogels. Microporous Mesoporous Mat. 109: 470 – 477. 65.Hou, R. Z., Zhang, N., Li, G., Huang, Y. B., Wang, H., Xiao, Y. P., Liu, Y. J., Yang, Y., Zhao, L. and Zhang, X. Z. 2005. Synthesis of tripeptide RGD amide by a combination of chemical and enzymatic methods. J. Mol. Catal. B-Enzym. 37: 9 – 15. 66. http://www.just.edu.tw/~envirdep/nano/index.htm. 67.Huang, Y. B., Xiao, Y. P., Wang, H., Hou, R. Z., Zhang, N., Wu, X. X., Xu, L. and Zhang, X. Z. 2005. Chemo-enzymatic synthesis of tripeptide RGD diamide in organic solvents. J. Biotechnol. 116: 51 – 59. 68.Huang, H.-Y., Shieh, Y.-T., Shih, C.-M., Twu, Y.-K. 2010. Magnetic chitosan/iron (II, III) oxide nanoparticles prepared by spray-drying. Carbohydr. Polym. In press. 69.Jia, H., Ma, X., Wei, D., Dong, J. and Qian, W. 2008. Direct formation of silver nanoparticles in cuttlebone-derived organic matrix for catalytic applications. Colloid Surf. A-Physicochem. Eng. Asp. 330: 234 – 240. 70.Jia, H., Zhu, G. and Wang, P. 2003. Catalytic behaviors of enzymes attached to nanoparticles: The effect of particle mobility. Biotechnol. Bioeng. 84: 406 – 414. 71.Jiang, D. S., Long, S. Y., Huang, J., Xiao, H. Y. and Zhou, J. Y. 2005. Immobilization of Pycnoporus sanguineus laccase on magnetic chitosan microspheres. Biochem. Eng. J. 25: 15-23. 72.Jones, J. 1992. Amino Acid and Peptide Synthesis. New York. 73.Ju, H. -Y., Yang, C. -K., Yen, Y. – H., and Shieh, C. -J. 2008. Continuous lipase-catalyzed synthesis of hexyl laurate in a packed-bed reactor: optimization of the reaction conditions in a solvent-free system. J. Chem. Technol. Biotechnol. 84: 29 – 33. 74.Kang, Y. S., Risbud, S., Rabolt, J. F. and Stroeve, P. 1996. Synthesis and characterization of nanometer-size Fe_3O_4 and γ-Fe_2O_3 particles. J. Mater. Chem. 2209 – 2212. 75.Katz, E., Sheeney-Haj-Ichia, L., Buckmann, A. F. and Willner, I. 2002. Dual biosensing by magneto-controlled bioelectrocatalysis. Angew. Chem. Int. Ed. 41: 1343-1346. 76.Khmel ‘ Nitski, Y. L., Dien, F. K., Semenov, A. N., Martinek, K., Veruovi?, B. and Kubanek, V. 1984. Optimization of enzyme catalyzed peptide synthesis in a “ water - water-immiscible organic solvent ” biphasic system. Tetrahedron 40: 4425 – 4432. 77.Kim, W. C., Kim, S. J., Lee, S. W. And Kim, C. S. 2001. Growth of ultrafine NiZnCu ferrite and magnetic properties by a sol-gel method. J. Mang. Magn. Mater. 226-230: 1418-1420. 78.Kim, D. H., Lee, S. H., Im, K. H., Kim, K. N., Shim, I. B., Lee, M. H. and Lee, Y. K. 2006. Surface-modified magnetite nanoparticles for hyperthermia: preparation, characterization, and cytotoxicity studies. Curr. Appl. Phys. 61: 242 – 246. 79.Kisee, H., Fujimoto, K. and Noritomi, H. 1988. Enzymatic reactions in aqueous-organic media. VI. Peptide synthesis by α-chymotrypsin in hydrophilic organic solvents. J. Biotechnol. 8: 279 – 290. 80.Kuo, T. M., and Gardner, H. W. 2002. Lipid Biotechnology. p. 387 – 398. Marcel dekker. New York. 81.Lacava, L. M., Lacava, Z. G. M., Azevedo, R. B., Chaves, S. B., Garcia, V. A. P., Silva, O., Pelegrini, F., Buske, N., Gansau, C., Da Silva, M. F. And Morais, P. C. 2002. Use of magnetic resonance to study biodistribution of dextran-coated magnetic fluid intravenously administered in mice. J. Magn. Magn. Mater. 252: 367-369. 82.Laskowski M. 1955. Chymotrypsinogens and chymotrypsins. Methods Enzymol. 2: 8-26. 83.Levison, P. R., Badger, S. E., Dennis, J., Hathi, P., Davies, M. J., Bruce, I. J. and Schimkat, D. 1998. Recent developments of magnetic beads for use in nucleic acid purification. J. Chromatogr. A 816: 107-111. 84.Li, G. Y., Huang, K. L, Jiang, Y. R., Ding, P. and Yang, D. L. 2008. Preparation and characterization of carboxyl functionalization of chitosan derivative magnetic nanoparticles. Biochem. Eng. J. 40: 408 – 414. 85.Liu, P., Tian, G. L., Lee, K. S., Wong, M. S. and Ye, Y. H. 2002. Full enzymatic synthesis of a precursor of bioactive pentapeptide OGP (10-14) in organic solvents. Tetrahedron Lett. 43: 2423 – 2425. 86.Liu, W., Zhang, S. and Wang, P. 2009. Nanoparticle-supported multi-enzyme biocatalysis with in situ cofactor regeneration. J. Biotechnol. 139: 102 – 107. 87.Lu, X., Mao, H., Chao, D., Zhang, W. and Wei, Y. 2006. Ultrasonic synthesis of polyaniline nanotubes containing Fe_3O_4 nanoparticles. J. Soild State. Chem. 179: 2609-2615. 88.Ma, Z. and Liu, H. 2007. Synthesis and surface modification of magnetic particles for application in biotechnology and biomedicine. China Particuology 5: 1 – 10. 89.Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, Jr. C. G. and Amundson, C. H. 1990. Immobilized lipase reactors for modification of fats and oils. A review. J. Am. Oil Chem. Soc. 67: 890 – 910. 90.Matijo?yt?, I., Arends, I.W.C.E., de Vries, S. and Sheldon, R.A. 2010. Preparation and use of cross-linked enzyme aggregates (CLEAs) of laccases. J. Mol. Catal. B-Enzym. 62: 142 – 148. 91.Mehta, R.V., Upadhyay, R.V., Charles, S. W. and Ramchand, C. N. 1997. Direct binding of protein to magnetic particles. Biotechnol. Tech. 11: 493 – 496. 92.Mendes, J. S., Silva, J. S., Ferreira, A. L. O. and Silva, G. F. 2007. Simulation of Process Interesterification in Fluidized Bed Bioreactor for Production of Biodiesel. Comput Aided Chem. Eng. 27: 1803-1808. 93.Merrifield, R. B. 1963. Solid-phase peptide synthesis. Ⅰ. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85: 2149 – 2154. 94.Mikhaylova, M.., Kim, D. K., Bobrysheva, N., Osmolowsky, M., Semenov, V., Tsakalakos, T. and Muhammed, M. 2004. Superparamagnetism of magnetite nanoparticles: dependence on surface modification. Langmuir 20: 2472-2477. 95.Mishima, K., Matsuyama, K., Baba, M. and Chidori, M. 2003. Enzymatic dipeptide synthesis by surfactant-coated α-chymotrypsin complexes in supercritical carbon dioxide. Biotechnol. Prog. 19: 281 – 284. 96.Morihara, K. and Oka, T. 1977. α-Chymotrypsin as the catalyst for peptide synthesis. Biochem. J. 163: 531 – 542. 97.Myers, R. H., and Montgomery, D. C. 2002. Response Surface Methodology: Process and Product Optimization Using Design Experiments. (2nd Eds.). Wiley. New York. 98.Nielsen, S. N., Yang, T., Xu, X. and Jacobsen, C.

2006. Production and oxidative stability of a human milk fat substitute produced from lard by enzyme technology in a pilot packed-bed reactor. Food Chem. 94: 53 – 60. 99.Ogino, H., Yamada, M., Watanabe, F., Ichinose, H., Yasuda, M. and Ishikawa, H. 1999. Peptide synthesis catalyzed by organic solvent-stable protease from Pseudomonas aeruginosa PST-01 in monophasic aqueous-organic solvent systems. J. Biosci. Bioeng. 88: 513 – 518. 100.Pan, C. P., Hu, B., Li, W., Sun, Y., Ye, H. and Zeng, X. 2009. Novel and efficient method for immobilization and stabilization of β-D-galactosidase by covalent attachment onto magnetic Fe3O4-chitosan nanoparticles. J. Mol. Catal. B-Enzym. 61: 208-215. 101.Pan, D., Luo, Y. and Tanokura, M. 2004. Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of Lactobacillus helVeticus JCM1004. Food Chem. 91: 123 – 129. 102.Parkash, G. and Srivasatava, A. K. 2007. Azadirachtin production in stirred tank reactors by Azadirachta indica suspension culture. Process Biochem. 42: 93-97. 103.Pedroche, J., Yust, M. M., Lqari, H., Megias, C., Giron-Calle, J., Alaiz, M., Vioque, J. and Millan, F. 2007. Obtaining of Brassica carinata protein hydrolysates enriched in bioactive peptides using immobilized digestive proteases. Food Res. Int. 40: 931 – 938. 104.Pieters, B. R., Williams, R. A. and Webb, C. 1992. Magnetic carrier technology. p. 248-286. Williams R.A. ed. Colloid and Surface Engineering: Applications in the Process Industries. Butterworth-Heinemann, Oxford, England. 105.Pyle, B. H., Broadaway, S. C. and McFeters, G. A. 1999. Sensitive Detection of Escherichia coli O157:H7 in Food and Water by Immunomagnetic Separation and Solid-Phase Laser Cytometry. Appl. Environ. Microbiol. 65: 1966 – 1972. 106.Rosa, C. L., Milardi, D., Amato, E., Pappalardo, M. and Grasso, D. 2005. Molecular mechanism of the inhibition of cytochrome c aggregation by Phy-Gly. Arch. Biochem. Biophys. 435: 182 – 189. 107.Saiyed, Z. M., Sharma, S., Godawat, R., Telang, S. D. and Ramchand, C. N. 2007. Activity and stability of alkaline phosphatase (ALP) immobilized onto magnetic nanoparticles (Fe3O4). J. Biotechnol. 131: 240 – 244. 108.Sanghi, R. and Verma, P. 2009. Biomimetic synthesis and characterisation of protein capped silver nanoparticles. Bioresour. Technol. 100: 501 – 504. 109.Santra, S., Tapec, R., Theodoropoulou, N., Dobson, J., Hebard, H. and Tan, W. H. 2001. Synthesis and characterization of silica-coated iron oxide nanoparticles in microemulsion: the effect of nonionic surfactants. Langmuir 17: 2900 – 2906. 110.Schuster, M., Aaviksaar, A., Haga, M. and Jakubke, H. D. 1990. Enzyme-catalyzed peptide synthesis in ice. Tetrahedron 46: 8093 – 8102. 111.Sergeeva, M. V., Paradkar, V. M. and Dordick, J. S. 1997. Peptide synthesis using proteases dissolved in organic solvents. Enzyme Microb. Technol. 20: 623 – 628. 112.Serralheiro, M. L. M., Prazeres, D. M. F. and Cabral, J. M. S. 1994. Dipeptide synthesis and separation in a reversed micellar membrane reactor. Enzyme Microb. Technol. 16: 1064-1073. 113.Serralheiro, M. L. M., Prazeres, D. M. F. and Cabral, J. M. S. 1999. Continuous production and simultaneous precipitation of a dipeptide in a reversed micellar membrane reactor. Enzyme Microb. Technol. 24: 507-513. 114.Shaw, S. -Y., Chen, Y. -J., Ou, J. -J. and Ho, L. 2006. Preparation and characterization of Pseudomonas putida esterase immobilized on magnetic nanoparticles. Enzyme Microb. Technol. 39: 1089 – 1095. 115.Shen, H. Y., Tian, G. L., Ye, Y. H. and Wang, J. 2005. Non-coded amino acids as acyl donor substrates for peptide bond formation catalyzed by thermoase in toluene. J. Mol. Catal. B-Enzym. 37: 26 – 29. 116.Suslick, K. S., Fang, M. and Hyeon, T. 1996. Sonochemical synthesis of iron colloids. J. Am. Chem. Soc. 118: 11960 – 11961. 117.Sweeney, P. J. and Walker, J. M. 1993. Proteolytic Enzymes for Peptide Production. p. 277-281. Methods in Molecular Bipology. Vol. 16. Burreli, M. M. ed. Humana Press, Totowa, NJ. 118.Tang, Z. -X., Qian, J. -Q. and Shi, L. E. 2006. Characterizations of immobilized neutral proteinase on chitosan nano-particles. Process Biochem. 41: 1193 – 1197. 119.Tanyolac, D. and Ozdural, A. R. 2001. BSA adsorption onto magnetic polyvinylbutyral microbeads. J. Appl. Polym. Sci. 80: 707 – 715. 120.Tardioli, P. W., Sousa, Jr. R., Giordano, R. C. and Giordano, R. L. C. 2005. Kinetic model of the hydrolysis of polypeptides catalyzed by AlcalaseR immobilized on 10% glyoxyl-agarose. Enzyme Microb. Technol. 36: 555 – 564. 121.Taylor, M. D. and Amidon, G. L. 1995. Peptide-Based Drug Design: Controlling Transport and Metabolism, American Chemical Society, Washington, D.C. 122.Thomson, D. 1982. Response surface experimentation. J. Food Process Preserv. 6: 155 – 188. 123.Thust, S. and Koksch, B. 2003. Protease-catalyzed peptide synthesis for the site-specific incorporation of α-fluoroalkyl amino acids into peptides. J. Org. Chem. 68: 2290 – 2296. 124.Tong, X. D., Xue, B. and Sun, Y. 2001. A novel magnetic affinity support for protein adsorption and purification. Biotechnol. Prog. 17: 134-139. 125.Tougu, V., Meos, H., Haga, M., Aaviksaar, A. and Jakubke, H. D. 1993. Peptide synthesis by chymotrypsin in frozen solutions. Free amino acids as nucleophiles. FEBS Lett. 329: 40 – 42. 126.Trusek-Holownia, A. 2008. Production of protein hydrolysates in an enzymatic membrane reactor. Biochem. Eng. J. 39: 221-229. 127.Unen, D. J., Engbersen, J. F. J. and Reinhoudt, D. N. 1998. Large acceleration of α-chymotrypsin-catalyzed dipeptide formation by 18-crown-6 in organic solvents. Biotechnol. Bioeng. 59: 553 – 556. 128.Vabeno, J., Lejon, T., Uhd Nielsen, C., Steffansen, B., Chen, W., Ouyang, H., Borchardt, R.T. and Luthman, K., 2004. Phe-Gly Dipeptidomimetics designed for the di-/tripeptide transporters PEPT1 and PEPT2: Synthesis and biological investigations. J. Med. Chem. 47: 1060 – 1069. 129.Vigneaud, V. D., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P. G. and Gordon, S. 1953. The synthesis of an octapeptide amide with the hormonal activity of oxytocin. J. Am. Chem. Soc. 75: 4879 – 4880. 130.Wa, S. R., Huang, J. S. and Yan, H. S. 2006. Size-controlled preparation of magnetite nanoparticles in the presence of graft copolymers. J. Mater. Chem. 16: 298 – 303. 131.Wandrey, C. and Wichmann, R. 1987. Production of L-amino acids in the membrane reactor. Biotechnol. 1: 85 – 92. 132.Wang, S., Tan, Y., Zhao, D. and Liu, G. 2008. Amperometric tyrosinase biosensor based on Fe3O4 nanoparticles – chitosan nanocomposite. Biosens. Bioelectron. 23: 1781 – 1787. 133.Wang, X., Chen, C., Liu, H. and Ma, J. 2008. Preparation and characterization of PAA-PVDF membrane-immobilized Pd-Fe nanoparticles for dechlorination of trichloroacetic acid. Water Res. 42: 4656 – 4664. 134.Wang, Y., Wang, X., Luo, G. and Dai, 2008. Y. Adsorption of bovin serum albumin (BSA) onto the magnetic chitosan nanoparticles prepared by a microemulsion system. Bioresour. Technol. 99: 3881-3884. 135.Woo, K., Hong, J., Choi, S., Lee, H., Ahn, J., Kim, C. S. and Lee, S. W. 2004. Easy synthesis and magnetic properties of iron oxide nanoparticles. J. Mater. Chem. 16: 28143 – 218. 136.Wu, Y., Wang, Y., Luo, G. and Dai, Y. 2009. In situ preparation of magnetic Fe3O4-chitosan nanoparticles for lipase immobilization by cross-linking and oxidation in aqueous solution. Bioresour. Technol. 100: 3459-3464. 137.Xia, Y. Q., Guo, T. Y., Song, M. D., Zhang, B. H. Zhang, B. L. 2006. Selective separation of quercetin by molecular imprinting using chitosan beads as functional matrix. React. Funct. Polm. 66: 1734-1740. 138.Xie, W. and Ma, N. 2009. Immobilized lipase on Fe3O4 nanoparticles as biocatalyst for biodiesel production. Energy Fuels. 23: 1347-1353. 139.Xing, G. W., Li, F. Y., Ming, C. and Ran, L. N. 2007. Peptide bond formation catalyzed by R-chymotrypsin in ionic liquids. Tetrahedron Lett. 48: 4271 – 4274. 140.Yamaura, M., Camilo, R. L., Sampaio, L. C., Maced, M. A., Nakamura, M. And Toma H. E. 2004. Preparation and characterization of (3-aminopropoyl) triethoxysilane-coated magnetite nanoparticles. J. Magn. Magn. Mater. 279: 210-217. 141.Yokozeki, K. and Hara, S. 2005. A novel and difficient enzymatic method for the production of peptides from unprotected starting materials. J. Biotechnol. 115: 211 – 220. 142.Yong, Y., Bai, Y., Li, Y., Lin, L., Cui, Y. and Xia, C. 2008. Preparation and application of polymer-grafted magnetic nanoparticles for lipase immobilization. J. Magn. Magn. Mater. 320: 2350 – 2355. 143.Yu, S. H., Fujino, T. and Yoshimura, M. 2003. Hydrothermal synthesis of ZnFe2O4 ultrafine particles with high magnetization. J. Magn. Magn. Mater. 256: 420-424. 144.Zhang, X. Z., Wang, X., Chen, S., Fu, X., Wu, X. and Li, C. 1996. Protease-catalyzed small peptide synthesis in organic media. Enzyme Microb. Technol. 19: 538 – 544.