Enzymatic Synthesis of Short Peptides in Heterogeneous Mixtures of Substrates

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Oligopeptides are becoming increasingly important in view of their biological activities, which include antibiotic, hormonal, enzyme inhibitory, immunomodulating, and sensory properties.[1] Enzyme-catalyzed peptide synthesis, with its well-documented advantages,[2] has attracted much interest as an alternative to conventional chemical methodologies.[3] However, in spite of significant advances made through the application of enzymes in aqueous organic solvent and especially low-water organic-solvent-based media[4] and the successful synthesis of a number of oligopeptides,[5] the synthetic usefulness of this approach still remains rather limited. To a large extent this is due to the low solubility of many amino acid (AA) derivatives in those organic solvents which are regarded as being most suitable for the application of enzymes,[6] resulting in significantly lower productivities as compared to solution-phase chemical methodologies. This communication demonstrates that this inherent limitation can largely be avoided by performing the reaction in a heterogeneous substrate mixture.

Initially, the subtilisin Carlsberg-catalyzed syntheses of the model dipeptides L-Phe·AANH_2 from L-PheOEt and L-AANH_2 were studied in organic solvents in which the acyl acceptors L-AANH_2 were only sparingly soluble. Thus, using equimolar substrate concentrations of 0.25 M, dipeptide yields of 32 and 33%, 30 and 29%, and 24 and 27% were obtained in dichloromethane and ethyl acetate using L-LeuNH_2, L-MetNH_2, and GlyNH_2 as acyl acceptors, respectively. It was found that neither the kinetics nor the yields were significantly affected when the syntheses were performed in heterogeneous suspensions as compared to homogeneous solutions (Figure 1).[7] Furthermore, it was possible to totally dispense with the requirement for a bulk solvent and to perform the reactions in equimolar mixtures of substates consisting of solid L-AANH_2 dispersed in liquid L-PheOEt. Under these conditions, immobilized subtilisin catalyzed the above reactions, and dipeptide yields of 83, 75, and 36% were obtained using L-LeuNH_2, L-MetNH_2, and GlyNH_2 as acyl acceptors, respectively. Using this methodology, productivities of 0.36–0.81 g/g of reaction mixture were obtained for these dipeptides, as compared to 0.015–0.030 g/g typically obtained in solution or suspension, even at substrate concentrations as high as 0.25 M.

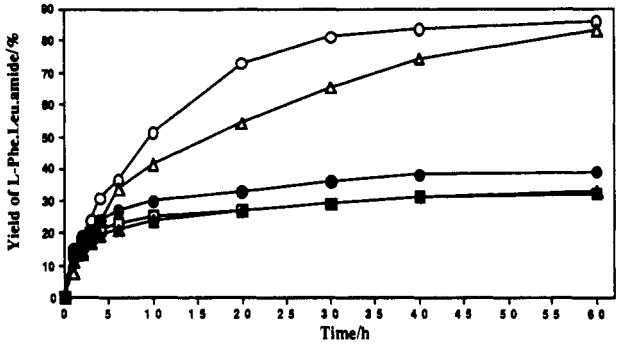

Figure 1. Enzymatic synthesis of L-Phe·LeuNH_2 catalyzed by immobilized subtilisin (Table I, note a). Homogeneous: ▲, 15 mM substrates in dichloromethane containing 0.25% v/v water; ●, 100 mM substrates in acetonitrile containing 3.5% v/v water. Heterogeneous: □, 250 mM substrates in dichloromethane containing 0.5% v/v water, with L-LeuNH_2 largely suspended; △, equimolar mixture of substrates, no solvent added; ○, equimolar mixture of substrates containing 10% w/w of triethyleneglycol dimethyl ether (TGDME).

We then investigated the more general situation where both substrates were solids in the pure state. It was found once again that immobilized proteases catalyzed the required reactions in equimolar mixtures of the substrates, provided that the substrates formed eutectic mixtures[8] or that small amounts of organic adjuvants[8] were added. A wide range of inert organic liquids could be used as adjuvants, including hydrocarbons, alcohols, ketones, alkyl and aryl esters, linear polyethers, alkoxyalkyl esters, and polyols. In order to establish whether catalysis took place in the liquid or the solid state, the subtilisin- and α-chymotrypsin-catalyzed synthesis of *N*-TFAc-L-Tyr·GlyNH_2 from *N*-TFAc-L-TyrOEt and GlyNH_2 was studied. Both of these substrates were solids, and mixtures of these did not form eutectics. In the absence of adjuvant, no reaction was observed, whereas upon the addition of 30% w/w of 2-methoxyethyl acetate, dipeptide yields of 36 and 55% were obtained for subtilisin and α-chymotrypsin, respectively, indicating that liquid-phase catalysis was involved.[9]

Finally, the synthetic utility of this methodology was confirmed

(1) (a) Lipmann, F. *Acc. Chem. Res.* **1973**, *6*, 361. (b) Wolfe, S.; Demain, A. L.; Jensen, S. K.; Westlake, D. W. S. *Science* **1984**, *226*, 1386. (c) Steward, M. W.; Howard, C. R. *Immunol. Today* **1987**, *8*, 51. (d) Smith, J. A.; Pease, L. G. *Crit. Rev. Biochem.* **1980**, *8*, 315.

(2) (a) West, J. B.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1986**, 117. (b) Wong, C.-H.; Matos, J. R.; West, J. B.; Barbas, C. F. Developments in Industrial Microbiology, Suppl. 3. *J. Indust. Microb.* **1988**, *29*, 171. (c) Fruton, J. S. *Adv. Enzymol.* **1982**, *53*, 239. (d) West, J. B.; Wong, C.-H. *Tetrahedron Lett.* **1987**, *28*, 1629. (e) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272. (f) Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802. (g) Kullmann, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL, 1987. (h) Fruton, J. S. *Advances in Enzymology*; Wiley: New York, 1982; Vol. 53, p 239. (i) Jakubke, H.-D.; Kuhl, P.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85. (j) Morihara, K. *Trends Biotechnol.* **1987**, *5*, 164. (k) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 453. (l) Nakanishi, K.; Kimura, Y.; Matsuno, R. *Synthetic Peptides in Biotechnology*; Alan R. Liss Inc.: New York, 1988; p 173.

(3) (a) Barany, G.; Merrifield, R. B. *The Peptides*; Academic Press: New York, 1982; Vol. 2, p 1. (b) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957.

(4) (a) Klibanov, A. M. *Trends. Biochem. Sci.* **1990**, *14*, 141; *Acc. Chem. Res.* **1990**, *23*, 114. (b) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259. (c) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194. (d) Schwarz, A.; Steinke, D.; Kula, M.-R.; Wandrey, C. *Biotechnol. Appl. Biochem.* **1990**, *12*, 188.

(5) (a) Gill, I.; Vulfson, E. N. *J. Chem. Soc., Perkin Trans. 1* **1992**, *6*, 667. (b) Kullmann, W. *Proc. Nat. Acad. Sci. U.S.A.* **1982**, *79*, 2840. (c) Kayahara, H.; Ohashi, A.; Tadasa, K.; Kato, S. *Agric. Biol. Chem.* **1990**, *54*, 1325. (d) Isowa, Y.; Ohmori, M.; Sato, M.; Mori, K. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2766. (e) Takai, H.; Sakata, K.; Nakamizo, N.; Isowa, Y. *Peptide Chemistry*; Protein Research Foundation: Osaka, Japan, 1981; p 213. (f) Clapes, P.; Mata-Alvarez, J. *J. Chem. Technol. Biotechnol.* **1989**, *45*, 191.

(6) (a) Akiyama, A.; Bednarski, M.; Kim, M.-J.; Simon, E. S.; Waldham, H.; Whitesides, G. M. *CHEMTECH* **1988**, 627. (b) Khmelnitsky, Y. L.; Levashov, A. V.; Klyachko, N. L.; Martinek, K. *Enzyme Microb. Technol.* **1988**, *10*, 710.

(7) The solubilities of the amides in dichloromethane and ethyl acetate were below 25 mM, whereas L-PheOEt was freely soluble in these solvents.

(8) The term eutectic[11] refers to a low-melting-point mixture obtained on mixing two or more components together. The term adjuvant refers to organic liquids added in small quantities in order to effect dispersion of substrates and to modify the physical properties of the reaction medium. In the present case, eutectics which were semiliquid at room temperature were obtained upon mixing the substrates together or upon the addition of small quantities of adjuvants, and the immobilized proteases readily catalyzed peptide synthesis in these mixtures. In some cases, eutectics were not formed even upon the addition of adjuvants, and lower peptide yields of 30–45% were obtained using such dispersions of substrates in up to 60% w/w of the adjuvant.

(9) In addition, no reactions were observed in mixtures below their eutectic temperatures.

(10) Vulfson, E. N.; Ahmed, G.; Gill, I.; Goodenough, P. W.; Kozlov, I. A.; Law, B. A. *Biotechnol. Lett.* **1990**, *12*, 597.

(11) Murrell, J. N.; Boucher, E. A. *Properties of Liquids and Solutions*; Wiley: Chichester, U.K., 1982.

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Table I. Protease–Catalyzed Synthesis of Oligopeptides

acyl donor	acyl acceptor	product[a]	scale (mmol)	yield (%)	mp (°C)	$[\alpha]^{20}$ [b]	FAB-MS $(M + H)_{obsvd}$[c]	FAB-MS $(M + H)_{calcd}$
PheOEt	L-LeuNH$_2$	**1**	2.6	73	125–126	−22.9	278.1870	278.1868
PheOEt	D-LeuNH$_2$	**2**	2.6	77	116–118	+43.0	278.1867	278.1868
PheOEt	L-MetNH$_2$	**3**	2.6	64	148–149	−12.8	296.1421	296.1433
N-CBZ-TyrOEt	L-LeuNH$_2$	**4**[d]	1.46	56	195–197	−15.0	428.2196	428.2185
N-CBZ-TyrOEt	D-LeuNH$_2$	**5**[d]	1.46	61	120–122	+23.5	428.2191	428.2185
N-CBZ-TyrOEt	L-AlaOBn	**6**[d]	1.46	52	159–161	−13.5	477.2010	477.2025
N-Bnd-PheOEt	L-LeuNH$_2$	**7**[e]	1.42	43	(semisolid)	−14.0	366.2216	366.2181
N-Ac-TyrOEt	Gly-GlyOEt	**8**[f]	3.10	44	195–196	+31.9	366.1693	366.1665
N-TFAc-TyrOEt	Gly-GlyOEt	**9**[f]	3.10	38	173–175	+21.8	420.1396	420.1382
N-CBZ-TyrOEt	Gly-GlyOEt	**10**[f]	3.10	45	169–171	+8.3	458.1941	458.1927

[a] Preparations: equimolar quantities of acyl donor and free base of acyl acceptor (**1–7**) or hydrochloride of acyl acceptor (**8–10**) were mixed together. *N,N*-Diisopropylethylamine, adjuvant, and water were then added to the substrate mixture as required (see below). α-Chymotrypsin (**6**, **8–10**) or subtilisin Carlsberg (**1–5**, **7**) adsorbed on Celite at a loading of 40 mg/g and 50 mg/g, respectively, as previously described,[10] was added to a final concentration of 200 mg/g, and the mixture was incubated in an open vial at 37 °C for 60 h. Products were purified by reverse-phase low-pressure liquid chromatography on Sorbsil RP18/C200 using a methanol–water gradient. Satisfactory elemental analyses (±0.6% for C, H, N) were obtained for all products. In addition, all products were fully characterized by 400-MHz ^{1}H NMR and 100-MHz ^{13}C NMR spectroscopy. [b] c = 1.0, MeOH. [c] 8 keV Xe, glycerol matrix. [d] 10% w/w of TGDME was added to the reaction mixture. In the absence of adjuvant, yields of 47, 53, and 44% were obtained for **4**, **5**, and **6**, respectively. [e] 5% w/w of TGDME was added to the reaction mixture. A yield of 35% was obtained in the absence of adjuvant. [f] 15% w/w of TGDME, 6% w/w of water, and 1 equiv of *N,N*-diisopropylethylamine was added to the reaction mixture. In the absence of adjuvant, yields of 38, 30, and 33% were obtained for **8**, **9**, and **10**, respectively. In the absence of water, yields were below 10%. Full experimental details are available in the supplementary material.

by the preparation of a range of di- and tripeptides, including the N-terminal and C-terminal fragments of enkephalin, in overall yields of 38–77% (Table I). In conclusion, this work clearly demonstrates that subtilisin and α-chymotrypsin can be successfully used for synthetic purposes in heterogeneous mixtures of substrates. This methodology offers a high productivity, approaching the theoretical maximum, together with the high specificity associated with biocatalysis.

Supplementary Material Available: Experimental procedures and characterization data for compounds **1–10**; photographs showing equimolar mixtures of *N*-TFAc-L-TyrOEt and GlyNH$_2$ (Plate A) and *N*-TFAc-L-TyrOEt and GlyNH$_2$ with 30% w/w 2-methoxyethyl acetate (Plate B); Figure 1, showing the ratioed absorbance spectra of the eutectic liquid phase at selected time intervals (12 pages). Ordering information is given on any current masthead page.