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USE OF NONPROTEASES IN PEPTIDE SYNTHESIS

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Summary: Practical procedures have been developed for synthesis of peptides using lipases and esterases as catalysts.

We describe here a new strategy for enzymatic peptide synthesis using an esterase without amidase activity as a catalyst and N-acyl amino acid esters and amino acid esters as substrates in ether or ethyl acetate containing 5-10% aqueous buffer or in pyridine as solvent.

A number of proteases have been used as practical catalysts in peptide synthesis[1], particularly in synthesis of small peptides. Their applications so far, however, are still quite limited because of three major problems: first, the peptides formed could be hydrolysed again by the same enzymes; second, many peptides containing unusual amino acids cannot be prepared by this strategy as proteases generally accept only L-amino acids (although there are some exceptions[2]); third, proteases are generally not stable in anhydrous environments where the reaction would give peptides in high yield in a kinetically or thermodynamically controlled approach. In order to overcome these drawbacks, we have initiated a study of enzymes without amidase activity as catalysts for peptide synthesis. Like protease-catalysed hydrolysis of esters or amides, many esterase-catalysed reactions proceed through an acyl-enzyme intermediate followed be deacylaction with water or other nucleophiles. Porcine pancreatic lipase (PPL)[3], *Candida cylindracea* lipase (CCL), and pig liver esterase[3b] are such enzymes, all being used as catalysts for kinetic resolution of alcohols, acids, and esters[4]. The lipases are also active and stable in very high concentrations of organic solvent[5]. These acyl-enzyme forming catalysts could be useful in peptide synthesis, if they would accept amines as a nucleophile during the deacylation step.

Table 1 summarizes experiments with the enzymes under various conditions. In general, N-protected amino acid methyl esters are poor substrates for the lipases.

We suspect that the high yields in the first two entries may be due to protease contaminants of crude PPL used in these experiments, as the results of the first five experiments exactly parallel what would be expected had chymotrypsin been the catalyst (see Ref. 2). From experiments on the hydrolysis of free amino acid esters by PPL and CCL, we determined the esters of longer, unbranched alcohols to be better substrates for hydrolysis (unpublished results), and that these esters might be better acyl donors. Such acyl donors were found to be ineffective in a biphasic reaction system (as in condition A, Table 1), but in pyridine, peptide synthesis could be seen to occur, albeit slowly (entries 7-10). It was thought that designing an acyl donor more closely resembling the natural substrate (triglyceride) might increase efficiency. Amino acid-1-glyceryl esters also have the added advantage of increasing the water solubility of the acyl donor, thus increasing the concentration of the donor at the enzyme active site.

N-protected amino acid glyceryl esters were derived from the respective solketal (1,2-glycerol acetonide) esters. In a typical synthesis, 10 mmol of Z-L-Phe-OH was dissolved in CH_2Cl_2, to which a catalytic amount of dimethyl-amino-pyridine and 2 equivalents of solketal were added. The reaction mixture was cooled to -10°C, and 1.1 equivalents of DCC were added. The reaction was allowed to continue overnight, and then was quenched by the addition of acetic acid. After filtration of the DCU and washing of the organic repeatedly with 0.1M Na_2CO_3, drying, then removal of the solvent afforded the solketal ester as an oil in 66-87% yield. The acetonide function was removed by dissolving the ester in MeCN/1N HCL (4:1, v/v) and stirring for 8 hours. The solvent was removed and the residue taken up in ethyl acetate, washed quickly with cold 0.1M Na_2CO_3, the solvent dried, then removed, affording the glyceryl ester in nearly quantitative yield.

We have found that entrapment of the lipases in Amberlite XAD-8 improved reaction times. In a typical procedure, 300 mg of lipase was dissolved in 2 ml 0.1M phosphate buffer (pH8). XAD-8 was added to form a thick slurry and allowed to sit for one hour, then filtered. Conditions for peptide syntheses are summarized in Table 1.

In experiments with PLE, we found the free enzyme to be a poor catalyst for synthesis. Only low amounts of the enzyme (< 5 ug confined to 0.1 ml of buffer in a biphasic system) was at all effective, and then the reaction would stop with only 10% of the acyl donor reacted, presumably due to interfacial denaturation of the enzyme. Covalent attachment of the enzyme to Eupergit C^6, however provided an effective, if slow catalyst.

We believe we have shown that these enzymes have the potential for being effective catalysts for synthesis of peptides containing usual and unusual amino

acids. N-phenylacetyl-L-cysteinyl-D-valine, for example, can be converted to penicillin G directly catalysed by a cyclase[7].

TABLE 1. NONPROTEASE CATALYSED PEPTIDE SYNTHESES.

#	Acyl Donor(M)	Acyl Acceptor(M)	Conditions (Enzyme)	Yield(%) (Rxn. Time)
1	Z-L-Phe-OMe(.5)	L-Ala-OBu^s(1.5)	A (PPL)	85 (16H)
2	Z-L-Phe-OMe(.5)	D-Ala-OBu^s(1.5)	A (PPL)	60 (16H)
3	Z-D-Phe-OMe(.5)	L-Ala-OBu^s(1.5)	A (PPL)	<10 (16H)*
4	Z-D-Phe-OMe(.5)	D-Ala-OBu^s(1.5)	A (PPL)	<10 (16H)*
5	Z-Gly-OMe (.5)	L-Ala-OBu^s(1.5)	A (PPL)	<1 (10D)*
6	Z-Gly-OOct (.5)	L-Ala-OBu^s(1.5)	A (PPL)	<1 (10D)*
7	For-Gly-OOct(.5)	Gly-PhH	B (PPL)	20 (5D)*
8	For-Gly-OOct(.5)	Gly-PhH	B (CCL)	26 (5D)*
9	Vl-Gly-OOct(.5)	Gly-PhH	B (PPL)	48 (5D)*
10	Vl-Gly-OOct(.5)	Gly-PhH(1.5)	B (CCL)	60 (5D)*
11	Z-L-Phe-Glc(0.1)	Glyal(0.4)	C (CCL)	19 (16H)*
12	Z-D-Phe-Glc(0.1)	Glyal(0.4)	C (CCL)	42 (16H)*
13	Z-L-Phe-Glc(0.1)	L-Ala-OPr^i(0.4)	C (CCL)	24 (3D)
14	Z-L-Phe-Glc(0.1)	D-Ala-OPr^i(0.4)	C (CCL)	22 (3D)
15	Z-Gly-Glc(0.1)	Glyal(0.4)	C (CCL)	55 (16H)
16	Z-L-Pro-Glc(0.1)	Glyal(0.4)	C (CCL)	7 (14D)*
17	Z-L-Tyr-OMe(0.2)	L-Met-NH_2,HCl(0.8)	D (PLE)	11 (16H)*
18	Z-L-Tyr-OMe(0.2)	L-Met-NH_2,HCl(0.8)	E (PLE)	66 (2W)
19	Pha-L-Cys(SSBu^t)OMe(0.1)	D-Val-Bzl(0.3)	A (CCL)	50 (3D)[8]

CONDITIONS. A:Ether, 10% 0.1M phosphate buffer (pH 8.0), 50mg enzyme. B: Pyridine, 1% H_2O, 50 mg enzyme. C:EtOAc, enzyme absorbed to XAD-8 (65 mg/1g absorbant), 400 mg absorbed enzyme. D: Ether, 10% phosphate buffer (pH 8.0), 3 mg soluble enzyme. E: Same as D, but enzyme attached to Eupergit C (19 mg/ 1g polymer). 200 mg of enzyme/polymer used.
NOTES. All reaction volumes 2 ml, and performed at room temperature. * Indicates not all of starting acyl donor reacted. All yields determined by reversed phase HPLC, conditions previously reported[2]. Unless otherwise stated, all amino acids used as free amines.
ABBREVIATIONS. Z, carbobenzyloxy; Bu^s, sec-butyl; Oct, n-octyl; PhH, phenyl-hydrazide; For, N-formyl; Vl, N-Valeroyl; Glc, 1-glyceryl ester; Glyal, glycinal dimethyl acetal; Pr^i, iso-propyl; Pha, phenylacetyl; Bzl, benzyl; SBu^t, tert-butyl mercapto.

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