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ENZYME-CATALYZED PEPTIDE SYNTHESIS IN BIPHASIC AQUEOUS-ORGANIC SYSTEMS

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SUMMARY: A preparative protease-catalyzed peptide synthesis in biphasic aqueous-organic systems is described. This approach using both free as well as immobilized proteases as catalysts provides high yields and includes the possibility to re-utilize the enzymes.

In 1937 Bergmann and Fraenkel-Conrat[1] were able to show that papain can be used for catalyzing amide bond formation. Since that time, numerous papers have appeared concerning the reversal of enzymic peptide hydrolysis[2]. Especially, during the last four years there has been a revival of protease-catalyzed peptide synthesis obviously caused by the interest in an alternative to chemical methods[3-5]. To guarantee a sufficient solubility of the substrates, most enzymic syntheses have been carried out in buffered homogeneous aqueous-organic mixtures. Furthermore, Homandberg et al.[6] have shown that the addition of organic cosolvents shifts peptide bond equilibria toward synthesis. There is no doubt that the use of solvents which are miscible with water will significantly decrease the catalytic activity of the proteases. Caused by these arguments and reports from other fields of enzyme-catalyzed syntheses[7,8] we have studied the influence of water-immiscible organic solvents on the protease-catalyzed peptide synthesis. Due to the minimum contact with the organic solvent the protease will not be damaged by the nonaqueous solvent. On the other hand, the solvent should promote the phase-transfer of the substrates to the enzyme.

Enzymic syntheses were carried out at room temperature in a buffer-organic heterogeneous system using Leu-NH_2 in all cases as amino component and different N-protected peptide derivatives as carboxyl components. The precipitate formed during α-chymotrypsin-catalyzed coupling (Table 1) was

washed on a sintered glass filter with water, 1 M HCl, water, 5% $NaHCO_3$, water and, if necessary, with small amounts of the organic solvent. After the purity of the peptide had been checked by t.l.c., identification was made in usual manner. All elemental analyses were within acceptable limits.

Table 1: α-Chymotrypsin-catalyzed peptide synthesis in aqueous-organic solvent systems[a)]

Donor ester	Organic solvent %(v/v)		Enzyme (mol)	Time (min)	Yield (%)
Ac-Leu-Phe-OMe	dichloromethane	75	0.05	20	79
Ac-Leu-Phe-OMe	1,2-dichloroethane	75	0.05	20	82
Ac-Leu-Phe-OMe	trichloroethylene	75	0.05	20	84
Ac-Leu-Phe-OMe	carbon tetrachloride	75	0.05	20	81
Ac-Leu-Phe-OMe	ethyl acetate	75	0.05	20	72
Ac-Leu-Phe-OMe	nitromethane	75	0.05	20	79
Ac-Leu-Phe-OMe	dimethylformamide	30	0.20	20	40
Z-Ala-Phe-OMe	carbon tetrachloride	50	0.30	90	72
Z-Ala-Phe-OMe	trichloroethylene	50	0.20	15	83
Boc-Leu-Phe-OMe	petroleum ether/trichloroethylene (2:1)	60	0.60	120	86
Boc-Ala-Phe-OMe	" "	60	0.60	120	76
Boc-$(Gly)_2$-Phe-OMe	" "	60	0.60	60	60

a) The reaction mixture contained 0.2 mmol donor ester, 0.2 mmol Leu-NH_2 and 0.2 M $Na_2CO_3/NaHCO_3$ buffer (pH 10) in a total volume of 2 ml for Ac-Leu-Phe-OMe and 1 ml for Z-Ala-Phe-OMe. For the tert.-butyloxycarbonyl-peptide esters a 10-fold excess of Leu-NH_2 in a total volume of 2.5 ml was used. Crystalline α-chymotrypsin was purchased from SPOFA (Prague).

In comparison with the good results obtained in the synthesis of Ac-Leu-Phe-Leu-NH_2 using water-immiscible organic solvents, the yield of the reaction in the presence of dimethylformamide was rather low. Especially, tert.-butyloxycarbonyl-peptide methyl esters could be coupled with success using chymotrypsin as a catalyst in the biphasic aqueous-organic system. To the best of our knowledge, chymotrypsin-catalyzed syntheses with such Boc-derivatives have not yet been reported in the literature.

Furthermore, we could establish that immobilized chymotrypsin catalyzes peptide bond formation with better results in a medium containing high concentrations of water-immiscible organic solvents than in the presence of methanol

or dimethylformamide as cosolvents (Table 2). This technique using insoluble catalysts to accelerate aqueous-organic phase reactions shows considerable potential for practical application.

Table 2: Peptide synthesis using immobilized α-chymotrypsin[b)]

Donor ester	Organic solvent % (v/v)		Total volume (ml)	Silica-bound enzyme (mg)	Time (h)	Yield (%)
Ac-Leu-Phe-OMe	CH_2Cl_2	60	2.5	100	1.5	64
Ac-Leu-Phe-OMe	CH_2Cl_2	60	2.5	125	1.5	75
Boc-Leu-Phe-OMe	CH_3OH	36	2.2	150	2	10
Boc-Leu-Phe-OMe	CCl_4	50	2.0	200	4	36
Z-Ala-Phe-OMe	DMF	35	2.0	150	2	31
Z-Ala-Phe-OMe	CCl_4	56	2.7	200	2	70

b) The reaction mixture contained 0.2 mmol donor ester, 0.2 mmol Leu-NH_2 and 0.2 M Na_2CO_3/$NaHCO_3$ buffer (pH 10) in a total volume indicated in the table. Chymotrypsin was immobilized on γ-succinamidopropyl silica (macroporous; pore diameter 20 nm) via the N-hydroxysuccinimid ester method. The content of chymotrypsin was 20 mg/g silica. The reaction mixture was stirred at room temperature. After filtration the peptide was removed from the silica-supported enzyme by a short treatment with methanol.

In contrast to our results Vann and Weetall[9] were not successful in peptide synthesis using protected amino acid substrates and immobilized enzyme in 78 % (v/v) ethanol.

One of the main advantages of our approach is the possibility to re-utilize the enzyme for further coupling reactions after simple phase separation. In the synthesis of the model peptide Ac-Leu-Phe-Leu-NH_2 we were able to show that with both the buffer phase of free chymotrypsin as well as the immobilized enzyme using the biphasic solvent system 0.2 M Na_2CO_3/$NaHCO_3$ buffer - 75 % (v/v) dichloromethane the yield decreased only by about 5 % after two re-utilization experiments.

Generally, the use of biphasic aqueous-organic solvent systems should be applicable to other protease-catalyzed coupling reactions. Table 3 indicates first results using papain as a catalyst in the syntheses of some model peptides. In the biphasic system 0.2 M McIlvaine buffer (pH 5.5) - carbon

tetrachloride and ethyl acetate, respectively, the yields obtained are significantly higher than those in methanol.

Table 3: Papain-catalyzed peptide synthesis in aqueous-organic systems[c)]

Solvent % (v/v) \ C-Component	Ac-Leu-Phe-OH	Boc-Leu-Phe-OH	Boc-Phe-Leu-OH	Boc-Ile-Phe-OH
	Yield of peptide amide (%)			
CH_3OH 15	25	30	56	21
$CH_3COOC_2H_5$ 25	49	61	73	61
CCl_4 25	60	78	71	43

c) The reaction mixture (2 ml) contained 0.2 mmol of the C-component, 0.2 mmol Leu-NH_2, 50 mg papain (50 nkat/mg), 0.1 ml mercaptoethanol and 0.2 M McIlvaine buffer (pH 5.5). The reaction mixture was stirred at 30°C for 20 h. Purification and identification was made as described above.

Studies on optimization of the preliminary results, the extension to other proteases and scaling-up experiments are in progress.

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