Peptide Synthesis Catalyzed by Chymotrypsin in Organic Solvents

J. M. Ricca and D. H. G. Crout *
Department of Chemistry, University of Warwick, Coventry CV4 7AL

When suspended in an organic solvent, α-chymotrypsin catalyzed peptide bond formation. Dipeptides containing D-amino acid components have been prepared. The importance of the water content of the system has been investigated.

Recently, great interest has been shown in the use of proteases to catalyse peptide bond formation.[1] The drawbacks inherent to catalysis in an aqueous environment (unfavourable thermodynamic equilibrium, narrow substrate specificity, and undesirable proteolysis of the peptide) have been overcome by the use of proteases in biphasic aqueous organic mixtures,[2] reverse micelles,[3] or chemically modified enzymes in organic solvents.[4] However, recently it has been found that enzymes can be catalytically active in anhydrous organic solvents and, under these conditions, show a new range of properties, *e.g.* relaxed stereoselectivity and modified substrate specificity.[5] Although the use of chymotrypsin as a suspension in organic solvents for esterification[6] and transesterification,[5c] is well documented, the enzyme has never been used to catalyze peptide bond formation under these conditions.

In the present study based on an initial observation by Dastoli *et al.*,[7] we show that chymotrypsin is catalytically active in a wide range of hydrophobic solvents and that it can be used to synthesize peptides in high yields by aminolysis of esters.

In a typical experiment, to a solution of the protected L-amino acid ester and the amino acid amide (40mM; 1:1 molar ratio) in anhydrous dichloromethane (distilled over calcium hydride immediately prior to use) was added 1 mg/ml of chymotrypsin, followed by the addition of 0.25% (v/v) of water. The resulting suspension was then stirred at room temperature for a certain period of time. In all cases investigated, the desired compound precipitated during the course of the reaction. The solvent was then evaporated under reduced pressure, the residue was thoroughly washed with water, and the product was recrystallized from hot methanol. This procedure was carried out on a 0.4—4 mmol scale. No activity was found with less than 0.2% of water, indicating that a minimal amount of water is absolutely essential for enzymatic catalysis. This essential water presumably allows the enzyme to maintain its native conformation, as well as sufficient flexibility, and hence to retain its catalytic activity.[5b,d]. In no case was any peptide formation detected in the absence of the enzyme. Under the aforementioned conditions, no hydrolysis of the final peptide was observed, indicating that the added water is not available for hydrolysis but is tightly bound to the enzyme. To examine specificity at the P'_1 position (notation of Schechter and Berger[8]), experiments were performed using various amino acid derivatives as nucleophiles. The results are summarized in Table 1.

Hydrophobic and bulky amino acid amides as nucleophiles were found to be suitable substrates for peptide synthesis, but the imino acid derivative, L-prolinamide, was not accepted as a substrate under any of the conditions tested. It is worth noting that amino acid esters (*e.g.* methyl L-leucinate) were not readily accepted as nucleophiles. It was also found that tyrosine derivatives were far the best substrates for the enzyme at the P_1 subsite (Table 2).

Other amino acid esters gave the desired dipeptide in high yield but at a much lower rate. This indicates the importance of the phenolic group for the binding of the substrate to the active site. These results show that conformational changes have occurred, during the transition from water to the organic solvent, leading to an enhanced specificity for tyrosine. This effect was confirmed by the use of *N*-benzyloxycarbonyl-3,4-dihydroxy-L-phenylalanine ethyl ester as a donor ester, which led to the synthesis of the corresponding dipeptide at a much greater rate than with the corresponding phenylalanine ester. As indicated by *X*-ray crystallography, at the bottom of the hydrophobic site, there is a tightly bound water molecule.[10] As long as some water has to be displaced from the hydrophobic pocket by the substrate, one can envisage that the hydroxy group of hydroxylated aromatic amino acid esters might replace a water molecule in a putative hydrogen bond network, resulting in the observed difference in rates. Surprisingly,

Table 1. Effect of the nucleophile specificity on synthesis

Donor ester	Nucleophile	Product	Yield (%)	Reaction time (h)
Ac-L-TyrOEt	L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	96	6
Ac-L-TyrOEt	L-LeuNH$_2$	Ac-L-Tyr-L-LeuNH$_2$	95	6
Ac-L-TyrOEt	L-LeuOMe	—	0	72
Ac-L-TyrOEt	L-ValNH$_2$	Ac-L-Tyr-L-ValNH$_2$	92	18
Ac-L-TyrOEt	L-AlaNH$_2$	Ac-L-Tyr-L-AlaNH$_2$	84	18
Ac-L-TyrOEt	L-MetNH$_2$	Ac-L-Tyr-L-MetNH$_2$	86	12
Ac-L-TyrOEt	L-ProNH$_2$	—	0	72
Ac-L-TyrOEt	L-LysOBu^t	Ac-L-Tyr-α-L-LysOBu^t	86*	18
Z-L-TyrOEt	Gly-GlyOEt	Z-L-Tyr-Gly-GlyOEt	88	18

Z = benzyloxycarbonyl

* Only the natural peptide linkage (involving the α-NH2 group of lysine) was formed. The dipeptide was characterized using 400 MHz ^{1}H n.m.r. spectroscopy. Formation of the peptide bond through the α-NH2 resulted in a downfield shift of the α-proton from 3.45 p.p.m. (in the free amino acid derivative) to 4.50 p.p.m. in the dipeptide. No change in the chemical shift of the ε-protons (2.95 p.p.m.) was observed. This result can be correlated with the synthesis of similar derivative using subtilisin suspended in anhydrous t-pentyl alcohol in which only the unnatural ε-linkage was obtained.[9]

Table 2. Effect of the nature of the donor ester

Donor ester	Nucleophile	Product	Yield (%)	Reaction time (h)
Ac-L-TyrOEt	L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	96	6
Z-L-TyrOEt	L-PheNH$_2$	Z-L-Tyr-L-PheNH$_2$	94	6
Bz-L-TyrOEt	L-PheNH$_2$	Bz-L-Tyr-L-PheNH$_2$	86	12
Ac-L-PheOEt	L-PheNH$_2$	Ac-L-Phe-L-PheNH$_2$	90	24
Boc-L-PheOEt	L-PheNH$_2$	Boc-L-Phe-L-PheNH$_2$	0	72
Ac-L-TrpOEt	L-PheNH$_2$	Ac-L-Trp-L-PheNH$_2$	88	48
Z-L-DopaOEt	L-PheNH$_2$	Z-L-Dopa-L-PheNH$_2$	90	12
Bz-L-AlaOEt	L-PheNH$_2$	Bz-L-Ala-L-PheNH$_2$	68	48
Z-L-Leu-L-PheOEt	L-PheNH$_2$	—	0	72
Z-L-Leu-L-LeuOMe	L-PheNH$_2$	—	0	72

Z-L-DopaOEt = *N*-benzyloxycarbonyl-3,4-dihydroxy-L-phenylalanine ethyl ester; Z = benzyloxycarbonyl; Boc = tert-butyloxycarbonyl; Bz = benzoyl.

Table 3. Stereoselectivity of chymotrypsin suspended in dichloromethane.

Donor ester	Nucleophile	Product	Yield (%)	Reaction time (h)
Ac-L-TyrOEt	D-PheNH$_2$	Ac-L-Tyr-D-PheNH$_2$	94	12
Ac-L-TyrOEt	D-LeuNH$_2$	Ac-L-Tyr-D-LeuNH$_2$	93	12
Ac-L-TyrOEt	D,L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	48	12
		Ac-L-Tyr-D-PheNH$_2$	47	
Ac-D-TyrOEt	L-PheNH$_2$	Ac-L-Tyr-D-PheNH$_2$	0	72
Ac-D-PheOEt	L-PheNH$_2$	Ac-L-Tyr-D-PheNH$_2$	0	72
Ac-D-TrpOEt	L-PheNH$_2$	Ac-L-Tyr-D-PheNH$_2$	0	72

dipeptides (as acyl donors) were never involved in synthesis, indicating again that the P_1 subsite has been modified. This was further demonstrated by the use of the BOC protecting group, which again led to negative results. The P_1 subsite seems to accommodate single amino acid derivatives with *N*-protection ranging from acetyl to benzyloxycarbonyl, but excludes dipeptides and amino acid derivatives bearing bulky protecting groups. This contrasts with the behaviour of the enzyme in water.

The stereoselectivity of the enzyme suspended in dichloromethane was found to be modified as shown in Table 3.

Involvement of D-amino acid derivatives as nucleophiles has been reported in reactions catalysed by chymotrypsin under alkaline conditions, but the reaction was slow[11] (approximately 10% as fast as that using L-amino acid derivatives).

In summary, chymotrypsin suspended in hydrophobic organic solvents permits preparation of diverse peptides. The isolation of the desired compounds is made very simple and the enzyme can eventually be recovered without loss of activity. The stereoselectivity of the enzyme has not been drastically altered as in the case of subtilisin.[5c] Nevertheless, various D-amino acid derivatives were readily incorporated at the P'_1 position. The specificity at the P_1 subsite becomes more stringent towards single *N*-protected L-amino acid esters, and high specificity for hydroxylated aromatic amino acid esters is shown.

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