Papain Catalysed Peptide Synthesis: Control of Amidase Activity and the Introduction of Unusual Amino Acids

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Procedures for the papain catalysed synthesis of peptides containing D-amino acids and derivatives with control of the enzyme's amidase activity have been developed.

Since our report of α-chymotrypsin and the Met(*O*)-(192)-modified enzyme as a catalyst for the preparation of dipeptides containing D-amino acids,[1] we have investigated the proteolytic enzyme papain (E.C. 3.4.22.2) as another more versatile catalyst for synthesis of unusual peptides. The primary specificity of papain is for a bulky aliphatic or aromatic group in the P-2 position.[2] This requirement is easily met with the use of common amine protecting groups such as benzoyl (Bz), benzyloxycarbonyl (Z), and t-butyloxycarbonyl (Boc). Utilizing such protecting groups, papain has been shown to catalyse the coupling of L-amino acids where the N-protected donor is any L-form amino acid or ester except proline, and the amine component is an L-amino acid ester or amine.[3] Problems in enzymatic peptide coupling arise owing to the reversible nature of the catalysis and high specificity which usually precludes efficient couplings involving D- or unusual amino acid derivatives, peptides containing which are often of special interest as pharmaceuticals or as sweeteners.[1]

By utilizing the kinetic approach to enzymatic peptide synthesis[1a] with papain, we are able to enhance greatly the relative esterase activity *vs.* the amidase activity of the enzyme by using a large proportion of organic solvent (40% methanol) and high pH[3b,4] (pH 9). Papain at pH 9 acts as an esterase with an activity of 10^3 times that of amidase. Under these conditions, the coupling of a Z-amino acid ester and an amine component (the nucleophile) under papain catalysis should be much faster than the enzyme-catalysed hydrolysis of amide bonds, if the activity of the water can be reduced. Further, after coupling the resulting peptide is safe from subsequent hydrolysis by the enzyme.

With high nucleophile concentration (*ca.* 1 M), we have indeed found that papain can effectively catalyse dipeptide formation where the nucleophile is a D-amino acid ester or other amine derivative despite previous reports to the contrary[3b,5] (see Table 1). These couplings were effected by the addition of 3 mmol of a L-amino acid ester and 6 mmol of a D-amino acid ester to 6 ml of a solution containing 0.2 M carbonate buffer, 40% methanol, and 30 μl 2-mercaptoethanol with adjustment to pH 9. Papain (20 mg, a twice-recrystallized preparation used as received from Sigma) was added and the solution was stirred vigorously for 3 h. Subsequent extraction with chloroform and washing yielded the desired dipeptides characterized by comparison of their optical rotation and n.m.r. spectra with the spectra of chemically prepared peptides. Note that only a two-fold excess of nucleophile is necessary in comparison with a sixteen-fold excess in the α-chymotrypsin catalysed synthesis.[1a]

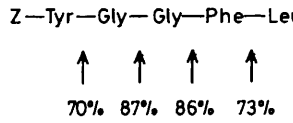
Of particular interest is the peptide Z-L-Asp(OBzl)-D-Ala-OPr^i (OBzl = $PhCH_2O$) which upon hydrogenolysis yields the dipeptide L-Asp-D-Ala-OPr^i. This dipeptide has been prepared chemically and shown to be a sweetner with a potency that is 125 times greater than sucrose.[6]† Owing to low yields

Table 1. Unusual peptides prepared by papain catalysed reactions.

Acyl donor	Nucleophile	Product	Yield /%	$[\alpha]_D^{25}$ (*c* 10, MeOH)
Z-GlyOEt	D-AlaOMe	Z-Gly-D-AlaOMe	92	+28.6
	D-AlaOPr^i	Z-Gly-D-AlaOPr^i	86	+28.8
	D-LeuOMe	Z-Gly-D-LeuOMe	89	+20.6
	D-PheOMe	Z-Gly-D-PheOMe	55	−9.4
	D-ValOMe	Z-Gly-D-ValOMe	61	+15.8
	ε-Aminocaproic acid methyl ester	Z-Gly-ε-AspOMe	63	Achiral
Z-PheOMe	Glycinal dimethyl acetal	Z-Phe-glycinal dimethyl acetal	73	−16.6
Z-Asp(OBzl)-OBzl	D-AlaPr^i	Z-Asp(OBz)-D-AlaOPr^i	86[a]	−8.4[b]

[a] Coupling facilitated using papain immobilized in XAD-8 with 4-methylpentan-2-one as solvent. [b] *C* 5, dimethylformamide.

† Pfizer Inc. in New York has recently prepared a number of chemically synthesized L-Asp-D-Ala derivatives with different protecting groups at the carboxy end of D-Ala. All of them are sweeter than sucrose and more stable than aspartame under high temperature (T. M. Brennan and M. E. Hendrick, *US Patent*, 4 411 925, 1983). These dipeptides could be made by papain catalysis as described here.



Scheme 1

using the aforementioned aqueous enzyme system, a system utilizing 4-methylpentan-2-one as the solvent with immobilized papain in XAD-8 [a neutral cross-linked poly(methyl methacrylate) from Sigma] was developed.‡ By minimizing the water content, which competes with the amino acid nucleophile in the deacylation of the enzyme, yields can be increased to 86%. In a representative preparation of immobilized papain, crude papain (1 g) from Sigma was stirred with 50 ml 0.1 M phosphate buffer, pH 7, and 4 g XAD-8 for 6 h. Papain entrapped in XAD-8 was filtered and dried under vacuum over KOH for 12 h. This immobilized enzyme (1 g) was added to a flask charged with 1 mmol Z-Asp(OBzl)OBzl, 3 mmol D-Ala-OPr^i, 3 ml 4-methylpentan-2-one, and 30 μl 2-mercaptoethanol. The mixture was stirred for about 2 days until all the Z-Asp(OBzl)OBzl was consumed as monitored by t.l.c. The immobilized enzyme was recovered by filtration. Ethyl acetate (20 ml) was added to the filtrate and the solution was subsequently washed with sodium hydrogen carbonate solution (50 mM), water, and HCl (0.1 M), and dried and concentrated to yield an oil which crystallized from ethyl acetate–hexane yielding the dipeptide in 86% yield. There has been much recent comment in the literature that ultra low water systems can be used for synthetic organic reactions with enzymes. It is worth pointing out that this work uses a miscible aqueous–organic solvent or immiscible aqueous–organic solvent (the XAD-8 system) with great success.

Stepwise synthesis of enkephalinamide illustrates the feasibility of this approach towards polypeptide synthesis. Enkephalinamide was synthesized from C to N terminus with the coupling of Z-Phe-OMe and Leu-NH_2, hydrogenolysis of the N protecting group and the coupling of the resulting N-free dipeptide with the subsequent Z-amino ester, hydrogenolysis *etc.* as in Scheme 1. In these syntheses, each of the first two couplings involves two equivalents of the amine component while each of the last two couplings involves two equivalents of the ester components. The numbers indicate the isolated yield for the corresponding couplings.

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‡ A similar system was reported for the synthesis of an aspartame precursor with thermolysin, though the water content of the system is not applicable for synthesis using the kinetic approach.[7]

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