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5(4H)-OXAZOLINONES AS ACYL DONORS IN PAPAIN-CATALYZED PEPTIDE FRAGMENT CONDENSATIONS

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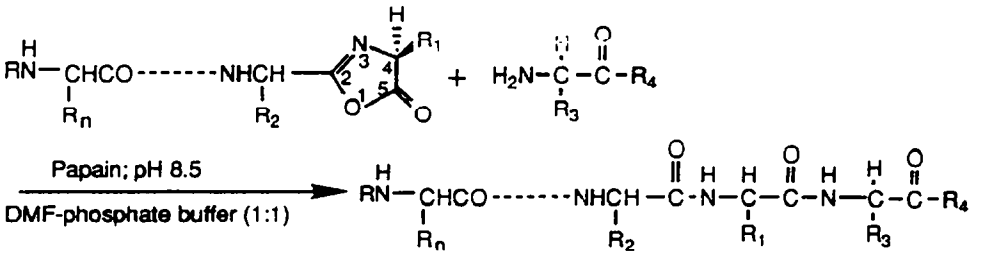
Summary: Papain, a thiol protease was shown to utilize 5(4H)-oxazolinones of peptides as acyl donors in peptide segment condensations. The effectiveness of this methodology is illustrated by the successful coupling of oxidized insulin B chain (30 residues) to angiotensin III (7 residues) in 59% yield.

Enzymatic methods for peptide synthesis[1] constitute an important supplement to conventional chemical synthetic methodology. While many significant advances[2] have been made, enzymatic peptide synthesis has not yet attained the stage of a standardized procedure. For each coupling, the reaction conditions must be carefully adjusted to maintain the position of equilibrium favoring synthesis and to minimize hydrolysis of the acyl-enzyme intermediate as well as the newly-formed peptidic product.[3] As a consequence, heretofore studies have been largely confined to the optimization of reaction conditions for the sequential assembly of amino acids to form small peptides.[4] Since peptides of moderate sizes (10-20 amino acid residues) can now be readily prepared using commercial peptide synthesizers[5], a more advantageous use of enzyme technology is in the condensation of peptide segments for the semisynthesis of larger peptides and proteins.

Enzymatic condensation of long peptides is difficult and is often achieved by assisting bond formation using either fragments that form noncovalent complexes[2b,6] or an excess of the nucleophilic component.[7] While the transpeptidation approach using activated acyl esters as donors is generally preferred in enzymatic syntheses[3], the chemoselective introduction of a suitable leaving group onto the C-terminal carboxyl in a long peptide is not a trivial undertaking.[8] However, we recently disclosed that this limitation could be ameliorated by the use of 5(4H)-oxazolinones as acyl donors, which was successfully applied to the α-chymotrypsin-catalyzed peptide segment couplings.[9] To further examine the scope of this approach, we now report our results using the thiol protease, papain, for the synthesis of long peptides.

As activated esters of N-acylamino acids hydrolyze through the corresponding 5(4H)-oxazolinones[10], the latter may be considered as activated internal esters of N-acylamino acids. Like the serine proteases, papain can also catalyze the enantioselective cleavage of certain 5(4H)-oxazolinones at useful rates via the formation of an acyl enzyme intermediate.[11] For example, the L-enantiomer of *DL*-4-methyl-2-phenyloxazolin-5-one[11] was preferentially cleaved with an E value[12] of 6. Papain has been widely used in peptide synthesis[13] because it has a relaxed substrate specificity at the P_1 and P_1' sites although it prefers arg and lys at the P_1 position.

For our initial studies we used 2-phenyl-4-benzyloxazolin-5-one[14] as the acyl donor and various nucleophiles as acyl acceptors in the papain-catalyzed syntheses of small peptides (Table 1). It is evident that



this protease was able to accommodate a wide variety of nucleophiles at the P_1' position as acyl acceptors to form dipeptides. The yields ranged from 43 to 80%, which is a reflection of the efficiency with which the acyl acceptors intercept the acyl enzyme intermediate. The extent of hydrolysis of the acyl enzyme is indicated by the amount of N-benzoylphenylalanine formed, which was quantitatively determined by HPLC. No significant amount of 2-phenyl-4-benzyloxazolin-5-one hydrolysis was detected in the absence of papain. The optimum reaction media was found to be DMF/phosphate buffer, pH 8.5 (1:1), which differed from those used for the α-chymotrypsin-catalyzed peptide syntheses [acetonitrile/phosphate buffer (1:1)].[9] Under these conditions, the L-oxazolinones were relatively stable and did not racemize, for only a single product was observed. In contrast, when (±)-2-phenyl-4-benzyloxazolin-5-one was used as the substrate (entries 1 and 2 of Table 1), two diastereomeric products, easily separated by HPLC, were formed with each acyl acceptor indicating papain has a modest stereochemical preference for the L-enantiomer. Entries 8-11 reveal that protection of the C-terminal carboxyl function of the acyl acceptor was not required, for similar yields of coupling products were obtained, but the reaction rate proved to be somewhat slower.

A representative procedure for the condensation of *N*-trifluoroacylated[15] oxidized bovine insulin B chain [*N*-TFA-FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYTPK(*N*-TFA)A] to angiotensin III (RVYIHPF) were as follows: TFA-oxidized insulin B chain (0.14 μmol) was dissolved in a mixture of acetic anhydride and dioxane (1:1). After the reaction mixture was stirred at 0°C[16] for 1.5 h, the solvents were evaporated under reduced pressure at 0°C and the residue (oxazolinone of TFA-insulin B chain) was dissolved in a mixture of *N,N*-dimethylformamide and pH 8.5, 0.2 M phosphate buffer containing 8 mM DTT and 4 mM EDTA (1:1). To this solution was added angiotensin III (0.28 μmol) and 4 μl of papain at a concentration of 25 μg/μl, and the resulting mixture was incubated at 24°C with stirring. The progress of the reaction was monitored by removing an aliquot of the reaction mixture at regular intervals and analyzing it by HPLC on a Waters C_{18} (4 μm) Nova-Pak reverse phase column. The column was eluted with a gradient of water-acetonitrile containing 0.1% TFA (0-100% of acetonitrile in 60 min) at a flow rate of 1 ml/min. The retention times of TFA-oxidized insulin B chain and angiotensin III were 22.5 min and 19 min respectively. The condensed peptide product appeared at 21.6 min and was isolated and further purified by rechromatographing on the same column. The amino acid analysis of the product gave the following: Asp(1) 0.41; Glu(3) 2.42; Ser(1) 1.06; Gly(3) 2.80; His (3) 3.17; Arg(2) 1.6;

TABLE 1. Peptide fragment coupling using papain.[a]

Entry	Acyl donor (as oxazolinones)	Acyl acceptor	Time (min)	Yield[b] (%)	Product[c]
1	*N*-Bz-(*D,L*-)-Phe	Ala-NH_2	30	40 19	*N*-Bz-Phe-Ala-NH_2 *N*-Bz-Phe-(*D*-)-Ala-NH_2
2	*N*-Bz-(*D,L*-)-Phe	Glu-NH_2	30	62 18	*N*-Bz-Phe-Glu-NH_2 *N*-Bz-Phe-(*D*-)-Glu-NH_2
3	*N*-Bz-Phe	Ala-NH_2	30	62	*N*-Bz-Phe-Ala-NH_2
4	*N*-Bz-Phe	Glu-NH_2	30	80	*N*-Bz-Phe-Glu-NH_2
5	*N*-Bz-Phe	Phe-NH_2	30	43	*N*-Bz-Phe-Phe-NH_2
6	*N*-Bz-Phe	Arg-NH_2	30	58	*N*-Bz-Phe-Arg-NH_2
7	*N*-Bz-Phe	Ser-NH_2	30	48	*N*-Bz-Phe-Ser-NH_2
8	*N*-Bz-Phe	Gly-Leu-OH	40	51	*N*-Bz-Phe-Gly-Leu-OH
9	*N*-Bz-Phe	Ala-Ala-Ala-OH	40	34	*N*-Bz-Phe-Ala-Ala-Ala-OH
10	*N*-Ac-AAA (1.8 mM)	EVF-OH (8.5 mM)	80	64	*N*-Ac-AAAEVF-OH
11	*N*-Ac-AAA (1.8 mM)	RVYIHPF-OH (3.6 mM)	80	74	*N*-Ac-AAARVYIHPF-OH
12	*N*-TFA-insulin chain B (0.7 mM)	Angiotensin III (1.4 mM)	80	59	*N*-TFA-FVNQHLC(SO_3H)-GSHLVEALYLVC(SO_3H)-GERGFFYTPK(*N*-TFA)A-RVYIHPF (*N*-TFA-insulin chain B - angiotensin III)

[a]Abbreviations: Ac, acetyl; Bz, benzoyl; TFA, trifluoroacetyl. All amino acids are of the *L* configuration unless otherwise stated. The peptides used were as follows: angiotensin III, RVYIHPF; N-TFA-insulin B chain (bovine), *N*-TFA-FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYTPK(*N*-TFA)A. Experimental conditions (unless otherwise stated) were: acyl donor, 10 mM; acyl acceptor, 50 mM; reaction media: DMF/pH 8.5, 0.2 M phosphate buffer, 8 mM DTT, 4 mM EDTA (1:1); papain, 1 mg/ml; 24°C. The progress of the reaction was monitored by HPLC analyses [Waters C_{18} (4 μm) Nova-Pak reverse phase column (8 x 100 mm); gradient (0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 40 min at a flow rate of 1 mL/min)]. Amino acid analyses were performed using the Waters Pico-Tag system.

[b]The yields were estimated from HPLC analyses and were based on the acyl donor.

[c]The amino acid analyses of **8**, **10** and **11** were as follows: **8**, G(1) 1.27; L(1) 1; F(1) 1.04. **10**, E(1) 0.8; A(3) 2.81; V(1) 1; F(1) 0.99. **11**, H(1) 0.88; R(1) 0.91; A(3) 3.17; P(1) 1.20; Y(1) 1.02; V(1) 1; I(1) 0.99; F(1) 1.01.

Thr(1) 0.42; Ala(2) 2.13; Pro(2) 2.56; Tyr(3) 2.77; Val(4) 4; Ile(1) 0.97; Leu(4) 3.87; Cya(2) 1.43; Phe(4) 3.83; and Lys(1) 1.26.

In conclusion, we have extended the usefulness of this approach by demonstrating that the thiol protease, papain, also effectively utilized 5(4H)-oxazolinones of peptides as acyl donors in oligopeptide synthesis. The application of this methodology to the enzymatic condensation of modified peptides is currently under investigation.

Acknowledgment

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15. *N*-Trifluoroacetylations of peptides were as follows: to bovine insulin B chain (0.2 mg), dissolved in 0.2 mL of 0.1 M $NaHCO_3$, was added 0.1 mL of *S*-ethyl trifluorothioacetate, and the mixture was stirred vigorously at 24°C for 3h. The solvents were evaporated to dryness under reduced pressure and the residue was dissolved in 0.1 mL of water and purified by HPLC.
16. The formation of the mixed anhydride and all subsequent operations were carried out at 0°C; at ambient temperatures, racemization of the peptide oxazolone was noted.

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