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ONE-POT TRIPEPTIDE SYNTHESES FROM THREE SINGLE AMINO ACID DERIVATIVES CATALYZED BY PAPAIN

Carlos F. Barbas, III, and Chi-Huey Wong*
Department of Chemistry, Texas A&M University
College Station, Texas 77843, U.S.A.

Summary: Procedures have been developed for the syntheses of tripeptides from three distinct amino acid derivatives in one step using papain as a catalyst.

Enzymes, namely proteases and lipases , have been utilized with great success in the coupling of two amino acid derivates or peptide fragments.[1-3] Though much research has been devoted to this endeavor, there are no reports extending the applicability of these catalysts towards the coupling of three distinct amino acid or peptide derivatives, in one step, to form a peptide with a pre-defined amino acid sequence.[2] We wish to report here the rationale, applicability, and limitations of the use of papain as such a catalyst.

Recently, we have reported the facile coupling of acyl donor residues with D-amino acid nucleophiles in protease catalyzed reactions.[3] Though this coupling proceeds in high yield when the acyl donor is glycine, anhydrous conditions were necessary for the coupling of L-Asp and D-Ala derivatives.[3b] Utilizing the same aqueous system previously reported[3b] with D-Leu-OMe as nucleophile and Z-Gly-OEt, Z-Ala-OEt, and Z-Phe-OMe as acyl donors, the yield of dipeptide was 92%, 22%, and 1% respectively. These results may be indicative of steric communication between the P_1 and P_1' residues and the binding sites when P_1' is a D-amino acid since this decrease in yield with increasing steric bulk in P_1 has not been observed in the coupling of L-amino acids.[4] This observation as well as the known S_2 subsite primary specificity[5] of papain suggested that it should be a catalyst for one-pot tripeptide synthesis from three amino acid derviatives. We have accomplished such a synthesis according to eq. 1 where AA_1 is a hydrophobic amino acid, AA_2 is Gly, AA_3 is a D-amino acid ester, Z (benzyloxycarbonyl) is a hydrophobic protective group.

$$Z\text{-}AA_1\text{-}OR_1 + AA_2\text{-}OR_2 + D\text{-}AA_3\text{-}OR_3 \xrightarrow{\text{papain}} Z\text{-}AA_1\text{-}AA_2\text{-}D\text{-}AA_3\text{-}OR_3 \quad (1)$$

$$\text{Z-AA}_1\text{-OR}_1 + \text{AA}_2\text{-OR}_2 \longrightarrow \underset{P_2\ P_1\ \ P_1'}{\text{Z-AA}_1\text{-AA}_2\text{-OR}_2} \xrightarrow{\text{D-AA}_3\text{-OR}_3} \underset{P_3\ P_2\ \ P_1\ \ \ \ P_1'}{\text{Z-AA}_1\text{-AA}_2\text{-D-AA}_3\text{-OR}_3} \quad (2)$$

The reaction mechanism was proposed to involve the initial formation of the acyl enzyme, Z-AA_1-E, followed by deacylation by AA_2-OR_2 where the S_2 subsite is occupied by the Z-group (eq. 2). In formation of the second acyl enzyme, Z-AA_1-AA_2-E, the S_2 subsite is occupied by the side-chain of AA_1 which should be hydrophobic. A low yield was seen when Gly was at this position since it lacks the side-chain necessary for hydrophobic bonding to the S_2 subsite. Deacylation with D-AA_3-OR_3 should produce the tripeptide. The synthesis of Z-Phe-Gly-D-Leu-OMe was first accomplished where R_1 = R_3 = methyl and R_2 was varied. The identity of R_2 was varied since it plays an important role in moderating the relative nucleophilicity of AA_2-OR_2 as well as the acyl donating ability of the intermediate Z-AA_1-AA_2-OR_2. HPLC analysis indicates that the synthesis follows the proposed mechanism. The tripeptide ester is accumulated because papain can not accept an acyl donor with a C-terminal D-amino acid. Further, under alkaline conditions (pH 9-10) the enzyme has very weak peptidase activity.[3b] As shown in Table I, entries 1 through 4, the yield of tripeptide is optimized where R_2 = isopropyl. Poor yields with the benzyl ester are most likely attributable to non-productive binding within the hydrophobic groove of papain.[5] The relative ratios and absolute concentrations of the three constituents were varied to give an optimized yield when Z-AA_1-OR_1:AA_2-OR_2:D-AA_3-OR_3 = 0.5:1:1M

The reactions are approximately 90% complete in 3 hrs. but are accompanied by traces of Z-AA_1-AA_2-AA_2-OR_2 and Z-AA_1-AA_2-AA_2-OH. Extending the reaction time to 12 hrs. allows for further conversion of these compounds to the desired tripeptides. This conversion is facilitated by the use of methanol as cosolvent since it has little effect on the relative amidase and esterase activities of the enzyme.[6] Methanol also acts to regenerate the acyl donating ester (Z-AA_1-AA_2-OMe) in the absence of bound amine nucleophile. With this capacity, methanol has been shown to be the most effective of alcohols since alcohols with longer alkyl chains, which are therefore more hydrophobic, bind to the enzyme in a manner which decreases the efficiency of ester regeneration.[7] Indeed, a 2-fold increase in yield was observed with methanol as cosolvent as compared with the use of a solvent incapable of this activity, e.g. dioxane. The desired tripeptides are extremely poor substrates for hydrolysis by papain, making their formation kinetically irreversible. Thus, extention of the reaction time enhanced rather than degraded the yield of the tripeptide. The presence of the dipeptide Z-AA_1-D-AA_3-OR_3 was not detected as examined by HPLC even though papain is competent in forming this bond to some extent in the absence of AA_2-OR_2. After 12 hrs. the tripeptide was easily seperated from the two acidic co-products Z-AA_1-OH and Z-AA_1-AA_2-OH by flash chromotography with ethyl acetate: methanol = 8:2 as eluent.

Table I: Peptides Prepared in Papain Catalyzed Reactions

	Z-AA_1-OMe	AA_2-OR_2	D-AA_3-OR_3	Product	% Yield[a]	m.p.
1[b]	Z-Phe-OMe	Gly-OEt	D-Leu-OMe	Z-Phe-Gly-D-Leu-OMe	37	155-156
2	"	Gly-OPr^i	"	"	55	"
3	"	Gly-OAllyl	"	"	21	"
4	"	Gly-OBzl	"	"	2	"
5[c]	"	Gly-OPr^i	"	"	67	"
6	"	"	D-Ala-OMe	Z-Phe-Gly-D-Ala-OMe	57	136-136
7	"	"	D-Val-OMe	Z-Phe-Gly-D-Val-OMe	60	143-144
8	"	"	D-Phe-OMe	Z-Phe-Gly-D-Phe-OMe	62	125-126
9	"	"	D-Met-OMe	Z-Phe-Gly-D-Met-OMe	44	122-123
10	Z-Leu-OMe	"	D-Leu-OMe	Z-Leu-Gly-D-Leu-OMe	65	66-68
11	Z-Val-OMe	"	"	Z-Val-Gly-D-Leu-OMe	41	77-78
12	Z-Ala-OMe	"	"	Z-Ala-Gly-D-Leu-OMe	69	63-65
13	Z-Phe-OMe	Ala-OPr^i	"	Z-Phe-Ala-D-Leu-OMe	0	163-165
14	"	β–Ala-OPr^i	"	Z-Phe-β–Ala-D-Leu-OMe	0	139-141

[a]Yields based on the limiting reagent were determined by HPLC using Beckman C_{18} column, 0.1 M acetate eluent, pH 3, with a linear gradient of methanol: methanol was increased from 50% to 100% in 10 min. and maintained at 100% for 5 min.. Methanol was then reduced to 50% over 5 min.. All amino acids are of the L-form unless specifically noted. All products were identical with respect to m.p., 200 MHz ^1H NMR, and HPLC retention times as compared to authentic samples prepared using mixed carbonic anhydride methodology[8]. Abbreviations: Z: benzyloxycarbonyl, Pr^i: isopropyl, Et: ethyl, Bzl: benzyl.

[b]Synthetic conditions for entries 1-4 are as follows: 0.5 M D-Leu-OMe·HCl, 0.125 M Z-Phe-OMe, 0.25 M AA_2-OR_2·HCl, 36% methanol and 9% methyl isobutyl ketone by volume, 0.25 M Tris, 3μL β –mercaptoethanol, 100 mg crude papain(EC 3.4.22.2, type II from Sigma), 2 mL total volume, pH 9 with vigorous stirring for 5 hrs..

[c]Optimized synthetic procedure for entries 5-14: Two mmoles each of AA_2-OR_2·HCl and D-AA_3-OR_3·HCl were dissolved in 0.8 mL methanol and 0.385 mL of 1.3 M Tris to which 3 μL of β–mercaptoethanol was added. The pH was adjusted to 9.5 with 10 M NaOH. Z-AA_1-OR_1, 1 mmole, was added followed by 150 mg crude papain. The mixture was shaken for 1 min., then 0.2 mL methyl isobutyl ketone was added. The mixture was then stirred vigorously for 12 hrs. at room temperature.

Entries 5-9 demonstrate the residues allowed at the AA_3 position , while 5 and 10-12 demonstrate wide variability allowed at the AA_1 position. Variation at the AA_2 position , entries 13 and 14, was unsuccessful. Though the yields of tripeptide are not high, it is important to recognize that the enzyme has catalyzed what is formally a three step synthesis using solution phase chemistry, in just one step. Thus, a yield of 69% in one step translates into a yield of 88% in each of three steps. Perhaps more importantly, only one purification step is necessary, the expensive amine protecting group on AA_2 is eliminated, and the integrity of the product is not degraded by free amino acid impurities in the starting materials. This procedure appears to be quite general and should be useful for syntheses of a number of tripeptides.

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