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Mode of Attack by Crude Papain on Racemic Z-Dipeptides That Contain a β-Alanine Residue during Anilide and Phenylhydrazide Syntheses

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A crude extract of papain exclusively attacked the carbonyl of an α-amino acid residue of Z-β-Ala-DL-Ala and Z-DL-Ala-β-Ala during catalyzed reactions with aniline or phenylhydrazine as the nucleophiles. The optical purities of the resultant, insoluble products, Z-β-Ala-L-Ala-NHPh, Z-β-Ala-L-Ala-NHNHPh, Z-L-Ala-NHPh, and Z-L-Ala-NHNHPh, were all substantially above 90%.

In papain-catalyzed reactions with nucleophiles, attack on a reactive Z dipeptide[1] can occur either at the carboxyl terminal[2] or at the carbonyl group[2,3] of the amide structure that joins the two amino acid residues. It was the purpose of the current study to examine the reactions of aniline and phenylhydrazine with the two racemic Z dipeptides that combine β-alanine with DL-alanine, when a crude papain extract was used as the catalyst. Although β-alanine is a well-known residue of the natural dipeptides, carnosine or anserine, it is not a component residue of proteins. Crude papain[4] contains a mixture of sulfhydryl proteases,[5,6] Enz–SH. It has frequently been utilized as a chiral, catalytic agent for resolutions of N-blocked DL-amino acids,[7,8] using a variety of nucleophiles.[8-11]

The reaction of Z-β-Ala-DL-Ala with RNH_2 in the presence of papain gave the Z-β-Ala-L-Ala-NHR derivatives with the peptide bond intact. Optical purities of the resultant anilide or phenylhydrazide were both approximately 99%.

Z-β-Ala-CO-NH-DL-Ala-COOH

$$\xrightarrow[\text{papain}]{RNH_2} \underset{\text{(insoluble)}}{\text{Z-β-Ala-CO-NH-L-Ala-CO-NHR}} + \underset{\text{(soluble)}}{\text{Z-β-Ala-CO-NH-D-Ala-COOH}}$$

On the other hand, with the β-Ala residue as the carboxyl terminal residue, the attack occurred at the peptide structure to give Z-L-Ala-NHR plus β-Ala. Respective optical purities of the anilide and phenylhydrazide were about 94 and 99%.

Z-DL-Ala-CO-NH-β-Ala-COOH

$$\xrightarrow[\text{papain}]{RNH_2} \underset{\text{(soluble)}}{\text{Z-D-Ala-CO-NH-β-Ala-COOH}} + \underset{\text{(insoluble)}}{\text{Z-L-Ala-CO-NHR}} + \underset{\text{(soluble)}}{NH_2\text{-β-Ala-COOH}}$$

Similar reactions were encountered when the L enantiomers, Z-β-Ala-L-Ala and Z-L-Ala-β-Ala, replaced the corresponding racemic modifications. Important details are summarized in Tables I and II. As might have been anticipated, on the basis of the absence of a β-Ala residue in proteins, crude papain did not catalyze such reactions with Z-β-Ala or Z-β-Ala-β-Ala. Furthermore, Z-β-Ala-D-Ala and Z-D-Ala-β-Ala were equally unproductive when appropriately tested.

An investigation of the pH dependence of yield was made for reactions of Z-β-Ala-Gly and Z-Gly-β-Ala with NH_2Ph and NH_2NHPh. Again, the nucleophilic attack was made exclusively on the carbonyl of the α-amino acid residue. pH optima are shown for the sole, insoluble products: Z-β-Ala-Gly-NHPh (pH 4.75); Z-β-Ala-Gly-NHNHPh (pH 4.25); Z-Gly-NHPh (pH 4.50); Z-Gly-NHNHPh (pH 4.25).

Thin-layer chromatography[2] on plastic plates coated with silica gel established that each successful catalysis yielded an insoluble product with a single structure. R_f values of reference compounds are recorded in Table III.

Experimental Section

Preparation of Active Crude Papain. The crude, active papain necessary for these experiments was prepared by a slight modification of the procedure outlined by Bennett and Niemann.[4]

Reactions of Z-β-Ala-L-Ala and Z-β-Ala-DL-Ala with Aniline and Phenylhydrazine. A mixture of 0.5000 g of Z-β-Ala-L-Ala, 0.26 mL of aniline or phenylhydrazine, 0.1000 g of papain, 0.1000 g of L-cysteine·HCl·H_2O, 25 mL of 0.50 buffer at pH 4.5, and 2 mL of hexamethylphosphoramide was filtered and then incubated at 40 °C. At appropriate time intervals, the solid reaction product was removed by suction filtration, washed with distilled water, dried in the incubator for several days, and weighed. When necessary, the solid was treated with carbon in methanol and filtered four times by suction filtration, with the terminal filtration through a fritted glass funnel. Sufficient methanol was used each time to remove all product from the funnel. Purified product was isolated either by rotary evaporation under reduced pressure or else evaporation in a Petri dish under the hood: % N Calcd for Z-β-Ala-L-Ala-NHPh 11.38, found 11.10; % N Calcd for Z-β-Ala-L-Ala-NHNHPh 14.68, found 14.32. Reactions of Z-β-Ala-DL-Ala were done on four times the scale of Z-β-Ala-L-Ala. Mixture melting points with corresponding products from Z-β-Ala-L-Ala showed no change.

The Behavior of Z-L-Ala-β-Ala and Z-DL-Ala-β-Ala toward Analine and Phenylhydrazine. For Z-L-Ala-β-Ala the solution contained 0.5000 g of Z-L-Ala-β-Ala, 0.52 mL of aniline or phenylhydrazine, 0.1000 g of papain, 0.1000 g of L-cysteine·HCl·H_2O, 25 mL of 0.50 M buffer at pH 4.5, and 2.0 mL of hexamethylphosphoramide. Following filtration, the solution was incubated at 40 °C. After appropriate time intervals, insoluble product was removed by suction filtration, dried for several days in the incubator, and then weighed. A mixture melting point for the anilide product with known Z-L-Ala-NHPh[2,7] exhibited no change. Similarly, the Z-L-Ala-NHNHPh from this study when mixed with known compound[12] showed no change in melting point. After purification: % N Calcd for Z-L-Ala-NHPh 9.39, found 9.62; % N Calcd for Z-L-Ala-NHNHPh 13.41, found 13.52. Reactions of Z-DL-Ala-β-Ala were performed in exactly twice the quantities used for Z-Ala-β-Ala. After purification: % N Calcd

Table I. Insoluble Products from Z-β-Ala-L-Ala and Z-β-Ala-DL-Ala

Product (mp, °C)	Incubation period, h	Wt, g	$[\alpha]^{25°C}_D$ in pyridine	% L enantiomer
Z-β-Ala-L-Ala-NHPh (187–188)	0–48	0.215	−48.0°	100
	48–168	0.382		
Z-β-Ala-Ala-NHPh[a] (187–188)	0–48	0.321	−47.1°	99
	48–168	0.448		
Z-β-Ala-L-Ala-NHNHPh (181–182)	0–48	0.375	−46.6°	100
	48–168	0.357		
Z-β-Ala-Ala-NHNHPh[a] (181–182)	0–48	0.447	−45.2°	99
	48–168	0.617		

[a] The product from Z-β-Ala-DL-Ala may contain some D enantiomer. Hence, Ala is used rather than L-Ala in designating the compound.

Table II. Insoluble Products from Z-L-Ala-β-Ala and Z-DL-Ala-β-Ala

Product (mp, °C)	Incubation period, h	Wt, g	$[\alpha]^{25°C}_D$ in pyridine	% L enantiomer
Z-L-Ala-NHPh (160–161)	0–24	0.2305	−36.3°	100
	24–48	0.0754		
	48–72	0.0118		
Z-Ala-NHh[a] (161–162)	0–24	0.2811	−31.7°	94
	24–48	0.0688		
Z-L-Ala-NHNHPh (153–155)	0–24	0.1373	−31.8°	100
	24–48	0.0506		
Z-Ala-NHNHPh[a] (153–155)	0–24	0.0979	−31.1°	99
	24–48	0.1033		
	48–168	0.1188		

[a] The reactant was Z-DL-Ala-β-Ala. Therefore, Ala, rather than L-Ala, is used to designate the product.

Table III. R_f Values of Standard Reference Compounds for Solvent Systems[a] of Methanol–Chloroform–Hexane

Compound	R_f value at 25 °C
Z-Gly-NHPh	0.28
Z-L-Ala-NHPh	0.36
Z-β-Ala-Gly-NHPh	0.04
Z-β-Ala-L-Ala-NHPh	0.16
Z-Gly-NHNHPh	0.17
Z-L-Ala-NHNHPh	0.28
Z-β-Ala-Gly-NHNHPh	0.06
Z-β-Ala-L-Ala-NHNHPh	0.10

[a] Volume proportions: for all anilides, 3:19:19; for all phenylhydrazides, 2.5:20:15.

for Z-Ala-NHPh 9.39, found 9.51; % N Calcd for Z-Ala-NHNHPh 13.41, found 13.37. Mixture melting points with known Z-L-Ala-NHPh[2,7] or Z-L-Ala-NHNHPh[12] displayed no change.

The Failure of Z-β-Ala, Z-β-Ala-β-Ala, Z-β-Ala-D-Ala, and Z-D-Ala-β-Ala as Suitable Substrates. Similar experiments were devised for attempted catalysis of reactions of these four potential substrates with aniline and phenylhydrazine as those used for the successful reactions. Reaction products were not obtained.

The pH Dependence of Yield for Reactions of Z-β-Ala-Gly or Z-Gly-β-Ala with Aniline and Phenylhydrazine. The general procedures have been outlined previously for similar experiments for other substrate combinations.[9–12] Hexamethylphosphoramide was added as a solubilizing agent for these Z-dipeptides. After incubation at 40 °C, single, insoluble reaction products were formed in each case. For each product, the pH optimum is given first, followed by the pH range that permitted at least one-half of the maximum yield to be obtained, then the incubation period, and finally the nitrogen analysis for new compounds. Z-β-Ala-Gly-NHPh: pH optimum 4.75; pH range 3.6–5.5; 6 days; % N Calcd 11.83, found 11.62. Z-β-Ala-Gly-NHNHPh: pH optimum 4.25; pH range 3.6–5.9; 6 days; % N Calcd 15.13, found 15.07. Z-Gly-NHPh: pH optimum 4.50; pH range 4.0–4.8; 4 days; mixture melting point with known Z-Gly-NHPh[9] no change. Z-Gly-NHNHPh: pH optimum 4.25; pH range 3.4–4.8; 4 days; mixture melting point with known Z-Gly-NHNHPh[7,12] no change. The melting points for Z-β-Ala-Gly-NHPh 182–183 °C; for Z-β-Ala-Gly-NHNHPh 164–165 °C.

Thin-Layer Chromatography of Solid Reaction Products Formed as a Result of Papain Catalysis. In order to confirm that each product obtained as an insoluble solid was solely the result of maintenance of the peptide structure or singularly the result of its cleavage, thin-layer chromatography was employed in a manner similar to procedures recently described.[2] The thin-layer plates plastic coated with silica gel were Baker-flex, silica gel IB2. A short wavelength UV light source was used to locate positions of migrated spots.

Determination of Optical Rotations of Optically Active Products from Reactions Catalyzed by Crude Papain. All optical rotations were determined by means of a Rudolph Model 80 high-precision polarimeter. Water-jacketed tubes were used, either 1 or 2 dm in length, depending on the substance being studied. The temperature was controlled at 25 °C by means of a constant-temperature bath. The concentration was about 1 g per 100 mL of solution. Eastman Spectrograde pyridine was used as the solvent.

Source of Potential Substrates. Z-β-Alanine and Z-β-alanyl-β-alanine were purchased from Sigma Chemical Co., St. Louis, Mo. All other Z dipeptides were made available through Dr. P. Grogg of Biosynthetika, Liestal, Switzerland.

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Registry No.—Papain, 9001-09-6; Z-β-Ala-L-Ala, 56120-15-1; Z-β-Ala-DL-Ala, 63250-94-2; aniline, 62-53-3; phenylhydrazine, 100-63-0; Z-β-Ala-L-Ala-NHPh, 63250-95-3; Z-β-Ala-L-Ala-NHNHPh, 63250-96-4; Z-L-Ala-β-Ala, 41273-31-8; Z-DL-Ala-β-Ala, 63250-97-5; Z-L-Ala-NHPh, 42166-73-4; Z-L-Ala-NHNHPh, 28861-55-4; Z-β-Ala-Gly, 58171-88-3; Z-Gly-β-Ala, 13029-38-4; Z-β-Ala-Gly-NHPh, 63250-98-6; Z-β-Ala-Gly-NHNHPh, 63250-99-7; Z-Gly-NHPh, 6833-09-6; Z-Gly-NHNHPh, 21855-71-0.

References and Notes

(1) Z is the accepted abbreviation for *N*-benzyloxycarbonyl.
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