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THIN-LAYER CHROMATOGRAPHY FOR DETECTION OF PEPTIDE CLEAVAGE OR INTEGRITY DURING REACTIONS OF THE Z-ALANYL-GLYCINES WITH ANILINE OR PHENYLHYDRAZINE UNDER PAPAIN CATALYSIS

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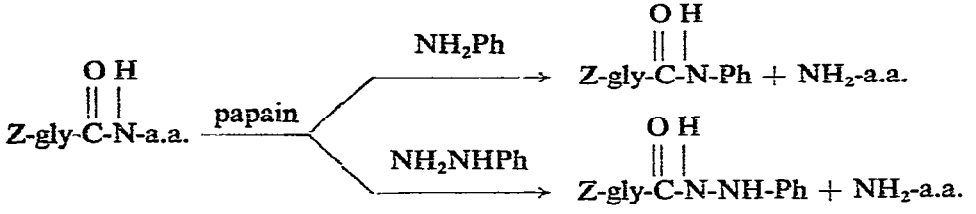
SUMMARY

Z-L-Ala-gly and Z-DL-ala-gly can yield anilides and phenylhydrazides through peptide cleavage or peptide integrity during papain-catalyzed reactions with aniline (NH_2Ph) or phenylhydrazine (NH_2NHPh). Since Z-D-ala-gly yielded only uncleaved Z-D-ala-gly-NHPh or Z-D-ala-gly-NHNHPh, these were used as standards in thin-layer chromatography (TLC) for detecting integrity. Known Z-L-ala-NHPh and Z-L-ala-NHNHPh were the standards for cleavage. Depending on the time of incubation, cleaved or unsplit products, or both, were readily detected. The solvent systems that were used for TLC were also effectively employed for separations of reasonable amounts of mixtures of cleaved and uncleaved products through thick-layer chromatography on ChromAR. This was followed by isolation of the separated components.

INTRODUCTION

During a study on the behavior of a select group of N-blocked dipeptides toward aniline (NH_2Ph) or phenylhydrazine (NH_2NHPh) under papain catalysis, it became essential to develop chromatographic procedures for detecting components formed concurrently that involved both peptide cleavage and integrity in certain of the insoluble products. The commonly used abbreviation for N-(benzyloxycarbonyl)- is Z, which was used as the acyl, N-blocking group in these Z-dipeptides. Z-Glycylglycine (Z-gly-gly), the three Z-glycylalanines (Z-gly-ala) and the three Z-alanylglycines (Z-ala-gly) were incorporated in the total program of research. It turned out that Z-gly-gly, Z-gly-L-ala, Z-gly-D-ala and Z-gly-DL-ala yielded only cleaved products, with resultant formation of insoluble Z-gly anilide or Z-gly phenylhydrazide. With a.a. designating the amino acid residue at the carboxyl terminal, cleavage would be represented as follows,

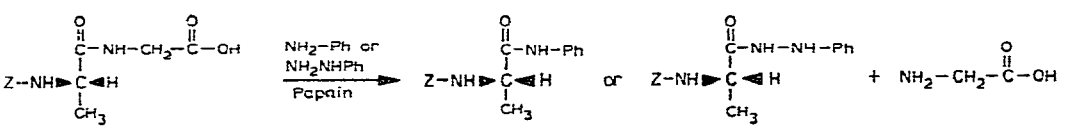


However, a more interesting situation became evident when the three Z-ala-gly were the Z-dipeptide reactants, namely, Z-L-ala-gly, Z-D-ala-gly, and Z-DL-ala-gly. In all instances, papain-catalyzed reactions did occur, but sometimes with singular peptide integrity, in other instances only with peptide cleavage, and in still other cases with both peptide cleavage and peptide integrity. When both cleavage and integrity were permissible for a given Z-dipeptide, each could take place independently during different, designated incubation time intervals, or both could occur simultaneously within an observed incubation period.

EXPERIMENTAL AND RESULTS

Standards for detecting Z-dipeptide cleavage or integrity

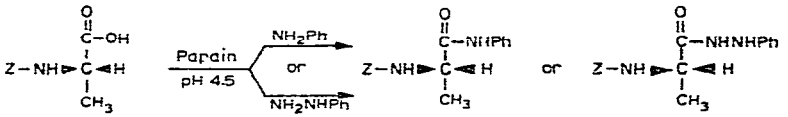
Since cleavage and integrity could take place during different incubation time intervals or simultaneously, it was imperative to resort to thin-layer chromatography (TLC) for rapid exposure of cleavage or integrity. This determined whether or not subsequent thick-layer chromatography on ChromAR was required for separation, isolation and identification of the components of the insoluble product mixture, formed within a particular incubation interval. All potential anilides or phenylhydrazides produced upon cleavage of any of the Z-ala-gly studied would be enantiomers or enantiomeric mixtures. A representative cleavage is shown for Z-L-ala-gly.



Z-L-ala-gly Z-L-ala anilide or phenylhydrazide

The extreme preference displayed by papain for the L enantiomer[1,2] during cleavage insures that even when Z-DL-ala-gly is the reactant, the product is composed chiefly of the L enantiomer.

Enantiomers have the same R_F value for a given solvent system on the flexible plastic plate coated with silica gel IB-F which was used for TLC. Hence, the standards selected for TLC detection of Z-dipeptide cleavage were Z-L-ala-NHPh[3,4], m.p. 161–163°, $[\alpha]_D^{25°} = -37.3°$ in pyridine and Z-L-ala-NHNHPh[5], m.p. 150–151.5°, $[\alpha]_D^{25°} = -33.5°$ in pyridine. These were prepared in optically pure condition through known papain-catalyzed reactions between Z-L-ala and NH_2Ph[3] or NH_2NHPh[5].

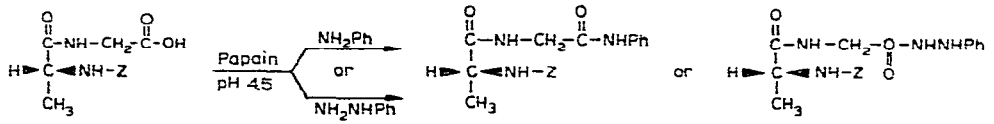


Optically pure Z-L-ala Z-L-ala anilide Z-L-ala phenylhydrazide
(TLC standards for Z-dipeptide cleavage)

Products were treated with carbon in methanol, filtered and recrystallized. The R_F value for Z-L-ala-NHPh was 0.32 at 25° for the solvent system methanol–chloro-

form–hexane (2:19:19). For Z-L-ala-NHNHPh, the R_F value was 0.28 at 25°, for the solvent system methanol–chloroform–hexane (2.5:20:15).

In selecting standards for Z-dipeptide integrity, Z-D-ala-gly anilide, m.p. 169–170°, $[\alpha]_D^{25}° = -13.6°$ in pyridine, and Z-D-ala-gly phenylhydrazide, m.p. 185–187°, $[\alpha]_D^{25}° = +12.2°$ in pyridine, were the preferred choices. Although the reaction between Z-D-ala-gly and aniline or phenylhydrazine proved to be rather slow, at the end of each short period of incubation only uncleaved products were formed with identical melting points over a period of seven days. They were readily identified by nitrogen analysis, optical rotations, and mixture melting points of products from sequential incubation periods.



Z-D-ala-gly

Z-D-ala-gly anilide or phenylhydrazide
(TLC standards for Z-dipeptide integrity)

Combined precipitates, from the sequential incubation periods for each product, were treated with activated carbon in methanol, filtered and recrystallized. The R_F value for Z-D-ala-gly anilide was 0.11 at 25°, when the solvent system was methanol–chloroform–hexane (2:19:19). For Z-D-ala-gly phenylhydrazide, the R_F value was 0.15 at 25°, when the solvent system was methanol–chloroform–hexane (2.5:20:15) at 25°. Comparative, schematic chromatograms for these TLC standards are exhibited in Figs. 1 and 2.

Detection of peptide cleavage or integrity

Reactions between Z-ala-gly and aniline or phenylhydrazine. The three Z-ala-gly, namely, Z-D-ala-gly, Z-L-ala-gly ,and racemic Z-DL-ala-gly, were subjected to reactions under papain catalysis with aniline and then with phenylhydrazine. As explained previously, Z-D-ala-gly did not undergo cleavage over a period of seven days, but produced either Z-D-ala-gly-NHPh or Z-D-ala-gly-NHNHPh, for the progressive incubation periods. These two compounds were employed as standards for establishing integrity during reactions of the other two Z-dipeptides. The variant reactions of Z-L-ala-gly for progressive incubation periods were considerably more interesting. After a few preliminary trial experiments for determining appropriate, sequential time intervals for collecting products after incubation, a satisfactory set of incubation periods for production of anilides (Fig. 1) from Z-L-ala-gly turned out to be 0–3 h, 3–6.5 h, 6.5–24 h, and 24–168 h. When products from each incubation period were chromatographed and set alongside the two standard chromatograms for the cleaved product, Z-L-ala-NHPh, and the uncleaved product, Z-D-ala-gly-NHPh, cleavage or integrity of Z-L-ala-gly was instantly revealed. Only Z-dipeptide integrity, Z-L-ala-gly-NHPh, occurred during 0–3 h (Fig. 1). Both integrity, Z-L-ala-gly-NHPh, and cleavage, Z-L-ala-NHPh, took place during the 3–6.5 h period. Cleavage alone, Z-L-ala-NHPh, was evident through the 6.5–24 h and 24–68 h periods.

When Z-DL-ala-gly was the reactant with aniline, a more complex situation was exposed. It is apparent from Fig. 1, by comparison with the TLC standards, that

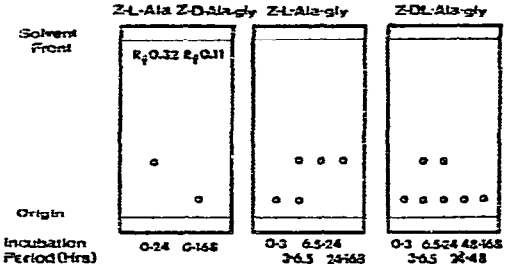


Fig. 1. TLC reproduction of papain-catalyzed products formed from aniline and Z-L-alanine or the Z-alanylglycines after various periods of incubation. Solvent system: methanol–chloroform–hexane (2:19:19).

only Z-dipeptide integrity was encountered for the incubation intervals 0–3 h, 24–48 h, and 48–168 h. On the other hand, both integrity and cleavage took place within the periods 3–6.5 h and 6.5–24 h. Recrystalization of the unsplit product from the 0–3 h period established it to be an enantiomeric mixture, preponderantly Z-L-ala-gly-NHPh, with m.p. 163.5–165° and $[\alpha]_D^{25°} = +9.5°$, in pyridine. By contrast, the uncleaved product from the 24–168 h period, m.p. 169.5–170.5° and $[\alpha]_D^{25°} = -13.1°$, in pyridine, proved to be almost entirely the enantiomeric Z-D-ala-gly-NHPh.

Similar situations were revealed when phenylhydrazine replaced aniline as the amino base. Since Z-D-ala-gly produced a single insoluble product, Z-D-ala-gly-NHNHPh for all incubation periods between 0–72 h, it was treated with carbon in methanol, filtered, and recrystallized, m.p. 185–187°, $[\alpha]_D^{25°} = +12.2°$, in pyridine, and used as the TLC standard for integrity for all other Z-dipeptide reactions. With Z-L-ala-gly as the reactant with phenylhydrazine, Fig. 2 discloses that only Z-L-ala-gly-NHNHPh was formed within the initial 0–3 h period. For 3–6.5 h, both integrity, Z-L-ala-gly-NHNHPh, and cleavage, Z-L-ala-NHNHPh, took place simultaneously. These were later confirmed by melting points and optical rotations. Again, the Z-DL-ala-gly reactant yielded a more interesting situation. Z-Dipeptide integrity was shown for three periods, *viz.* 0–3 h, 6.5–24 h, and 24–48 h. However, for the 0–3 h interval, the mixture of enantiomers consisted chiefly of Z-L-ala-gly-NHNHPh. By contrast, the enantiomeric mixture for 6.5–48 h displayed over 99% of the D enantiomer, Z-D-ala-gly-NHNHPh. Within the interval 3–6.5 h, when both cleavage and integrity

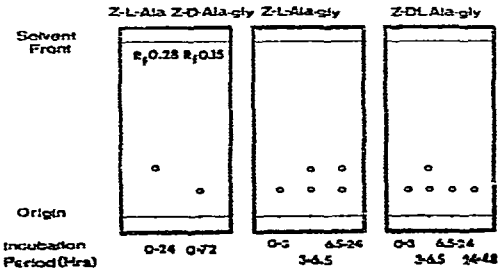


Fig. 2. TLC reproduction of papain-catalyzed products formed from phenylhydrazine and Z-L-alanine or the Z-alanylglycines after various periods of incubation. Solvent system: methanol–chloroform–hexane (2.5:20:15).

occurred, the split product was mainly Z-L-ala-NHNHPh and the unsplit product was substantially Z-L-ala-gly-NHNHPh.

Preparation of active papain

Dried papaya latex from the African Congo region, donated by the Wallerstein Company (Deerfield, Ill., U.S.A.) was processed by a slightly modified procedure of Bennett and Niemann[6] to yield dry, active papain.

Synthesis of standards. Z-L-Ala-NHPh[3,7] or Z-L-ala-NHNHPh[5] were prepared through papain-catalyzed reactions between Z-L-ala and aniline or phenylhydrazine. Z-L-Ala, 0.7965 g, and 0.52 ml of aniline or 0.60 ml of phenylhydrazine were dissolved in 36 ml of 0.50 *M* acetic acid buffer solution, pH 4.5, and 4 ml of hexamethylphosphoric triamide. To each solution was added 0.2000 g of L-cysteine·HCl·H_2O. Each solution was filtered and incubated at 40° in a glass-stoppered flask. At the end of 48 h the precipitates that were formed were removed by filtration, washed with 50 ml of cold water, dried at 40°, and recrystallized from methanol, resulting in Z-L-ala-NHPh, m.p. 161–163° and Z-L-ala-NHNHPh, m.p. 150–151.5°.

Z-D-Ala-gly-NHPh and Z-D-ala-gly-NHNHPh were prepared similarly from Z-D-ala-gly and aniline or phenylhydrazine. Each solution contained 1.0000 g of Z-D-ala-gly, dissolved in 36 ml of 0.50 *M* acetic acid buffer, pH 4.5, and 4 ml of hexamethylphosphoric triamide. To each solution was added either 0.52 ml of aniline or 0.60 ml of phenylhydrazine, plus 0.2000 g, each, of activated papain and L-cysteine·HCl·H_2O. Each solution was filtered, placed in a glass-stoppered flask and incubated at 40°. At the end of designated time intervals, over a period of several days, precipitates were removed by suction filtration and the filtrates were returned for incubation. The precipitates were washed with 30 ml of cold water and dried for 24 h at 40°. Since melting points and mixture melting points established that a single, uncleaved product was produced in each case, the combined precipitates for each were treated with activated carbon in methanol, filtered and recrystallized, resulting in Z-D-ala-gly-NHPh with m.p. 169–170°, $[\alpha]_D^{25}° = -13.6°$ and Z-D-ala-gly-NHNHPh had m.p. 185–187° and $[\alpha]_D^{25}° = +12.2°$.

TLC of standards. Flexible plastic plates coated with silica gel IB-F (7.5 × 15 cm) were used for establishing all thin-layer chromatograms. After preliminary experimentation, the satisfactory solvent used for the anilides was methanol–chloroform–hexane (2:19:19). After marking one spot at the origin line, Z-L-ala-NHPh was dissolved in the solvent and spotted five consecutive times, with drying each time, with the use of a capillary tube for spotting. At a second marked spot, Z-D-ala-gly-NHPh was similarly spotted with the same solvent. Three large filter papers were soaked with the solvent and evenly distributed around the inside wall of a large beaker to induce rapid chamber saturation with solvent vapor. Additional solvent was poured into the beaker, which was covered with a large watch glass and allowed to come to equilibrium at 25°. The chromatographic plate with the two spots was rapidly introduced into the beaker, which was again covered with the watch glass. The solvent was allowed to rise on the TLC plate to a level about 1 cm below the top. Removal of the plate was followed by immediately marking the solvent front and then drying. A short-wavelength UV lamp was used to locate the positions of the spots. The R_F value for Z-D-ala-gly-NHPh was 0.11, while the R_F value for Z-L-ala-NHPh was 0.32 at 25°. These are shown in Fig. 1.

For TLC standards in detecting integrity or cleavage during Z-dipeptide reactions with phenylhydrazine, the solvent used was methanol–chloroform–hexane 2.5:20:15). Similar chromatographic procedures disclosed for the standard Z-D-ala-gly-NHNHPh an R_F value of 0.15, while Z-L-ala-NHNHPh had an R_F value of 0.28 at 25°. Fig. 2 displays this record.

Reactions between Z-L-ala-gly or Z-DL-ala-gly and aniline or phenylhydrazine. Two solutions were made of Z-L-ala-gly. A 1.0000-g sample of the Z-dipeptide was dissolved in a mixture of 36 ml of 0.50 *M* acetic acid buffer, pH 4.5, and 4 ml of hexamethylphosphoric triamide. To one solution was added 0.52 ml of aniline and to the other was added 0.60 ml of phenylhydrazine. To each solution was then added 0.2000 g each of activated papain and L-cysteine·HCl·H_2O.

Two solutions were also made up for Z-DL-ala-gly, using a 1.0000 g sample of this Z-dipeptide. Other details were identical. Each of the four solutions was filtered and placed in separate glass-stoppered flasks and incubated at 40°. Precipitates were removed at designated time intervals, washed twice with 30 ml of cold water, each time, dried for 24 h at 40°, and weighed. The incubation time intervals are indicated in Figs. 1 and 2.

TLC detection of cleavage or integrity of Z-L-ala-gly and Z-DL-ala-gly anilides and phenylhydrazides. For each incubation period, an appropriate amount of the dried product from the reactants aniline and Z-L-ala-gly was dissolved in methanol–chloroform–hexane (2:19:19). Each solution was spotted five times at a marked position on the flexible plastic plate coated with silica gel IB-F. The same was done for dried products from aniline and Z-DL-ala-gly. Fig. 1 shows the results after chromatography and detection of cleavage or integrity, or both, by means of a short-wavelength UV lamp, as described previously.

Incubation period products were also collected for reactions between phenylhydrazine and either Z-L-ala-gly or Z-DL-ala-gly. Resultant chromatography with the use of the solvent system methanol–chloroform–hexane (2.5:20:15) are displayed in Fig. 2. When compared with the two standards, Z-L-ala-NHNHPh and Z-D-ala-gly-NHNHPh, cleavage, integrity or both cleavage and integrity were instantly evident.

Thick-layer chromatographic separations on ChromAR for mixtures of anilides or phenylhydrazides resulting from cleaved or unsplit Z-dipeptides

For those instances where TLC exposed both integrity and cleavage to occur during a set incubation interval, it was necessary to separate, isolate and identify the products in the dried product mixture collected from that incubation period. For mixed anilides, the same solvent system that was successful for TLC, Fig. 1, was employed for thick-layer chromatography. Similarly, for mixed phenylhydrazides, the same solvent system was used as had been employed for TLC (Fig. 2). A sheet of ChromAR, 10 cm × 20 cm, was repeatedly streaked at the origin line with a solution containing about 0.1000–0.2000 g of the mixed anilides or phenylhydrazides, dissolved in the solvent used for TLC. After thoroughly drying, the cloth-like sheet was pressed between two glass plates and placed in a desiccator. Solvent was introduced, the desiccator was closed, and chromatography was allowed to proceed until the position of the solvent front had moved close to the top of the sheet of ChromAR.

The ChromAR sheet was allowed to dry in the atmosphere and the zones of uncleaved and cleaved anilides were detected by means of a short-wavelength UV lamp. The same was true for mixed phenylhydrazides. Each zone was removed with scissors, then cut into small fragments and allowed to stand in 100 ml of chloroform, with occasional stirring, for 24 h. Suction filtration was followed by washing the ChromAR residue with more chloroform into the filtrate. The combined filtrate was evaporated to dryness in a rotary evaporator. Such solids were dried at 40° for 24 h.

It was also essential to recrystallize and identify the dried, single products, where only cleavage or only integrity, not both, were revealed by TLC. In the instance of Z-L-ala-gly, of course either Z-L-ala-NHPh or Z-L-ala-gly-NHPh were obtained as single products or else they were separated from a mixture of the two by ChromAR (Fig. 1). Also for Z-L-ala-gly, either Z-L-ala-NHNHPh or Z-L-ala-gly-NHNHPh were produced independently, or else they were separated from a mixture by CrhomAR (Fig. 2).

When Z-DL-ala-gly was the reactant with aniline (Fig. 1), the initial 0–3 h period showed that the Z-ala-gly-NHPh contained about 85% of the L enantiomer. The ChromAR separated mixture for the 3–6.5 h period indicated that the Z-ala-NHPh component contained about 98% of the L enantiomer, while the Z-ala-gly-NHPh portion included approximately 85% of the L enantiomer. The ChromAR separated mixture for the 6.5–24 h interval indicated that the Z-ala-NHPh was composed of about 98% of the L enantiomer but the Z-ala-gly-NHPh was composed of nearly 97% of the D enantiomer. The final product for the 24–168 h interval contained 98% of the D enantiomer in the single, uncleaved Z-ala-gly-NHPh.

For Z-DL-ala-gly and NH_2NHPh as the reactants (Fig. 2), the 0–3 h period contained Z-ala-gly-NHNHPh with 95% of the L enantiomer. For the ChromAR separated mixture of the 3–6.5 h interval, the Z-ala-NHNHPh was 89% of the L enantiomer, while the Z-ala-gly-NHNHPh was 93% of the L enantiomer. For the 6.5–48 h period, only uncleaved Z-ala-gly-NHNHPh was formed, composed of 99% D enantiomer.

DISCUSSION

It is apparent that for reactions with NH_2Ph or NH_2NHPh, Z-D-ala-gly is prevented from cleavage due to the D residue. Papain exhibits such a high degree of preference for an L residue[1,2] when the enzyme undergoes intermediate acylation, Enz-S-H → Enz-S-CO-R, that such acylation is forced to take place at the carboxyl of the glycine residue, with consequent Z-dipeptide integrity. Z-L-ala-gly reacts far more rapidly than the D enantiomer with NH_2Ph or NH_2NHPh during integrity or cleavage due to the influence of the L residue. When racemic Z-DL-ala-gly is the reactant, the D enantiomer cannot initially compete well with the L enantiomer for the active site of papain. Depletion of Z-L-ala-gly occurs rather early, by both cleavage and integrity. Then, the slower reacting Z-D-ala-gly takes over to yield a steady preponderance of uncleaved anilide or phenylhydrazide. During cleavage of Z-DL-ala-gly, only small quantities of Z-D-ala-NHPh or Z-D-ala-NHNHPh are mixed in with the far greater amounts of their L enantiomers. This is predictable on the basis of the chirality, conformation and catalytic mechanism of papain's action[1,2,8].

It should be pointed out that greater differences in R_F value are usually evident

during TLC of mixtures of cleaved and uncleaved anilides or phenylhydrazides from a specific Z-dipeptide in the solvent systems employed here (Figs. 1 and 2) than are shown between Z-amino acids and their corresponding Z-dipeptides for the conventional solvent systems used during their chromatography[9,10]. Furthermore, chromatography is fairly rapid (a little over 60 min) for the anilides or phenylhydrazides in the current work. For the Z-amino acids and Z-dipeptides nearly 3 h are required. The general TLC procedures used here are being employed advantageously in extentions of related work, with proper adjustments of solvent systems.

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