Transesterification Reactions Catalyzed by Papain*

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SUMMARY

The hydrolysis of a series of esters of benzoyl-l-arginine and glycylglycine by papain has been examined in mixtures of water with various alcohols in the pH range of 5 to 6. The presence of straight chain alcohols resulted in an apparent inhibition of acid release. It was shown that this inhibition could, in part, be accounted for by papain-catalyzed transesterification reactions between the various substrates and the alcohols studied. The products of transesterification, transamidation, and hydrolysis resulting on incubation of papain with esters of glycylglycine were separated and identified, and the kinetics of their formation were examined. Ficin was found to behave similarly to papain. The present study, in conjunction with earlier reports, suggests that the ability to catalyze alcoholytic reactions may be a property of many hydrolytic enzymes.

It is known from the work of a number of investigators that many proteolytic enzymes are inhibited by relatively low concentrations of normal aliphatic alcohols. In 1956, McDonald and Balls (1) showed that the liberation of titratable acid from 1-tyrosine ethyl ester by α-chymotrypsin was inhibited by a series of primary and secondary alcohols, and were able to show that the observable inhibition was due, at least in part, to chymotrypsin-catalyzed transesterification reactions. Chymotrypsin-catalyzed transesterification reactions have since been reported by a number of other investigators (2-4). Glazer showed that trypsin (5) and subtilisins (6) from the Carlsberg, Novo, and BPN' strains of Bacillus subtilis also readily catalyzed transesterification reactions.

Alcoholytic reactions are not restricted to the proteolytic enzymes. Indeed, the first instance recorded of such a reaction was the transformation of chlorophyll to ethyl chlorophyllide in the presence of the enzyme chlorophyllase noted by Tswett in 1908 (7) as explaining a result obtained by Borodin in 1882 (8). The plant proteases, papain and ficin, are known to be inhibited by alcohols (12, 13). Since no transesterification reaction involving a sulfhydryl proteolytic enzyme has as yet been reported, it was of interest to examine these two enzymes with respect to their ability to catalyze such reactions. It was found that both papain and ficin are capable of catalyzing transesterification reactions between ester substrates and a variety of straight chain aliphatic alcohols.

EXPERIMENTAL PROCEDURE

Materials—Papain was prepared by the method of Kimmel and Smith (14) and recrystallized three times. The protein was stored at 4°C as a crystalline suspension in saturated sodium chloride. Stock solutions were prepared either by dissolving a suitable aliquot of the suspension in distilled water, or, if a concentrated solution of the enzyme was required, by dialysis against distilled water. All of the enzyme solutions were kept in an ice bath and discarded after 24 hours. Less than 5% loss of activity was observed over this time period. Papain concentrations were determined spectrophotometrically by using \( E_{1}^{1\text{cm}} \) of 25.0 at 278 μM (15). When assayed in the presence of 0.004 M 2,3-dimercaptopropanol, with 0.05 M benzoyl-L-arginine ethyl ester as substrate at pH 5.5 and 37°C, the papain preparation had a \( C_{1} \) of 1.9 ± 0.1 (mean of 10 determinations).

A suspension of twice-crystallized ficin in 0.001 M ethylene-diaminetetraacetic acid, lot 660, was obtained from Worthington. Stock ficin solutions were obtained by the addition of suitable aliquots of the suspension to 0.05 M acetate buffer at pH 5.0. Traces of insoluble material were removed from the resulting solutions by high speed centrifugation and the solutions were stored in an ice bath. Ficin concentrations were determined spectrophotometrically on suitably diluted aliquots by using the relation \( A_{280} \times 0.44 = \text{milligrams of protein per ml} \) (16).

Chromatographically pure α-N-benzoyl-l-arginine methyl ester·HCl, lot N-1365, and α-N-benzoyl-l-arginine, lot G2356, were obtained from Mann and α-N-benzoyl-l-arginine ethyl ester·HCl, lot 611282, was from Calbiochem. Glycylglycine ethyl ester·HCl, lot R-4093, was obtained from Cyzco Chemical Corporation. Glycylglycine methyl ester·HCl, lot P2006, was obtained from Mann. This preparation was contaminated with between acetylcholine and various alcohols catalyzed by a cholinesterase from Pseudomonas fluorescens (11).

These observations suggested that many other hydrolytic enzymes may be capable of catalyzing alcoholytic reactions and that inhibition by normal aliphatic alcohols might be regarded as a suggestive indication in this connection.
The n-propyl, n-butyl, and n-amyl esters of glycglycine synthesized by the above procedure were found to be pure as judged by chromatography in n-butyl alcohol-acetic acid-water (200:30:75, v/v), n-butyl alcohol-pyridine-acetic acid-water (150:10:3:12, v/v), and by high voltage electrophoresis at pH 4.6.

Tetraglycine methyl ester and tetraglycine amyl ester were synthesized by the procedure described above for the preparation of glycglycine esters. The products were purified by chromatography in n-butyl alcohol-acetic acid-water (200:30:75, v/v). These derivatives were used solely for the comparison of the chromatographic and electrophoretic mobilities with those of the products of enzymatic transamidation reactions.

Analytical reagent grade alcohols were used throughout and were obtained from the following sources: methyl alcohol, J. T. Baker; ethyl alcohol, United States Industrial Chemical Company; n-amyl alcohol, Mallinckrodt; and the other alcohols used Matheson, Coleman, and Bell.

Determination of Esterase Activity—The rates of substrate hydrolysis were determined with the aid of a Radiometer pH-stat model TTT 1e, equipped with a model SBR2 recorder and a thermostated reaction vessel. The volume of the reaction mixture was 5 ml, and the titrations were performed at 37°C with either 0.025 or 0.25 N NaOH as the titrating agent. The details of the compositions of the various reaction mixtures used are given in the appropriate places in the text.

Chromatographic and Electrophoretic Separations—The various esters of glycglycine could all be separated by one-dimensional descending chromatography in n-butyl alcohol-acetic acid-water (200:30:75, v/v) on Whatman No. 3MM paper for 154 hours at pH 4.6. The chromatographic and electrophoretic mobilities of the various compounds examined are listed in Table I.

Separation of α-N-benzoyl-L-arginine and its methyl and butyl esters can be readily achieved by electrophoresis at pH 4.6 in Whatman No. 3MM paper at 60 volts per cm for 60 min. Under these conditions, α-N-benzoyl-L-arginine moved 9.1 cm, the butyl ester, 27.3 cm, and the methyl ester, 30.5 cm. The methyl and ethyl esters of α-N-benzoyl-L-arginine did not separate completely in this system.

Kinetics of Transamidation and Transmethylation—The kinetics of these reactions was followed by allowing the enzymatic reaction to proceed for various suitable time intervals and then stopping it by the addition of sufficient 1 N HCl to bring the pH to 2.4. The acidified mixtures were immediately frozen. Under these conditions, no detectable spontaneous hydrolysis of benzoyl-l-arginine esters occurred over a period of at least 4 hours of glycglycine esters over a period of at least 24 hours. At the end of the experiment, appropriate aliquots of the acidified mixtures were subjected to either electrophoretic or chromatographic separation. Several standards of different concentrations of the appropriate compounds were included on each chromatogram electrophoretogram. At the conclusion of the separation procedure, the papers were dried for 30 min at 60°C. In the case of the glycglycine compounds, the papers were then dipped in a drying oven at 80°C for 30 min; following this the col
development was allowed to go to completion by leaving the chromatograms in an ammonia-free atmosphere for 48 hours. The red bands were then cut out, the color was eluted with absolute methyl alcohol, and the amounts of the various components in the reaction mixture were determined from the absorbance at 500 mp. In this procedure, the color yields given by the various glycglycine esters and by glycglycine were equal within experimental error. The color yield of tetraglycine esters was assumed to be equal to that of tetracycline. In the case of separations involving α-N-benzoyl-L-arginine and its esters, the papers were dipped in a modified Sakauchi reagent (19) and the amounts of the various components determined qualitatively by comparison of color intensities with those given by known amounts of standards.

RESULTS

Hydrolysis of α-N-Benzoyl-L-arginine Esters by Papain in Water-Alcohol Mixtures—On incubation of the butyl and methyl esters of benzoyl-L-arginine with papain in water-alcohol mixtures, products of transesterification could be readily detected. The proportion of transesterification product to hydrolysis product observed under various conditions is given in Table II. The size of the alcohol used appeared to be an important factor in determining the extent of transesterification. Thus, from a consideration of solely the $K_m$ and $V_{max}$ values for the benzoyl-L-arginine esters (Table III), one would expect that the greatest accumulation of transesterification product would be found on incubation of the butyl ester with papain in presence of ethyl alcohol since the $K_m$ for benzoyl-L-arginine ethyl ester is considerably higher than that for the butyl ester and the $V_{max}$ values are approximately equal; whereas, in actual fact, it is found that methyl alcohol is considerably more effective than ethyl alcohol in the transesterification reaction. No accumulation of the butyl ester was detected on papain-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester in presence of 0.55 M n-butyl alcohol (Table II). This result is consistent with the low $K_m$ value of the butyl ester as compared to that of the ethyl ester and the large size of n-butyl alcohol. The relatively low solubility of n-butyl alcohol in water precluded studies in solutions of considerably higher alcohol content.

Hydrolysis of Glycglycine Esters by Papain in Aqueous Solution—In earlier work with trypsin (5), it was found that the rate of transesterification observed, at a given alcohol concentration, with "good" substrates, such as benzoyl-L-arginine ethyl ester, was lower than that observed with "poor" substrates, such as t-lysine methyl ester. This observation prompted a search for "poor" ester substrates of papain. The glycglycine esters were chosen for further study for a number of reasons. First, they are hydrolyzed by papain at rates considerably lower than the benzoyl-L-arginine esters. Second, the products of papain action could be completely and readily separated by suitable chromatographic and electrophoretic techniques. Last, the amounts of the various components of the reaction mixture could be accurately determined, following separation, by quantitative reaction with ninhydrin on paper.

Papain is known to be an efficient catalyst of transamidation reactions at alkaline pH values where a significant proportion of the α-amino group of peptides is in the uncharged form (21-24). To minimize these reactions, all of the studies with the glycglycine esters were performed at pH 5.0. At this pH, the α-amino group of glycglycine ethyl ester, $pK_1(NH_2^+)$ = 7.75 (25), and, of glycglycine, $pK_1 = 8.25$ (25), is 99.82% and 99.94% in the $NH_2^+$ form, respectively; nevertheless, papain-catalyzed transamidation reactions were found to proceed at a significant rate at this pH. Inasmuch as the over-all qualitative pattern of results was the same for the methyl, ethyl, $n$-propyl, $n$-butyl, and $n$-amyl esters of glycglycine, the discussion will deal mainly with the results obtained with glycglycine $n$-amyl ester.

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percentage of alcohol, by volume</th>
<th>Ratio of transesterification product to hydrolysis product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl-L-arginine butyl ester</td>
<td>50% methyl alcohol</td>
<td>~0.2</td>
</tr>
<tr>
<td>Benzoyl-L-arginine butyl ester</td>
<td>50% ethyl alcohol</td>
<td>~0.01</td>
</tr>
<tr>
<td>Benzoyl-L-arginine methyl ester</td>
<td>5% n-butyl alcohol</td>
<td>~0.01</td>
</tr>
<tr>
<td>Benzoyl-L-arginine ethyl ester</td>
<td>5% n-butyl alcohol</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-N-Benzoyl-L-arginine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl ester</td>
<td>0.0066 ± 0.0003</td>
<td>22.9 ± 1.1</td>
</tr>
<tr>
<td>α-N-Benzoyl-L-arginine</td>
<td>0.022 ± 0.003*</td>
<td>18.7 ± 1.9</td>
</tr>
<tr>
<td>ethyl ester</td>
<td>0.0039 ± 0.0005</td>
<td>23.6 ± 3.0</td>
</tr>
</tbody>
</table>

* A $K_m$ of 0.018 was reported for α-N-benzoyl-L-arginine ethyl ester by Smith and Parker (20) under the same conditions as above.

Unpublished observations.
Glycylglycine n-amyl ester was incubated with papain at pH 5.0 in aqueous solution, and the reaction mixture was analyzed at various times during the first 35% of substrate hydrolysis. The reaction mixture was found to contain, in addition to the substrate, tetraglycine n-amyl ester, glycylglycine, tetraglycine, and traces of hexaglycine. All of these products were identified by direct comparison with the authentic synthetic systems. On further incubation (50% hydrolysis), traces of higher glycine polymers appeared. When glycylglycine

experiment, it was found that tetraglycine was split to glycylglycine extremely slowly by papain under the above conditions. The results presented above can be readily accounted for by the following papain-catalyzed transesterification and hydrolytic actions.

\[
\text{Glycylglycine n-amyl ester} + \text{papain} \rightarrow [\text{glycylglycyl-papain}] + \text{n-amyl alcohol} \quad (1)
\]

\[
[\text{Glycylglycyl-papain}] + \text{H}_2\text{O} \rightarrow \text{glycylglycine} + \text{papain} \quad (2a)
\]

\[
[\text{Glycylglycyl-papain}] + \text{glycylglycine n-amyl ester} \rightarrow \text{tetraglycine n-amyl ester} + \text{papain} \quad (2b)
\]

Tetraglycine n-amyl ester + papain ⇌ [tetraglycyl-papain] + n-amyl alcohol (3)

\[
[\text{Tetraglycyl-papain}] + \text{H}_2\text{O} \rightarrow \text{tetraglycine} + \text{papain} \quad (4)
\]

During the initial stages of hydrolysis, Reactions 2a and 2b were found to proceed at comparable rates. The transamidation reaction (2b) is analogous to those described for papain a number of years ago by Fruton et al. (21–24), e.g.

Carbobenzoxyglycinamide + L-Leu-Gly ⇌ carboxbenzoxy-Gly-L-Leu-Gly + NH₃

The reaction

Glycylglycine n-amyl ester + glycylglycine \[\text{papain}\] \rightarrow \text{tetraglycine n-amyl ester}

does not appear to take place at a measurable rate at pH 5.0 even when glycylglycine is present in equimolar concentration with the ester substrate. Since Reaction 2b above, does proceed readily, it is clear that the ester substrate competes successfully with glycylglycine as a replacement agent under these conditions. This observation complements that of Mycek and Fruton (24), who showed that in an equimolar mixture of carboxbenzoxyclycinamide, Gly-Gly, and L-Leu-Gly at pH 7.3, L-Leu-Gly inhibited the reaction leading to carboxbenzoxyclycinamide by 75% while the reaction leading to carboxbenzoxyclycinamide-L-Leu-Gly proceeded at the same rate as in the absence of Gly-Gly.

No measurable amounts of free glycine or triglycine were detected in the reaction mixtures. Clearly, the ester linkage is hydrolyzed very much faster than the peptide linkages. Papain did not release glycine or triglycine from tetraglycine or glycylglycine. This is in accord with the known specificity of papain on peptide substrates. Papain cleaves the peptide bond adjacent to a free \(\alpha\)-carboxyl group extremely slowly (20).

The relative affinity of papain for the various glycylglycine esters under the conditions used for this study was examined by determining the initial rate of production of titratable acid for each of the esters over a wide range of substrate concentration. The observed base uptake was a measure of the sum of Reaction 2a and 3, i.e., of the rate of production of glycylglycine and tetraglycine. From the values given in Table IV, it may be seen that the relative rate of hydrolysis of the glycylglycine esters was in the strict order of chain length: n-amyl > n-butyl > n-propyl ethyl > methyl. Obviously, it is impossible to calculate from these data alone actual \(K_m\) and \(V_{max}\) values for these substrates.
in view of the transamidation reactions which proceed at rates comparable to those of the hydrolytic reactions.

**Hydrolysis of Glycylglycine Esters by Papain in Water-Alcohol Mixture**—Low concentrations of normal straight chain alcohols inhibited papain-catalyzed liberation of titratable acid from all of the glycylglycine esters studied. Representative results are shown in Table V. n-Amyl alcohol was found to be the most effective inhibitor followed by n-butyl, n-propyl, ethyl, and methyl alcohols in the order given.

Examination of reaction mixtures containing papain, a glycylglycine ester, and the various straight chain alcohols by electrophoresis and chromatography showed that in every case a transamidification reaction had occurred between the glycylglycine ester and the alcohol present. For example, incubation of glycylglycine n-amyl ester with papain in water-methanol solution resulted in the formation of glycylglycine methyl ester and tetraglycine methyl ester in addition to all of the products formed in aqueous solution, namely, tetraglycine n-amyl ester, glycylglycine, and hexaglycine (see Fig. 1). The formation of glycylglycine methyl ester and tetraglycine methyl ester can be accounted for by a combination of Reactions 1 and 3 (above) with Reactions 5, 6, and 7.

\[
\text{[Glycylglycyl-papain]} + \text{methanol} \rightleftharpoons \text{glycylglycine methyl ester} + \text{papain}
\]  
\[5\]

\[
\text{[Tetraglycyl-papain]} + \text{methanol} \rightleftharpoons \text{tetraglycine methyl ester} + \text{papain}
\]  
\[6\]

\[
\text{[Glycylglycyl-papain]} + \text{glycylglycine methyl ester} \rightleftharpoons \text{tetraglycine methyl ester} + \text{papain}
\]  
\[7\]

The kinetics of the reactions occurring during the early stages...
of the papain-catalyzed hydrolysis of the n-amyl and n-butyl ester of glycylglycine in water-methyl alcohol solutions were studied in detail. The results are presented in Fig. 2. From these results, it may be seen that the transesterification reaction (Reaction 5) proceeds at a rate comparable to that of the hydrolytic reaction (Reaction 2a) and to that of the transamidation reaction (Reaction 2b).

**Ficin-catalyzed Transesterification and Transamidation Reactions**—A brief survey was carried out of the action of ficin on the various glycylglycine esters in both aqueous and alcoholic solutions. The considerable similarity, which has long been recognized to exist between the mechanisms of action of these two enzymes (see Reference 26 for a review), was clearly evident in the present study. Ficin catalyzed all of the transamidation and transesterification reactions described above for papain (see Fig. 1, for example), and showed, qualitatively, the same pattern of inhibition by straight chain aliphatic alcohols.

**DISCUSSION**

In earlier studies (1–6), it has been shown that the “serine” enzymes (chymotrypsin, trypsin, and the subtilisins) are all efficient catalysts of transesterification reactions. The present investigation has shown that the “sulfhydryl” enzymes (papain and ficin) are also able to catalyze such reactions. This finding, taken in conjunction with reports of studies on a variety of hydrolytic enzymes (7–11), strongly suggests that many more hydrolytic enzymes will be found capable of using alcohols in place of water, and that the possibility of transesterification should be examined carefully whenever studies are performed in water-alcohol media.

The transesterification reactions appear to account for only about 20% of the observed inhibition by alcohols of papain-catalyzed release of titratable acid from esters of glycylglycine. The effect of the alcohols on the dielectric constant of the medium undoubtedly plays a major role in the inhibition as shown in earlier studies on papain by Stockell and Smith (12) and Smith, Finkle, and Stockell (27). The esters of benzoyl-L-arginine have a lower \( K_a \) and higher \( V_{max} \) values than the amide (28). The failure to observe alcoholytic reactions in earlier studies with papain (5, 12) in which benzoyl-L-arginine amide was used as substrate was undoubtedly in part due to the failure of the product of the alcoholytic reaction to accumulate under the particular conditions used. This emphasizes the importance of investigating a variety of combinations of substrates, alcohols, and pH in order to rule out alcoholytic reactions whenever alcohols are used in studies of the influence of dielectric constant on the catalytic process.

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