Phenolic Hydroxyl Ionization in Papain*

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Various properties of crystalline papain have been under investigation in this laboratory for several years in attempts to understand the specificity, mode of action, and other aspects of the behavior of this enzyme (1). Inasmuch as papain must be in its native state to retain its enzymic activity, a knowledge of the role of the amino acid side chains in maintaining conformation is essential. The difference spectrum obtained by comparison of papain in acid and neutral solution exhibits peaks at 279 and 287 mμ (2) and is suggestive of the involvement of the phenolic hydroxyl groups in hydrophobic or hydrogen bonding.

Crammer and Neuberger (3) developed a spectrophotometric procedure to investigate the ionization of phenolic groups in ovalbumin and insulin. Similar investigations have since been performed on poly-L-tyrosine (4), serum albumin (5), ribonuclease (6, 7), lysozyme (8, 9), chymotrypsinogen (10), trypsinogen (11), myosin, and the meromyosins (12). These studies have shown marked differences in the ionization behavior of phenolic groups in various proteins.

This paper presents an investigation of the behavior of the phenolic hydroxyl groups in papain by means of spectrophotometric titration of this protein in the range from pH 5.5 to 14.

EXPERIMENTAL PROCEDURE

Papain was prepared by the method of Kimmel and Smith (13). Ovalbumin, twice crystallized, salt-free, was obtained from Nutritional Biochemicals Corporation. Lysozyme was obtained from Armour and Company (Lot No. 003L1). Fresh stock solutions of twice recrystallized, salt-free lyophilized papain were prepared each day and stored at 4°C. Protein concentration was determined from the absorption at 278 mμ in an appropriately diluted solution. The value of ε278 was found to be 25.0 for anhydrous, ash-free papain. The value for the molecular weight of papain used in all calculations was 20,700.

A stock solution of sodium hydroxide was made by diluting a saturated solution with carbon dioxide-free water. The concentration was checked by titration against standard potassium hydroxide. Stock solutions for measurement were in the concentration range 1 to 2 x 10⁻⁶ M papain, and the pH was adjusted by careful addition of appropriate amounts of acid or base. Sodium chloride was used to adjust the ionic strength of each solution to either 0.01 or 0.2.

Measurements of pH were made with a Radiometer-Copenhagen type TTT18 autotitrator equipped with a type C electrode. Correction for sodium ion errors was made with the aid of a nomograph supplied with the instrument. Solutions of high pH (in the range 11.5 to 13.0) were NaOH solutions of known molality containing sufficient sodium chloride to bring the ionic strength to 0.2. The pH of these solutions was computed from the activity coefficients obtained from hydrogen electrode measurements by Tanford (14). The Radiometer autotitrator and the Cary spectrophotometer (see below) were both located in a room where the temperature was maintained at 27°C ± 0.2°C.

A Cary model 14 recording spectrophotometer was used for absorption measurements. Cylindrical tightly stoppered cells with a 10-mm light path were employed. The cells were matched with water over the range 400 to 220 mμ with the multipots on the instrument.

RESULTS

Ultraviolet Absorption Spectrum of Papain—The spectrum of papain in neutral solution, immediately after adjustment to pH 12, and immediately after adjustment to pH 13, is shown in Fig. 1, which shows that not all the phenolic hydroxy groups are ionized at pH 12 (Table I). In fact, the situation appears to be very similar to that described by Crammer and Neuberger (3) for ovalbumin.

It was observed that the absorbancy of papain solutions in the pH range of 12 to 12.5 changes with time. A typical plot of change in absorbancy as a function of time is shown in Fig. 2. The molar extinction at pH 12.2 increases from about 40,000 (obtained within 30 seconds of adjusting the pH) to 51,000 ± 1,000 at the end of approximately 60 minutes at 27°C. Adjustment of a solution from pH 6 to 13 is accompanied by an instantaneous change in the molar extinction from 12,500 to about 50,000. A slow increase in absorbancy at 295 mμ is then observed, a final value of 52,500 being reached in about 30 minutes at 27°C. Exposure to normal sodium hydroxide (near pH 14) for 2 or 3 minutes at 39°C results in a final molar extinction of 52,800 at 295 mμ.

It was essential to decide whether the time-dependent changes at 295 mμ observed above pH 12 were due to slow ionization of a few phenolic groups, or, whether they were due to some other cause such as an increase in light scattering caused by denaturation and aggregation. The ionization of the phenolic groups in tyrosine (3), polytyrosine (4), and in a number of proteins (3, 7) is accompanied by a large increase in extinction at 295 mμ, a considerably smaller change at 265 mμ, and a very small change at 278 mμ. It was observed that the wave length dependence of the increase in absorbancy of papain solutions follows that of tyrosine. It thus appears that the changes with the protein are due to a slow ionization of phenolic groups.

A similar situation has been reported for ribonuclease (7). In this protein, a slow ionization of three phenolic groups takes...
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![Figure 1](image1.png)

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>Conditions*</th>
<th>No. of ionized phenolic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.8</td>
<td>5 minutes at 27°</td>
<td>11.6</td>
</tr>
<tr>
<td>13.0</td>
<td>30 seconds at 27°</td>
<td>15.5</td>
</tr>
<tr>
<td>14.0</td>
<td>5 minutes at 39°</td>
<td>17.0</td>
</tr>
</tbody>
</table>

* All solutions were 0.2 M in NaCl.
† Normal sodium hydroxide solution.

place at pH values greater than 12, the wave length dependence of the process being very similar to that observed with papain. On the other hand, the slow change in absorbancy observed with bovine serum albumin at pH values greater than 12, shows the greatest change at 265 mp, a smaller one at 278 mp and the smallest at 295 mp, and has thus been attributed to a denaturation process (5).

**Change in Molar Extinction**—The molar extinction at 295 mp of solutions of papain in the pH range 5 to 8 is 12,500. This value was taken as the molar extinction of papain before the ionization of the phenolic groups. When the curves for absorbancy at 295 mp versus time at pH values greater than 12 are examined (e.g. Fig. 2), it is found that these approach the limiting value of 52,800. Amino acid analysis (1) as well as spectrophotometric determinations (see below) have indicated the presence of 17 tyrosine residues in the papain molecule. The total change in the molar extinction at 295 mp is 40,300. This corresponds to a change of 2,370 per tyrosine residue. This is in good agreement with the value of 2,300 for tyrosine at the same wave length (3). Tanford and Roberts (5) found the change at 295 mp per phenolic group to be 2,430 for bovine serum albumin. The value of 2,630 was obtained with ribonuclease (7).

**Reversibility**—The titration curve for papain shown in Fig. 3 can be fitted with a single S-shaped curve in the pH range 8.0 to 11.5. Over this pH range, the ionization is completely reversible. Exposure of papain to pH values higher than 12.0 leads to irreversible changes in the ionization behavior. The curve obtained by back titration of a solution which had been kept at pH 13 for a sufficient time to attain complete ionization equilibrium is also shown in Fig. 3.

After exposure to a pH value greater than 12 for a few minutes, the protein becomes insoluble at pH values below 9.5, (presumably, because of denaturation and aggregation), and the ionization reversal curve cannot be continued below this pH. The results obtained in the pH range 9.5 to 13.5 indicate that all the 17 tyrosine hydroxyl groups show normal ionization behavior after the exposure to pH values greater than 12 for sufficient time to attain equilibrium.

**Spectrophotometric Determination of Tyrosine and Tryptophan Content**—The ultraviolet absorption spectrum of papain in alkaline solution was studied under a variety of conditions. The methods of Goodwin and Morton (15) and of Benche and Schmid (16) were applied to the determination of the tyrosine to trypto-
Tyrosine to tryptophan ratio in papain and ovalbumin
Tyrosine to tryptophan ratios reported in the literature are 3.4:1 for papain (1) and 2.5:1 for ovalbumin (3).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conditions</th>
<th>No. of determinations</th>
<th>Tyrosine-tryptophan ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Benche and Schmid method</td>
</tr>
<tr>
<td>Papain</td>
<td>pH 13, 27°, 5 min</td>
<td>4</td>
<td>2.70 ± 0.10 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>pH 13, 27°, 17 hours</td>
<td>4</td>
<td>2.95 ± 0.05 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>pH 13.5, 39°, 90 min</td>
<td>2</td>
<td>3.25 ± 0.05 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Denatured at pH 1.7 at 92° for 5 min, then pH 13.5, 39°, 30 min</td>
<td>3</td>
<td>2.80 ± 0.10 ± 0.24</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>pH 13, 27°, 5 min</td>
<td>2</td>
<td>2.65 ± 0.05 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>pH 13, 27°, 17 hours</td>
<td>2</td>
<td>2.65 ± 0.05 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Denatured at pH 1.7 at 92° for 5 min, then pH 13, 27°, 5 min</td>
<td>2</td>
<td>2.65 ± 0.05 ± 0.05</td>
</tr>
</tbody>
</table>

DISCUSSION

Phenolic hydroxyl ionization in papain

The tyrosine to tryptophan ratio in lysozyme was also determined under various conditions. A tyrosine to tryptophan ratio of 0.28 ± 0.02 was obtained by the Benche and Schmid method after exposure of lysozyme to 0.1 N NaOH for 5 minutes at 27°.

This ratio changed to 0.49 ± 0.02 after exposure of this protein to 0.1 N NaOH for 17 hours at 27°.

The ionization of the phenolic groups of papain has been determined by spectrophotometric titration. The titration of 11 to 12 tyrosine residues in the pH range 9.0 to 12.0 has been found to be normal and reversible. These phenolic groups have an observed pK' value of 10.3 ± 0.1 with a calculated intrinsic pK value of 9.8 ± 0.1. A slow ionization of four groups takes place in the pH range 12 to 13. These residues are instantaneously ionized at pH 13. One to two residues ionize slowly above pH 13. The titration curve for the phenolic groups of papain, exposed to a pH greater than 12, is irreversible. The tyrosine and tryptophan content of papain has been determined spectrophotometrically and is in good agreement with that obtained by amino acid analysis.

REFERENCES

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