Hydrolysis of Irradiated Ovalbumin by Pepsin

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ABSTRACT Solid ovalbumin has been irradiated at three doses, 6.5, 25, and 40 megarads, under high vacuum. The native and irradiated samples have been hydrolyzed at pH 1.4 by pepsin, after centrifugation of the aggregates, if necessary. The number of bonds broken per ovalbumin molecule has been estimated by comparing the rate of protein destruction with the rate of formation of NH₃ groups. Both rates increase very much with the dose, but the number of bonds broken decreases. Sedimentation measurements show a strong shape modification of the soluble fraction in the case of the 25 and 40 megarad samples. The increase in asymmetry is bound to the increase in the rate of attack on γ-irradiated ovalbumin by pepsin. Infrared spectra of the aggregates show small difference from those of the native samples.

It was early recognized that proteins were very sensitive to electromagnetic waves of great frequencies. Already, in 1934, J. H. Northrop (1) studied the effects of ultraviolet light and gamma and beta rays (from radium) on the inactivation of pepsin. He supposed that a different amount of energy is necessary to inactivate pepsin at different wave-lengths but that it is also possible for energy to be absorbed without causing inactivation.

In this paper, we discuss some aspects of the effects of the very energetic gamma rays of a ⁶⁰Co source upon ovalbumin molecules irradiated in the solid state. Serological studies and thermal stability research (2) led to the conclusion that ovalbumin treated by ionizing radiations contains a broad range of protein molecules in which the secondary hydrogen-bonded structure is in various stages of unfolding. According to Linderström-Lang’s concept, an enzyme, like pepsin, should then act more profoundly on such a partially unfolded structure than it does on the native globular protein.

We have followed the action of gamma rays on the solid ovalbumin by measuring the rate of pepsin hydrolysis and also by ultracentrifugation.
EXPERIMENTAL

Irradiation of the Samples

The ovalbumin was recrystallized three times with anhydrous sodium sulfate by the method of Kekwick and Cannan (3), dialyzed free of salt as judged by protein nitrogen determinations, and lyophilized.

The lyophilized ovalbumin was placed under high vacuum in glass ampules of 16 m/m diameter, each of them containing 2 gm of protein. They were irradiated at 20°C in an homogeneous gamma ray field of a 160 curies 60Co source. The dose rate was estimated by ferrosulfate spectral dosimetry (G_{\gamma} = 15.5); it was of the order of magnitude of 1.10^6 rad/hour, and it was also calculated in terms of electron volts absorbed per protein molecule (ev/molecule), using the 44,000 molecular weight value and the atomic absorption coefficients for the photoelectric and Compton effects.

To reduce or avoid any modification after irradiation, the samples were kept at low temperature (−15°C) and analyzed as soon as possible. A fairly good vacuum was kept in the ampules after a 40 megarad integral dose and, despite a relatively strong odor of burned hairs, evolution of gas was negligible during the irradiation.

Hydrolysis by Pepsin

Native and irradiated samples were dissolved in twice distilled water to give solutions having a concentration of 4 per cent (grams in 100 ml); the pH was adjusted to 1.4 with \( \text{HCl} \) hydrochloric acid.

Owing to internal modifications, irradiated samples at a dose equal to or higher than 25 megarads were not completely soluble; it was necessary to centrifuge the undissolved part. Nitrogen microkjeldahl estimations carried on the perfectly clear supernatants give the percentage of ovalbumin remaining in solution (35 per cent for the 25 megarad irradiated sample and 30 per cent for the 40 megarad irradiated sample).

A solution containing 0.5 mg of pepsin per ml at pH 1.4 was added to these solutions so that for each gram of ovalbumin present there was 0.5 mg of pepsin. The temperature of hydrolysis was 36°C; one measurement was made at 30°C in order to compare the activity of our pepsin sample with published values (4).

In each experiment, 10 ml samples were removed after zero, 20, 40, 60, 80, and 100 minutes, neutralized to pH 4–5 with solid NaHCO\(_3\), and placed on a boiling water bath for at least 5 minutes. The samples were made up to 25 ml, filtered, and the clear filtrate put into the refrigerator. Solid irradiated ovalbumin does not give rise to appreciable amounts of non-protein nitrogen. Determinations of total nitrogen were made on 2 cm\(^3\) aliquots of these coagulated digests by the classical microKjeldahl procedure and the nitrogen values were converted to protein using the conversion factor 6.7 for native ovalbumin. The percentage of hydrolysis was accepted as being proportional to the ratio of peptides after hydrolysis to the total ovalbumin present before hydrolysis. The amino nitrogen formed during the hydrolysis was estimated
by a ninhydrin colorimetric micromethod (5), using a 0.5 cm³ aliquot of the correctly
diluted digests and referring to standards of glycine and native ovalbumin.
In both determinations (total and amino nitrogen), blanks and controls were run
on the uncoagulated digests.

**Sedimentation Measurements**

Sedimentation measurements were made at three concentrations in a Spinco model
E ultracentrifuge. Native ovalbumins and those irradiated at 6.5 megarads were
dissolved directly in a sodium acetate–acetic acid buffer at pH 3.95 and μ = 0.1.
PpH 1.4 soluble fractions of ovalbumins irradiated at 25 and 40 megarads were diluted
to pH 3.95 with a sodium acetate solution to get a final ionic strength of about 0.1.
Sedimentation constants were corrected for temperature taking into account only
the viscosity factor. If necessary, extrapolations to zero concentration of the sedi-
mentation constants were made by plotting the reciprocal value of the sedimentation
constant as a function of the concentration.

**RESULTS**

*Hydrolysis*

The percentage of hydrolysis and the amino nitrogen milliequivalent content
per gram of dissolved protein are presented in Figs. 1 and 2 as functions of the time expressed in minutes.

For the native protein, the two curves at 30°C agree with those found elsewhere (6). The initial rate of hydrolysis at 36°C for the native sample is about 25 per cent per hour while at 30°C it is only 16 per cent per hour.

With increasing doses of irradiation both initial rates increase strongly.

\[ \alpha = -\left[\frac{d[P]}{dt}\right]_0 \cdot \frac{I}{[P]} \]

\[ \beta = +\left[\frac{d[NH_2]}{dt}\right]_0 \cdot \frac{I}{[P]} \]

where \([P]\) is the total initial concentration of ovalbumin in the solutions submitted to the enzymatic hydrolysis and \([NH_2]\) is the concentration in amino groups which are formed by hydrolysis.

The ratio \(\beta/\alpha\) gives the number of bonds broken in the enzymatic process. All these ratios are summarized in Table I.

The weight ratio of the enzyme to the protein is constant and equal to \(5.10^{-4}\), so that at the start of the hydrolysis there is in each case 1 molecule of pepsin for about 1580 molecules of ovalbumin.

**Figure 2.** Milliequivalents of NH₂ groups as a function of the time in minutes.

From our experimental data, we have calculated the following initial ratios:
It is therefore possible to estimate the number of ovalbumin molecules transformed by one molecule of pepsin during 1 hour at the initial rate of hydrolysis. The results in function of the dose are indicated in Table II, where we also have noted the average time of attack on an ovalbumin molecule by one pepsin molecule.

**Sedimentation**

Sedimentation constants extrapolated to zero concentration and corrected

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature °C</th>
<th>Average No. of ovalbumin molecules destroyed per hr.</th>
<th>Average time of attack on one molecule in sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>250</td>
<td>14.2</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>395</td>
<td>9.1</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>555</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>790</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>1580</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Same as in Table I.

**Table III**

Constants and slopes of sedimentation

<table>
<thead>
<tr>
<th>Sample*</th>
<th>$S$, $10^4$ sec.⁻³</th>
<th>$K$, $10^{-26}$ (sec. gm/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>3.20</td>
<td>Zero</td>
</tr>
<tr>
<td>3</td>
<td>3.18</td>
<td>Zero</td>
</tr>
<tr>
<td>4</td>
<td>2.95</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>2.62</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Same as in Table I.
for temperature (20°C) are given in Table III. The slopes $K$ of the extrapolation lines, $I/S$ vs. $C$, are also given.

**DISCUSSION**

From the results given in Table I, it appears that the rate of ovalbumin hydrolysis by pepsin increases with the dose; at a dose of 180 ev/molecule it is at least four times greater than that of the native sample. This coefficient is certainly too small because, in Experiments 4 and 5, the absolute concentrations of ovalbumin and pepsin were almost four times lower than in the other experiments. However, the number of bonds broken per molecule, given by the ratio $\beta/\alpha$, does not depend on the concentration of the system: substrate-enzyme, and the experimental values show that this ratio tends to decrease a little with the dose.

The degradation of an irradiated ovalbumin molecule takes place with the appearance of a smaller number of NH$_2$ groups; one may say that this sensitivity of such a molecule to pepsin action has increased with the dose.

Furthermore, the results of ultracentrifugation are important because they show a net rise of the slope $K$ with the dose.

Such a change may induce an easier attack on ovalbumin molecules by pepsin. If there is no change in the molecular weight of the soluble fraction of the 40 megarad sample, a sedimentation constant of 2.65 and a slope $K$ of 0.40 are compatible with a frictional ratio of 1.65.

Moreover, this high asymmetry appearing with irradiation is probably responsible for the important aggregation observed after irradiation. Comparative infrared spectra of native and 40 megarad ovalbumins show only small differences in the general shape of the very large stretching vibrations of NH and CO groups. However, in the aggregates obtained after centrifugation and lyophilization of this sample, one notes the disappearance of the 1400 cm$^{-1}$ CH$_2$ deformation vibration, which is known to be very sensitive to the surrounding groups.

Dr. Dieu is an associate of Fonds National de la Recherche Scientifique (Belgium).

**REFERENCES**