STUDIES ON THE DENATURATION OF EGG ALBUMIN UNDER HIGH PRESSURE

By Keizo Suzuki*

A number of studies have been carried out on the denaturation of protein under high pressure. Bridgman¹ has reported that egg white coagulates completely at 7000 atm within 30 minutes, and Kiyama et al.² have confirmed that the coagulation occurs above 3880 kg/cm². Grant et al.³ and Suzuki et al.⁴ have found that SH groups are detected in the compressed sample of egg albumin. On the other hand, Johnson et al.⁵ and Tongura⁶ have reported that the thermal denaturation of proteins is retarded in a few examples by the pressure of about 1000 atm.

In order to discuss the nature of denaturation of proteins in relation to pressure, especially to discuss the relation between these two opposing roles of pressure, i.e. acceleration and retardation, more abundant and more quantitative informations should be required.

In the present research, egg albumin was used, as a typical globular protein, and its rate of denaturation under different pressures and temperatures was studied from the measurement of concentration change due to precipitation induced by denaturation.

Experimentals

Preparation of egg albumin Egg albumin was prepared from hen's egg white by the method of Sørensen and Hørup⁷, recrystallized three times, dialyzed against water until free from ammonium sulfate, and stored as the stock solution in a refrigerator.

This stock was diluted with water and acetate buffer of pH 4.8 (except for the studies of the effect of pH) to a given protein concentration and a buffer concentration (usually 0.1 M), and used as a test solution.

It was found that the rate of denaturation is very sensitive to the freshness of egg albumin, even if stored in a refrigerator, so that newly prepared egg albumin was used within a week.

High pressure apparatus and procedures The schematic layout of the compressing apparatus is shown in Fig. 1. The experiments were carried out in the part A of the figure.

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¹ P. W. Bridgman, J. Biol. Chem., 19, 511 (1914)
² R. Kiyama and T. Yanagimoto, This Journal, 21, 41 (1931)
³ E. A. Grant, R. B. Dow and W. R. Franks, Science, 94, 616 (1941)
⁷ S. P. L. Sørensen and M. Hørup, Compt. rend. trav. lab. Carlsberg, 12, 12 (1915/17), cf. Protein, Nucleic acid and Enzyme 2, 212 (1957)
where a test solution sealed into a polyvinyl chloride sack was charged. The reaction temperature was kept constant by circulating water from the thermostat through the jacket surrounding the vessel, but owing to the large heat capacity and heat conductance of the apparatus, the temperature in the vessel was often found to differ from that of the thermostat. So the temperature of the vessel was measured directly by means of the thermister inserted in a small side-chamber of the vessel K.

After a definite time under compression, the sack containing the test solution was taken out and the sample was filtered, and then the protein concentration of the filtrate was determined by the colorimetric measurement of biuret reaction\(^8\), corrected by the micro-Kjeldahl analysis of nitrogen\(^9\), by means of an electron-tube-photometer.

### Results

**Effect of pH** The effect of pH on the pressure denaturation of egg albumin was studied in the pH range 4.0~7.0. The effect was examined by applying about 5000 kg/cm\(^2\) for 10 minutes. In this experiment only, Merck's impalpable powder of egg albumin was used. The test sample contained ca. 0.7 mg/ml of protein nitrogen, and its pH was adjusted with HCl or NaOH without using a buffer solution. After the application of pressure, the pH of the solution was brought to 4.8 with acetate buffer. After centrifugation, the nitrogen concentration in the supernatant was determined by the micro-Kjeldahl analysis.

The results are shown in Fig. 2. The nitrogen concentration in the supernatant takes a minimum value at about pH 4.8, the isoelectric point of egg albumin, namely, the rate of denaturation by pressure apparently reaches maximum here. Therefore, to minimize the complication due to the effect of pH, all other runs were carried out at pH 4.8.

**Effect of pressing duration** A number of experiments were carried out on the effect of

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It was found that the optical density of biuret reaction is linear to the protein concentrations, so that the values of optical density are taken directly as the measure of protein concentration in this figure.

Pressing duration on the denaturation of egg albumin. A representative part given in Fig. 3 shows that the plots of the logarithm of the protein concentration in the supernatant against pressing duration constitute straight lines for the given pressures and temperatures, hence the denaturation of egg albumin by heat or pressure is of the first order.

Reversibility of denaturation Although it is found in a few examples that their denaturation by heat or pressure is reversible, yet under the present experimental conditions such a case is not found. No difference was found between the sample filtered immediately after the application of pressure and that filtered after being stored 2 or more days in the refrigerator at the isoelectric point or at more alkaline side of pH.

Effect of pressure The effect of pressure at constant pressing time was investigated at several temperatures. A given pressure was applied for 5 or 10 minutes to an egg albumin solution with M/10 acetate buffer of pH 4.8, containing ca. 0.5-1.5 mg of protein nitrogen per ml, and the remaining protein in the supernatant was determined. The results below 50°C as given in Fig. 4 show similar patterns: above a certain pressure (about 4000 kg/cm²), further increase in pressure results in a rapid increase of the precipitation up to a second point which varies with
Studies on the Denaturation of Egg Albumin under High Pressure

Effect of temperature

As shown in Fig. 4, the increase in temperature shifts the concentration-pressure curve to the right side in the range 10–40°C, namely it decreases the rate of denaturation. But the relation is reciprocal above this temperature. Another effect is that the increase in temperature lessens the slope of the curve slightly in the whole temperature range of the experiment.

Effect of temperature and pressure at relatively high temperature

Approximately above 60°C, the so-called thermal denaturation occurs at atmospheric pressure. Fig. 5 shows the effects of pressure at 65 and 70°C at a definite pressing duration. In this experiment, considerable amounts of protein coagulates before the pressure reaching to an appointed value, and after releasing pressure. These values are determined in a blank test and are subtracted from the experimental values. As shown in the figure, the increase in pressure rather increases the protein concentration in the supernatant below about 3000 kg/cm²; in other words, denaturation was retarded by pressure. But the curves corresponding to the range above 4000 kg/cm² show the similar patterns to the cases at lower temperatures; pressure causes a rapid decrease in protein concentration in the supernatant to zero, above a certain pressure.
Considerations

From the experimental results mentioned above, it seems that there are two features of denaturation processes under high pressure below and above 40°C respectively. As shown in Fig. 6, the plot of the logarithm of the rate constant against the reciprocal of the absolute temperature is found to be consisted of two straight lines for the pressure around 4500 kg/cm², which coincide together at the point corresponding to about 40°C.

![Fig. 6 Relations between logarithm of rate constant k (min⁻¹) and reciprocal of absolute temperature T, at pH 4.8](image)

The values of the activation energy $E$ are calculated by using the Arrhenius relation, from the slopes of each straight line which was obtained from two series of experiments carried out under 4500 and 5000 kg/cm² respectively. The values of $E$ calculated and listed in Table 1 show that under these high pressure conditions, the activation energy $E$ takes positive value in the range above 40°C and negative value in the range below 40°C.

<table>
<thead>
<tr>
<th>Pressure (kg/cm²)</th>
<th>Activation energy, kcal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10~40°C</td>
</tr>
<tr>
<td>4500</td>
<td>-24</td>
</tr>
<tr>
<td>5000</td>
<td>-25</td>
</tr>
</tbody>
</table>

The relation between the rate constant $k$ and pressure $P$ is represented by the equation:

$$\frac{d \ln k}{d P} = -\frac{\Delta V^*}{RT},$$

where $\Delta V^*$ is the molal volume change on activation, $P$ the pressure, $R$ the gas constant and $T$ the absolute temperature. So the values of $\Delta V^*$ can be calculated from the slope of each line obtained by plotting the logarithm of rate constant against the pressure, as shown in Fig. 7, and are listed in Table 2.

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta V^*$, cc/mole</td>
<td>-92</td>
<td>-63</td>
<td>-81</td>
<td>-79</td>
<td>-77</td>
<td>-55</td>
<td>-38</td>
</tr>
</tbody>
</table>
Studies on the Denaturation of Egg Albumin under High Pressure

In these high pressure regions above 4000 kg/cm², the values of $\Delta V^*$ take negative values in all cases, but their absolute values tend to decrease with the increase in temperature. Especially in the case where the thermal denaturation under atmospheric pressure is remarkable, the values of $\Delta V^*$ decrease much more.

In Fig. 8 the logarithm of rate constants are plotted against pressure in a wide range of pressure (0~1000 kg/cm²) at 65 and 70°C where evidently the thermal denaturation occurs (cf. Fig. 5). Probably as the effect of two types of denaturation due to heat and to pressure overlap, the linear relation does not hold. But for convenience, the activation energy $E$ at a certain pressure and molal volume on activation $\Delta V^*$ in a certain range of pressure are calculated from Fig. 8, the values being shown in Table 3. It is noticeable that the values of $E$ are positive and decrease with the increase in pressure, while the values of $\Delta V^*$, changing their sign below and above 3000~4000 kg/cm², decrease with the increase in pressure.

The kinetic results obtained above from the experiments and summarized in Fig. 9 indicate...
apparently three kinds of denaturation, namely, (I) that at relatively high pressure and low
temperature where $E<0$ and $\Delta V^*<0$, (II) that at relatively low pressure and high temperature
where $E>0$ and $\Delta V^*>0$, and (III) that at high pressure and high temperature where $E>0$
and $\Delta V^*<0$.

Whether these differences mean the different denaturation paths or whether these are ascribed
to a complicated activation mechanism of protein denaturation, is the most fundamental problem
and the author has some conceptions about this. But this classification is based merely on the
observation of the precipitation due to denaturation, and now the author, carrying out some
experiments, where SH groups, viscosities and electrophoretic behaviors of native and denatured
egg albumin are measured, will make full discussion later, taking all those results into consideration.

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