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# THE PROTEIN DENATURATION UNDER HIGH PRESSURE

## Horse Serum Albumin

By Chieko Suzuki, Kiyoshi Kitamura, Keizo Suzuki and Jiro Osugi

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The denaturation of horse serum albumin was studied under pressure up to 10,000 kg/cm² and at the temperature of 10 to 70°C. The extent of the denaturation was examined by measuring the solubility, the reactivity of sulfhydryl groups, and the susceptibility to proteolysis by the bacterial proteinase. Even if the serum albumin solution was compressed at 10,000 kg/cm² and at pH 4.8 adjusted with acetate buffer, only a slight opalescence was observed, and no precipitation was formed. The heat coagulation of serum albumin was remarkably retarded by the pressure up to 8,000 kg/cm². From the experiments of oxidation of the reactive sulfhydryl groups and of the proteolysis by proteinase, the following deductions were made: 1) The temperature coefficient of pressure denaturation is positive. 2) The amount of pressure-denatured serum albumin is at most only about a half that of heat-denatured serum albumin. Further, increasing the pressure above approximately 7,000 kg/cm² resulted in little or no increase in the amount of denatured protein. 3) Though measurable renaturation was not found within 3 hours after releasing pressure, the renaturation at the instance of releasing pressure may be possible. These results are considerably different from those obtained already for ovalbumin. Accordingly, it may be suggested that serum albumin considerably differs in its secondary or tertiary structures from ovalbumin.

## Introduction

We have studied the pressure denaturation of ovalbumin[1-3] and bovine carbonylhemoglobin [2,4,5] by the solubility test, and obtained the following results: (1), the reaction of pressure denaturation is of first order, and (2), the phenomena of the protein denaturation under pressure are classified into three groups according to the signs of thermodynamic functions of the denaturation rate, namely the increases in heat content, entropy and volume in activation process as is listed in Table 1.

Table 1 Classification of thermodynamic properties of denaturation rate under pressure.

| | Temp. (°C) | Pressure (kg/cm²) | $\Delta H^*$ | $\Delta S^*$ | $\Delta V^*$ |
|---|---|---|---|---|---|
| I | <30 | >4,000 | − | − | − |
| II | >40 | >3,000 | − | ± | − |
| III | >60 | <3,000 | + | + | + |

1) K. Suzuki, *This Journal*, **28**, 24 (1958)
2) K. Suzuki, *ibid.*, **29**, 91 (1959)
3) C. Suzuki and K. Suzuki, *J. Biochem.*, **52**, 67 (1952)
4) K. Suzuki and K. Kitamura, *This Journal*, **29**, 81 (1959)
5) K. Suzuki and K. Kitamura, *ibid.*, **29**, 86 (1959)

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Tongur and his co-workers[6] studied the effect of pressure on the thermal denaturation of serum albumin by measuring the solubility and the turbidity in view of chemical equibrium. The investigators of Institute of Pasteur[7] discussed the state of aggregation of denatured serum albumin by pressure from the measurement of streaming birefringence etc.. However, the detail of the denaturation of this protein under pressure still remains unclarified.

In the present study, the denaturation of horse serum albumin (HSA) under high pressure was investigated by measuring the solubility, the susceptibility to proteolysis by proteinase and the reactivity of sulfhydryl groups.

## Experimental

**Materials** HSA was prepared by the ammonium sulfate method from horse blood plasma and recrystallized three times, and its aqueous solution was dialyzed against running water. Crystalline bacterial alkaline proteinase was purchased from Nagase Sangyo Co. Lid., Japan.

**Procedures** The same high pressure apparatus as in the previous publications[1,3] was used. The protein solution of 0.4 to 1.0% was hydrostatically compressed at a constant pressure and temperature in the range of 1 to 10.000 kg/cm$^2$ and of 10 to 70°C, and then the change of properties caused by denaturation was examined by the following methods after releasing pressure:

(1) The solubility test: The denatured protein was brought to coaguration at the isoelectric point, pH 4.8 (0.1 *M* acetate buffer) and was filtered off, and then the amount of the remaining protein molecules in the filtrate were colorimetrically measured at the wavelength of 530 m$\mu$ by the Biuret reaction[8].

(2) The measurement of the reactive SH groups: Reactive SH groups were oxidized by potassium ferricyanide (PFC) and estimated from the amounts of the consumed oxidant[8]. A characteristic absorption of PFC is at the wavelength of 420 m$\mu$ where the absorption of potassium ferrocyanide is negligible. Therefore, the oxidized SH groups of protein were estimated by the measurement of the diminished value of absorbance at 420 m$\mu$[8]*. The pH of the sample solution was adjusted to 6.8 with phosphate buffer, where this oxidation reaction was specific. PFC was added in excess of the amounts needed to react with all of the sulfhydryl groups (final conc. 1/1,000 to 1/2,000 *M*). We examined two cases, A and B. In case A, PFC was mixed with a compressed protein solution, and in case B, a mixture of protein solution and PFC was compressed.

(3) The measurement of the susceptibility to proteolysis by proteinase: Proteolysis by pro-

6) V. S. Tongur and V. I. Kasatochkin, *Chem. Abst.*, **45**, 2039 (1951): *ibid.*, **47**, 12438 (1953)

7) E. Barbu, M. Macheboeuf, P. Rebeyrotte and P. Slizewiez, *Bull. Soc. Chim. biol.*, **34**, 724 (1952): E. Barbu, J. Basset and M. Joly *ibid.*, **36**, 323 (1954): E. Barbu and M. Joly, *Farad. Soc. Disc.*, **13**, 77 (1953)

8) S. Nakagawa, T. Kaminaga and S. Araya, *J. Biochem.*, **41**, 371 (1954)

* As the linear relationship between cystein concentration and the diminished value of absorbance $\Delta A$ at 420 m$\mu$ was obtained, $\Delta A$ is proportional to the amount of oxidized SH groups.

teinase extracted from *Bacillus subtilis* was examined by Okunuki's method[9] as reported in the previous paper[3]. To 1 ml of 0.4% HSA solution at pH 7.4 (0.1 *M* phosphate buffer), 2 ml of 0.05% proteinase solution was mixed. The digestion was carried out for 10 minutes at 30°C and stopped by mixing 2 ml of *M* trichloroacetic acid (TCA). After filtration, the concentration of the TCA soluble products of the digestion was measured by Folin's method[10]. We examined two case A′ and B′. In case A′, the proteolysis of the compressed HSA solution was performed at atmospheric pressure, and in case B′, a mixture of HSA and the proteinase solution was compressed, namely the proteolysis was performed under high pressure.

## Results and Discussion

**Solubility test** Only a slight opalescence was observed and no precipitation was formed, even if the protein solution (protein concentration 0.6%, pH 4.8 with 0.1 *M* acetate buffer) was compressed for 20 minutes at 10,000 kg/cm² and 25°C. This method is, therefore, not adequate to assess the extent of the pressure denaturation of serum albumin. However the above findings interest us in comparison to the facts that isoelectric ovalbumin and hemoglobin denature and coagulate almost perfectly after about 10 minutes at several thousands kilogram per square centimeter at room temperature.

The pressure effect on the thermal denaturation of HSA was also examined by the solubility test, and the results are shown in Fig. 1 together with those of ovalbumin[1] and carbonylhemoglobin[5] already reported by us. It shows that the thermal denaturation reaction of serum albumin is retarded by the pressure up to 8,000 kg/cm² and the effects are enhanced with increasing pressure, while in the thermal denaturation of ovalbumin and hemoglobin, the retardation effects are maximum at about 3,000 and 2,000 kg/cm² respectively. Therefore, it seems that the stability

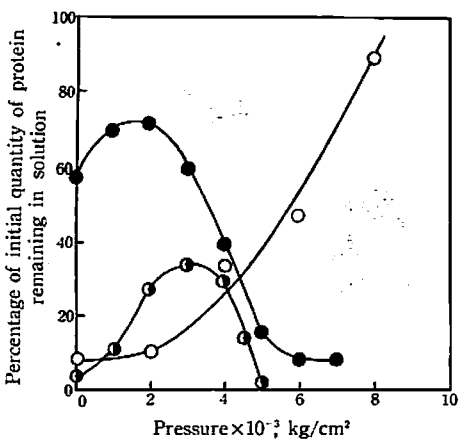


Fig. 1 Relationship between the percentage of the remaining soluble protein ($C/C_0 \times 100$) and applied pressure.
$C_0$: initial concentration of protein
$C$: protein concentration of protein

| | pH | Temp. (°C) | Pressing duration (min) | $C_0$ (%) |
|---|---|---|---|---|
| -○-: HSA | 4.8 | 70.0 | 10 | 0.69 |
| -◑-: ovalbumin | 4.8 | 70.0 | 20 | 0.50 |
| -●-: hemoglobin | 6.8 | 75.5 | 5 | 0.46 |

9) K. Okunuki, B. Hagihara, H. Matsuda and T. Nakayama, *J. Biochem.*, 43, 453 (1956)
10) B. Hagihara, *Koso Kenkyuho*, Edited by S. Akabori, Asakura-Shoten, Tokyo, Vol. I, P. 165 (1955)

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of the conformation of serum albumin considerably differs from those of the two other proteins.

**Oxidation of the reactive SH groups** The results in cases A and B are compared in Table 2. It shows that PFC is not consumed at all in mixing with the compressed HSA solution, but a large amount of PFC is consumed in compression of a mixture of HSA and PFC, though the effect of PFC on the pressure denaturation must be considered in case B. The facts show that SH groups which are once exposed under pressure are probably masked again owing to the refolding of peptide chain, the intermolecular association, or the formation of S–S bonds at the release of the pressure. These results differ from those of ovalbumin in which the SH groups were oxidized even in case A, though the amount of oxidized SH groups was less than in case B*.

Table 2 Absorbances and its diminished values $\Delta A$ at 420m$\mu$ in case A and B.

| | Absorbance at 420 m$\mu$ | $\Delta A$ |
|---|---|---|
| Native | 0.192 | |
| Pressed, in case A | 0.200 | −0.008 |
| Pressed, in case B | 0.096* | 0.096 |

Sample solutions were pressed for 10 minutes at 8,000 kg/cm², pH 6.8, and 25°C.
* The reading of absorbance was the same within 3 hours.

Fig. 2 shows the diminished value of absorbance at 420 m$\mu$ which is proportional to the amount of oxidized SH groups by PFC against applied pressure, in case B. It is noticiable that the reactivity of SH groups rapidly enhances with the increase of pressure above nearly 4,000 kg/cm², but further increase of pressure above 7,000 kg/cm² brings about little or no more effect on the reactivity of SH groups. In comparison with the diminished value of absorbance at 420 m$\mu$ of heat-denatured HSA after boiling for 10 minutes at 100°C with PFC, only about 40%

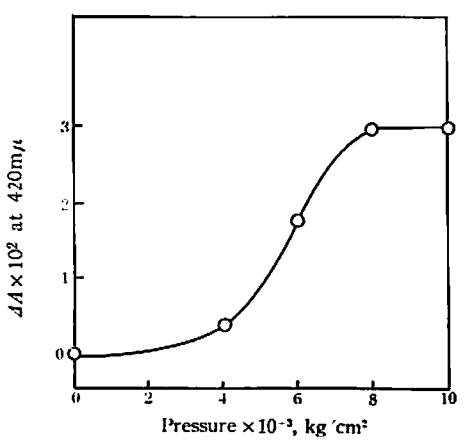


Fig. 2 Relation between the diminished value of absorbance at 420 m$\mu$ ($\Delta A$) and applied pressure, in case B. (see text)
0.88% protein solutions containing 1/2,000 *M* potassium ferricyanide were pressed for 10 minutes at pressures indicated at pH 6.8 and 25°C.

* Unpublished data by the present authors.

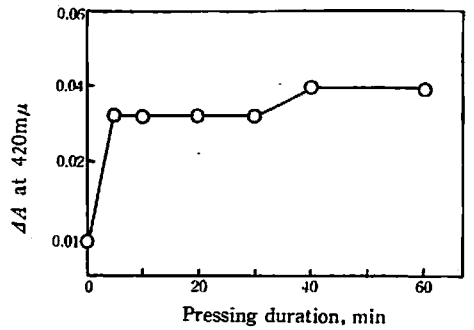


Fig. 3 Time course in the oxidation of SH groups. $\Delta A$ is diminished value of absorbance at 420 m$\mu$. Samples were pressed at 7,000 kg/cm$^2$ indicated at pH 6.8 and 15°C. Initial conc. of protein was 0.93% and that of potassium ferricyanide was 1/1000 *M*.

of SH groups oxidized in heat-denatured HSA are oxidized by pressure denaturation. The oxidized SH groups increased in steps with the increase of pressure duration as showh in Fig. 3, but its kinetics can not yet be understood. The same stepwise charater has also been observed in the thermal denaturation of serum albumin and ovalbumin[8] and the pressure denaturation of ovalbumin*.

The effect of temperature on the pressure denaturation examined by measuring the oxidized SH groups is shown in Fig. 4. It shows that the rate of denaturation increases with the rise of

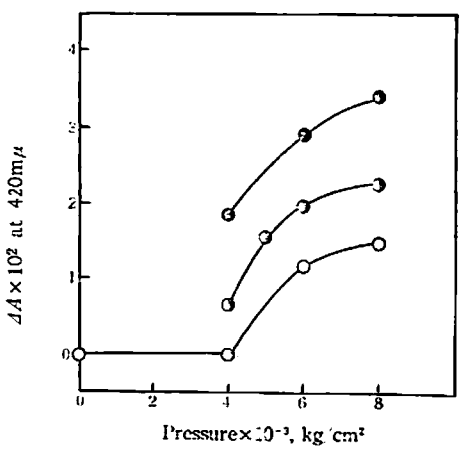


Fig. 4 Relationship between the diminished valus of absorbance at 420 m$\mu$ ($\Delta A$) and applied pressure at each temperature, in case B (see text)

Temp. (°C)

-○-: 10

-◑-: 30

-●-: 50

0.83% protein solutions containing 1/1,000 *M* potassium ferricyanide were pressed for 5 minutes at pH 6.8.

temperature. These results are different from those of ovalbumin and hemoglobin. These two proteins have negative temperature coefficients for the rate of pressure denaturation below 40 to 50°C[1,5]**.

**Proteolysis by proteinase** Fig. 5 is the plot of the relative amounts of hydrolysis products against applied pressure, which was estimated by the following equation:

$$\text{percentage of denatured protein} = \frac{D - D_n}{D_n - D_d} \times 100$$

* Unpublished data by the present authors.

** They are examined by measuring the solubility as an index.

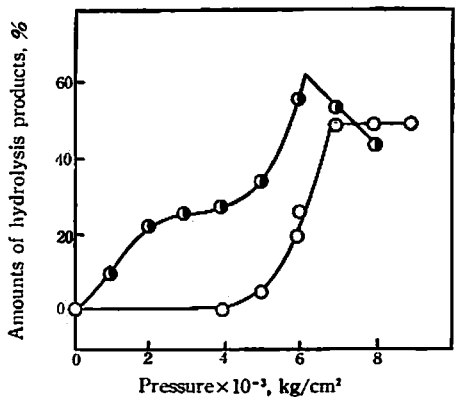


Fig. 5 Relationship between the relative amounts of hydrolysis products and applied pressure.
-○-: in case A', -◑: in case B' (see text)
0.4% protein solutions were pressed for 10 minutes at pressures indicated at pH 7.4, and 30°C.

where $D_n$, $D_d$ and $D$ are the absorbances of native, completely denatured and pressure-treated proteins, respectively. For $D_d$ was taken the value of heat-denatured HSA treated in boiling water for 10 minutes.

In case A', there is no difference between the susceptibility to proteolysis by proteinase of the native HSA and of the compressed HSA at pressure below 4,000 kg/cm². The proteolysis is promoted with increasing pressure above about 4,000 kg/cm² and further increase in pressure results in little or no more effect in the proteolysis. The recovery from the denatured state was not found within 3 hours after releasing pressure.

On the other hand, in case B', the proteolysis proceeds even at low pressure. The susceptibility to proteinase increases with increasing pressure until it reaches a constant value between 2,000 and 4,000 kg/cm², and then it increases with increasing pressure above 4,000 kg/cm² again. But the increase of pressure above 6,000 kg/cm² conversely results in the decrease of the hydrolysis products. Now, we will consider these three sections one by one. Below 4,000 kg/cm², the proteolytic reaction seems to be accelerated by pressure, since serum albumin is somewhat hydrolized by proteinase in even the native state in comparison with ovalbumin. The rapid increase in susceptibility with increasing pressure from 4,000 to 6,000 kg/cm² seems to depend on the large increase of the amount of denatured protein. The fact that the amount of hydrolysis products in case B' is much more than that in case A' may be interpreted from the following reasons: (1) Pressure denaturation may be accelerated by proteinase*, (2) the hydrolysis may be accelarated by pressure and (3) HSA molecules may regenerate from the denatured state immediately after releasing pressure. The last reason does not seem to be important, since the susceptibility to proteolysis of the renatured serum albumin from urea denatured state is equal to that of the fully denatured

* Proteolytic emzyme may cause denaturation by merely shifting an equilibrium between the native and the denatured forms of a protein as a result of attack by the emzyme on the denatured form, and a little on the native form, according to the following sequence:

Native protein $\rightleftharpoons$ Denatured protein
Denatured protein $\underset{\text{Enzyme}}{\rightleftharpoons}$ Hydrolysis products.[11]

11) W. Kauzmann, *Mechanism of Enzymic Action*, Edited by W. D. McElroy and B. Glass, P. 70 (1954)

protein[12]. It is clear that the decrease of hydrolysis products above 6,000 kg/cm$^2$ is due to the inactivation of proteinase itself by pressure*.

Table 3 shows the results of temperature effect on the pressure denaturation by measuring the susceptibility to proteolysis by proteinase in case A′. It is found from this table that the temperature coefficient of the denaturation process is positive and similar to the results shown in Fig. 3 which was obtained by measuring the oxidized SH groups.

Table 3 Temperature effect on pressure denaturation by measuring the susceptibility to proteolysis by proteinase in case A′.

| Pressure (kg/cm$^2$) | Temp. (°C) | Absorbance of the Folin color at 660 mμ |
|---|---|---|
| 6,000 | 10 | 0.225 |
| | 20 | 0.234 |
| | 30 | 0.240 |
| 6,500 | 10 | 0.227 |
| | 30 | 0.246 |

0.4% protein solutions were pressed for 10 minutes at pH 7.4.

In conclusion, the molecular structure of horse serum albumin may be assumed to be very different from that of ovalbumin, and the possibility of the renaturation immediately after the release of pressure is suggested**.

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*Department of Chemistry*
*Faculty of Science*
*Kyoto University, and*
*Faculty of Science and Engineering*
*Ritumeikan University*
*Kyoto, Japan*

12) F. W. Putnum, *The Proteins*, Edited by H. Neurath and K. Bailey, Academic Press, Vol. I, P. 807 (1953)

* Unpublished data by the present authors.

** After this study, the recovery of serum albumin from the denatured state immediately after releasing pressure was illustrated by measuring the turbidity under high pressure as an index. K. Suzuki, Y. Miyosawa and C. Suzuki, *Arch. Biochem. Biophys.*, In press.