Reprinted from ARCHIVES OF BIOCHEMISTY AND BIOPHYSICS **110**, 381—387 (1965)

Studies on Taka-Amylase A under High Pressure

III. Some Physicochemical Properties of Pressure-Denatured Taka-Amylase A[1]

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Received October 10. 1964

Some physicocemical properties of pressure-denatured Taka-amylase A (TAA) were studied by measuring the rate of sedimentation, optical rotation, diffusion, and viscosity, Pressure-denatured TAA which was compressed at pH 7.5 was polydisperse, but at pH 9.0 it was monodisperse. From the calculation of molecular weight, TAA (at pH 9.0) showed neither association nor dissociation by Pressure. The mobility of the pressure-denatured TAA was decreased in comparison with native TAA. The isoelectric point of the pressure-denatured TAA appears to be shifted slightly toward higher pH. The optical rotatory dispersion constant, λ_c was found to be 228 mμ (for native TAA it is 279 mμ) and from the value of b_0, the right helical content was found to be 10.8 % (native TAA has 15.9% helical content). This value corresponds to 68 % of the value for native TAA. The reduced viscosity of the pressure-denatured TAA was not dependent on the concentration of TAA, and the value was found to be 0.105 dl per gram (for naitve TAA it is 0.035 dl per gram). From the above findings, it may be said that TAA is not drastically altered by pr essure. Moreover, inactivation and recovery were acompanied by changes of conformation of TAA. If the changes of levorotation, $[\alpha]_{365}^{20}$, wree less than about—180° then some recovery was possible even if the activity was completly lost, but even small changes in rotation beyond this limit made any recovery of activity impossible.

1 Presented at the 16th Annual meeting of the Chemical Society of Japan, Tokyo, April, 1963.

2 This work was done at the Division of physical Chemistry, Institute for Protein Research, Osaka University, Osaka, as a visiting researcher.

In our earlier papers (1, 2) dealing with the high pressure inactivation and the recovery of activity of Taka-amylase A (Asp. Oryzae α-amylase; TAA), it was shown that, the pressure inactivation of TAA was observed at about 4000 and 9500 kg per square centimeter, the enzyme was completely inactivated by compression for 5 minutes. The activity of TAA inactivited by compression at 9500 kg per square centimeter for a short time (less than 60 minutes) was spontaneonsly restored after the pressure has been released, but after a prolonged compression at the same pressure, the activity of TAA could not be recovered. The extent of the inactivation and the recovery of activity was dependent on the concentration of enzyme, pH, temperature, and ionic strength. Moreover, the recovery of activity was found to be enhanced by a recompression at a low pressure.

The inactivation and the recovery of activity are undoubtedly accompanied by changes of molecular comformation of the enzyme; however, no available deta concerning the nature of pressure denatured protein have been reported in the literature, so far as we know. Accordingly, it seems necessary to obtain further information in order to clarify the mechanisms of pressure denaturation.

In the present work, changes of the configuration due to pressure denaturation were studied by measuring the rate of sedimentation, optical rotatory dispersion, diffusion, viscosity, and electrophoresis. It has been found that the conformational changes of TAA by high pressure treatment did not seem to be very drastic.

EXPERIMENTAL

Taka-amylase A used in the present work was prepared and purified according to the method of Akabori *et al.* (3) from

"Taka-diastase Sankyo," Rivanol treatment was repeated twice to eliminate the contamination of acid Taka-protease (4). An aqueous TAA solution was dialyzed against 0.1 *M* potassium chloride and stored as a stock solution in a refrigerator in about 5% solution. The concentration of TAA was estimated spectrophoto-metrically, assuming the extinction in water to be $E_{1cm}^{1\%}=22.1$ at 278.5 mμ. In the present experiment, the stock solution was dialyzed against distilled water and then diluted to a given concentration of enzyme with buffer solution. The high pressure apparatus and the procedures for denaturation were the same as previously reported by Suzuki (5). A TAA solution was sealed in a polyvinylchloride sack and compressed in a high pressure chamber. After a defined compression at several thousand kilograms at 30°, the sack was taken out, and sedimentation, diffusion, electrophoresis, optical rotation, viscosity, and activity of the protein were measured.

As it is known that the effect of the charge of protein on its sedimentation constant and diffusion coefficient are reduced if the solvent contains salt at an ionic strength greater than 0.1, the ionic strength of the solvent was kept at 0.15 in the present experments.

A Hitachi model UCA-1 was used to conduct sedimentation experiments at 20° at 55,430 rpm. The protein concentration for the sedimentation experiments was 1%.

A Hitachi model HT-B electrophoresis apparatus conbined with a Neurath-type diffusion cell, and a 5% enzyme concentration, were used to measure diffusion coefficients.

Prior to electrophoresis, the pressure-denatured TAA at pH 9.0 was dialyzed against a large volume of acetate buffer, phosphate bnffer, or veronal buffer for 24 hours in the refrigerator. Electrophoresis was conducted at varions pH values at 20° with a Tiselius-type apparatus equipped with a schlieren diagonal

system. The electric conductivity was measured at 20°.

Optical rotatory dispersion measurements were carried ont in the range of 330-550 mμ on a Rudolph photoelectric spectropolarimeter (model 200-S) at 20°.

Viscosity was measured by a viscometer of the Ostwald type with a flow time of 160-180 seconds for water at 20°.

Amylase activity was measured at pH 5.5 (0.1 *M* acetate buffer) at 30°; soluble starch was used as a substrate according to the method of Noelting and Bernfeld (6). The concentration of TAA for the activity measurements was 5×10^{-4}%.

RESULTS

Some physicochemical properties of pressure-denatured TAA. The sedimentation coefficients were measured at a protein concentration of 1% for both the native protein and the pressure-denatured TAA compressed at 9500 kg per square centimeter for 120 minutes at 30° at pH 7.5 and 9.0. Sedimentation patterns are illustrated in Fig. 1. The patterns of the native and the pressure-denatured TA A at pH 9.0 shows only one peak, but at pH

TABLE I

DIFFUSION COEFFICIENT, SEDIMENTATION COEFFICIENT, AND MOLECULAR WEIGHT OF NATIVE AND PRESSURE-DENATURED TAA

	*D*cm/sec	*S*20, W(s)	M.W.
Native TAA (pH 9.0)	6.3×10^{-7}	3.9	50,000
Pressure-denatured TAA (pH 9.0)	4.3×10^{-7}	3.5	66,000
Pressure-Denatured TAA (pH 7.5)		(f) 7.6 (s) 6.0	

7.5 they show double peaks. Table I shows the values of the

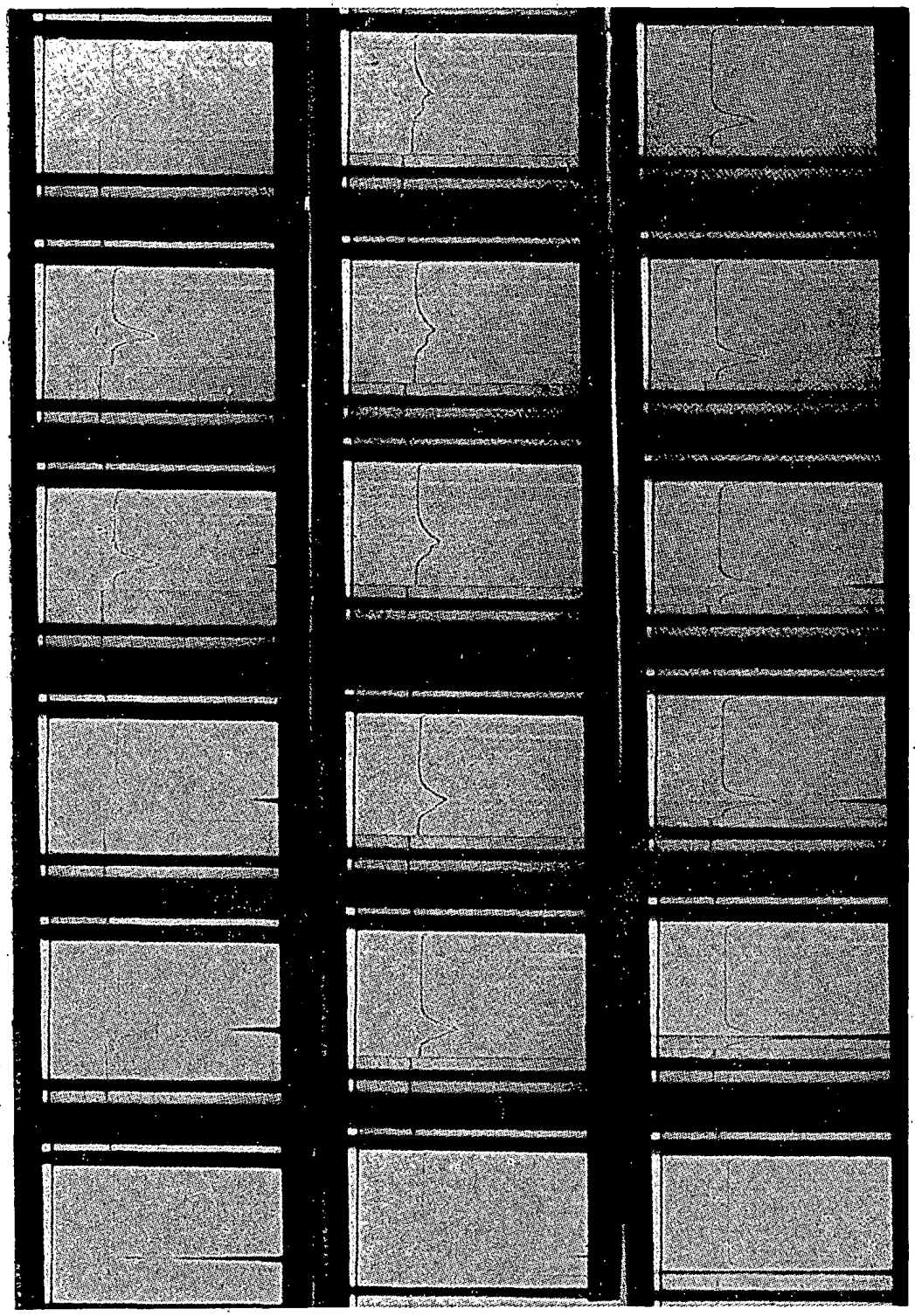

Fig. 1 Sedimentation diagrams of native TAA at pH 9.0 (top) and pressure-denatured TAA which was compressed at pH 7.5 (center) and pH 9.0 (bottom at 9500 kg/sp for 120 minutes at 30°. Photographs were taken at intervals of 10 minutes at a rotor speed of 55,430 rpm.

sedimentation coefficient.

These results were taken into consideration, and measurements of the diffusion coefficient, optical rotation, and viscosity were carried out at pH 9.0.

The diffusion coefficients were measured at 20° with a 0.5% TAA solution which was compressed at 9500 kg per square centimeter for 120 minutes at pH 9.0. The values of the diffusion coefficient are listed in Table I; D was calculated by the statistical method.

The electrophoresis was carried out at a pH range of 4.5—8.9 for native TAA and 5.4—8.9 for the pressure-denatured TAA. Native TAA at a pH lower than 4.0 and the pressuredenatured TAA at a pH lower than 5.0 showed some turbidity after dialysis for 24 hours at 5°. Therefore, the electrophoresis could not be carried out at lower pH values. Over the whole pH range examined, a single boundary was observed, but the peak of the pressure-denatured TAA became broad below pH 7.0. Table II lists the calculated mobilities. The mobilities of the press-

TABLE II

MOBILITIES OF NATIVE AND PRESSURE-DENATURED TAA AT VARIOUS pH's

pH	Mobility (cm^2/sec/volt 10^{-4})	
	Native TAA	Pressure-denatured TAA
4.5	0.41	
4.9	0.68	
5.4	0.66	0.43
6.1	0.71	0.49
7.5	0.78	0.58
8.9	0.79	0.59

ure denatured TAA were smaller than those of the native TAA over the whole pH range examined.

Optical rotatory dispersion measurements were carried out in the range of 330-550 mμ, and the results were plotted according to the Moffitt's equation (7, 8):

$$[M]_\lambda = \left(\frac{3}{n^2+2}\right)\left(\frac{M}{100}\right)[\alpha]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2+\lambda_0^2)^2}$$

where $[M]$ is effective residue rotation, α is specific rotation at wavelength λ, M is the mean residue weight of TAA [assumed to be 119.5 (9)], n is the refractive index of the solvent, λ_0 is a constant value [assumed to be 212mμ [(10)], b_0 is a constant considered to be related to the α-helical content. and a_0 is also a constant. The results are shown in Fig. 2. The plots show that the Moffitt's equation is obeyed, the b_0 values being —100° for native TAA and —60° for the pressure-denatured TAA.

The reduced viscosities of native and the pressure-denatured TAA were not dependent on the protein concentration (between about 0.25 and 1 %), and the values were found to be 0.035 and 0.105 dl per gram, respectively.

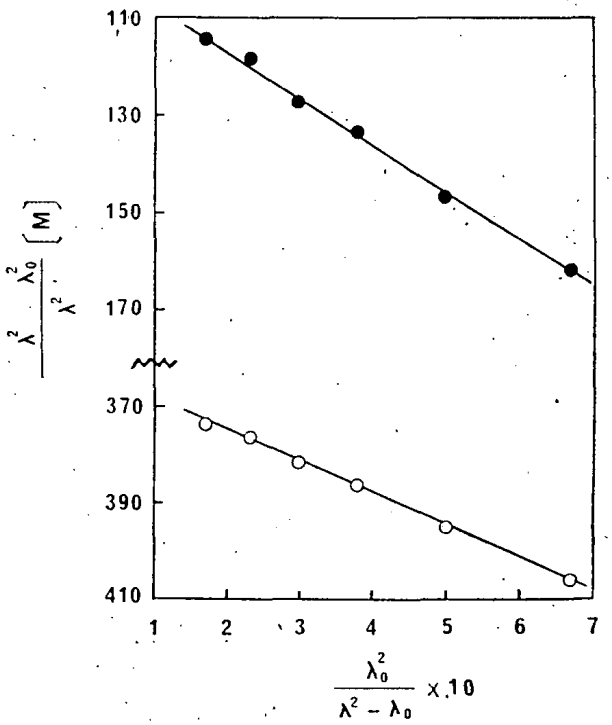

Fig. 2. Moffitt plots of native (-◍-) and pressure-denatured TAA (-○-) (compressed at 9,000 kg/sq cm at pH 9.0 for 120 minutes at 30°).

Changes of optical rotation and viscosity with inactivation and reactivation. As shown in earlier paper (2), inactivated TAA which was compressed for 10 minutes at 9500 kg per square centimeter (unbuffered aqueous solution of TAA was compressed, and after releasing the pressure the pH was adjusted to 7.5) could spontaneously recover its activity. After 60 minutes of compression, however, inactivated TAA could not recover its activity. This suggested that even though the activity is completely lost in both experiments, the extent of change of the TAA molecule must be different. In order to obtain some information about the relationship between activity and change of configuration of TAA, the following experiments were carried out.

Figure 3 shows the time course of the recovery of activity of the inactivated TAA after different durations of exposure to 9500 kg per square centimeter. The activity of TAA which was

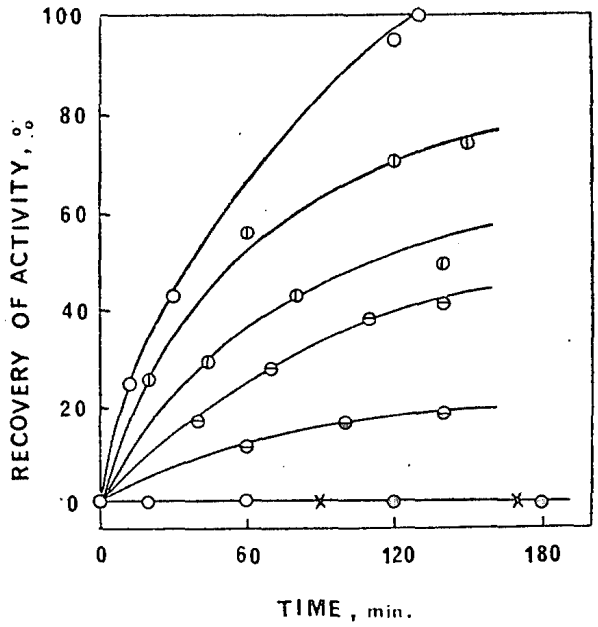

Fig. 3 Effect of pressure duration at 9500 kg/sq cm on recovery of activity.

Time course of recovery at 30° under atmospheric pressure of pressure-denatured TAA (1% of TAA, pH 9.0) (compressed at 9500 kg/sq cm at 30° for 5 minutes (-○-), 10 minutes (-◑-), 25 minutes (-◑-), 45 minutes (-◓-), 60 minutes (-◒-), 90 minutes (-●-), and 120 minutes (-×-), repectively).

compressed for 5 minutes was completely recovered, but no activity of TAA could be recovered if it is compressed for more than 90 minutes.

Figure 4 shows the change of optical rotation, $-[\alpha]^{20}_{365}$, with

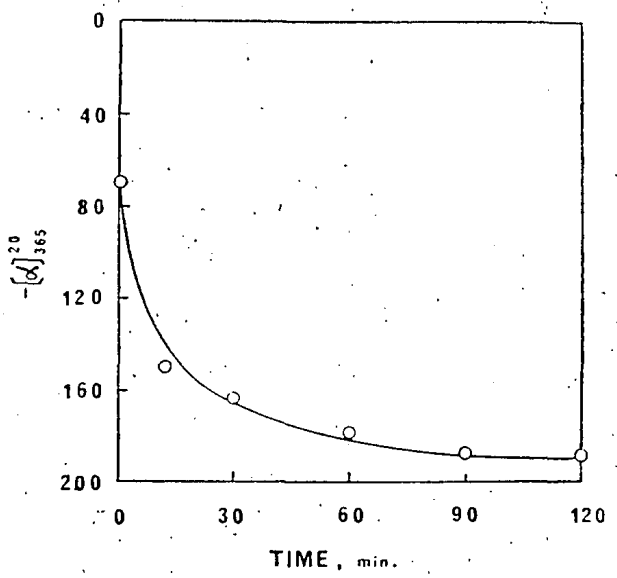

Fig. 4. Changes of optical rotation with pressure duration at 9500 kg/sq cm. TAA (1%, pH 9.0) was compressed at 9500 kg/sq cm at 30° for various durations. Immediately after the pressure was released, the optical rotation at wavelength 365 mμ was measured.

pressure duration at 9500 kg per square centimeter at pH 9.0. The specific rotation of native TAA was found to be −70°, and the levorotation for the pressure-treated TAA increased with the pressure duration. After 60 minutes of compression $[\alpha]^{20}_{365}$, was about −178°, but a partial recovery of activity could be still observed (cf. Fig. 3). The levorotation for the irreversibly denatured TAA which was compressed for a prolonged time at 9500 kg per square centimeter reached to −190°.

Figure 5 shows the changes of optical rotation, $-[\alpha]^{20}_{365}$, and

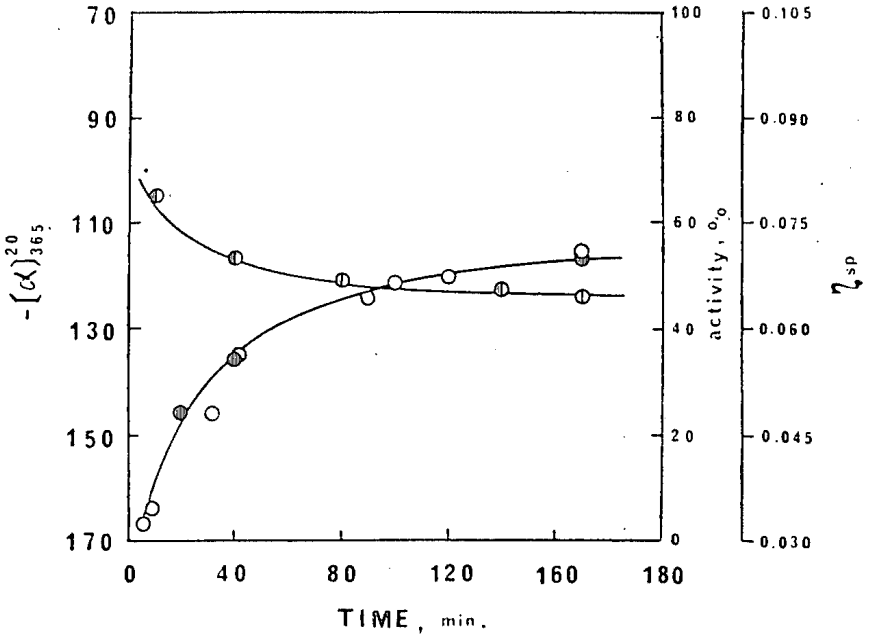

Fig. 5. Changes of optical rotation and viscosity with recovery of of activity. TAA (1%, pH 9.0) was compressed at 9500 kg/sq cm for 25 minutes. After releaing the presrure, optical rotation, $[\alpha]_{365}^{20}$, (-●-), viscosity (-◐-), and activity (-○-) were followed as a function of the time.

viscosity with the recovery of activity after compression for 25 minutes at 9500 kg per square centimeter at 30°. Reactivation parallels the recovery of specific rotation and viscosity. After 160 minutes under atmospheric pressure. activity, specific rotation, and viscosity were recovered to the extent of about 55, 55, and 38% of native TAA, respectively.

DISCUSSION

As shown in Fig. 1, the sedimentation patterns reveal that the pressure-denatured TAA at pH 7.5 shows a double peak. It might be attributed to the association of TAA molecules by high pressure. On the other hand, the pressure-denatured TAA at pH 9.0 shows monodispersity. Accordingly, we can calculate the molecular weight of the pressure-denatured TAA by using the Svedberg equation:

$$M=\frac{NkTs}{D(1-\bar{v}\rho_{\omega})},$$

where N is the Avogadro number, k the Boltzmann constant, T the absolute temperature, and ρ_{ω} the density of water, s the value of the sedimentation coefficient, and D the diffusion coefficient. The partial specific volume, $\bar{v}$, of the pressure-denatured TAA is assumed to be equal to that of the native TAA, 0.700 ml per gram (11). The calculated molecular weight of the native and the pressure-denatured TAA are 50,000[3] and 66,000, respectively.

Taking into account the errors involved in the use of value not extrapolated to infinite dilution for the sedimentation coefficients, the calculated molecular weight must be considered only approximate. Moreover, since the partial specific volume of the denatured protein is generally smaller than that of the native protein (15), the molecular weight of the pressure-denatured TAA may be lower than 66,000.

From this consideration, it seems likely that the pressure denaturation of TAA at pH 9.0 causes neither molecular association nor dissociation.

The mobility of the pressure-denatured TAA was decreased in comparison with that of native TAA over the whole pH range examined. From the trend of the mobility values, isoelectric point of the pressure-denatured TAA is probably shifted slightly to the alkaline side.[4] It may thus be suggested that the net charge of the TAA molecule decreases due to the pressure denaturation. It is difficult to give a full interpretation of these results, but it is assumed that some of the carboxyl or hydroxyl groups in the pressure-denatured TAA molecule may undergo abnormal dissociation which might be attributed to the intra-

3 Reported molecular weight of native TAA is from 51,000 to 54,000 (11-14).

or intermolecular hydroden bonds or ionic bonds.

From our present experiments on the optical rotatory dispersion, the optical rotatory dispersion constant, λ_c, was found to be 228 mμ for the TAA completely denatured by high pressure treatment. This value suggests that pressure-denatured TAA has a considerable helical content, and the right-handed helical content can be calculated from $-b_0/630$ (b_0 for 100% right-handed α-helix was assumed to be -630). These calculated values correspond to 16.0% for native TAA and 10.8% for the pressure-denatured TAA.

As for the viscosity, the increase in the intrinsic viscosity from 0.035 to 0.105 dl per gram suggests that the hydrodynamically effective volume of TAA does not increase so much as in the case of TAA denatured by reduction in 8 *M* urea (TAA denatured by reduction in 8 *M* urea is a perfectly randomly coiled single polypetide chain (17)). Comparisons of some physicochmical properties for native, pressure-denatured, and TAA denatured by other means are summarized in Table III.

TABLE III

SOME PHYSICOCHEMICAL PROPERTIES OF NATIVE, PRESSURE-DENATURED, AND TAA DENATURED BY OTHER MEANS

Properties	Native	Pressure-denatured	Acid-denatured[a]	8 *M* Urea-denatured[a]	Denature by reduction in 8 *M* urea[b]
Activity (%)	100	0	0	0	0
λc(mμ)	279	228	230	219	219
b_0	−100	−68	−70	—	—
[η] (dl/gm)	0.035	0.105	—	0.310	0.405

[a] Data reported by Takagi and Toda (18).

[b] Data reported by Isemura *et al.* (17).

In view of the above findings, it may be said that one of

4 The isoelectric point of native TAA is about 3.7 (16).

the most notable features of the pressure-denatured TAA molecule is that TAA is not so drastically altered by pressure treatmeant, and the geometry of the higher structure of molecule still remains to a considerable extent unchanged.

The extent of the destruction of molecule is similar to that of acid denaturation (18).

As shown in Fig. 3, TAA which was compreseed for 5 minutes at 9500 kg per square centimeter at pH 9.0 could recover its activity after releasing the pressure, whereas the extents of the recovery of activity were dependent on the duration of the pressure for durations of more than 5 minutes. After compression for 90 minutes or more. inactivated TAA could no longer recover its activity. The results obtained were different from earlier results (2), in that TAA inactivated by 60 minutes compression at 9500 kg per square centimeter could still recover to a considerable extent. These differences are believed to be caused by differences in the buffering conditions and by a difference in the concentrations of enzyme employed (in the earlier experiments, $2 \times 10^3\%$ enzyme solution was used, but in the present experiments the enzyme concentration was 1%).

As shown in Fig. 4, during the first 10 minutes of compression at 9500 kg per square centimeter the specific retation underwent a large change, whereas further compression produced only a gradual increase of levorotain. From the inclination of the curve it would appear that, during 5 minutes of compression at 9500 kg per square centimeter, the disorganization of the TAA molecule is rather slight in spite of the fact the activity is completely lost.

Recovery of activity was accompanied by the recovery of the structure, as shown in Fig. 5. Changes of optical rotation and viscosity were followed by the recovery of activity. If the change of the levorotation, $[\alpha]^{20}_{365}$, was less than about $-180°$,

then some recovery of activity was posible, but even a small change beyond this limit abolished all traces of recovery.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Professor T. Isemura, Director of Institute for Protein Research, Osaka University, for his kind guidance and encouragement throughout this work, and to Professor K. Suzuki, Ritumeikan University, Kyoto, for his valuable discussion. The author's gratitude is also due to Professor J. Osugi, Kyoto University, for his kind permission to use the high pressure apparatus.

REFERENCES

1. MIYAGAWA, K., AND SUZUKI, K., *Arch. Biochem. Biophys.* **105,** 297 (1964).
2. MIYAGAWA, K., SANNOE, K., AND SUZUKI, K., *Arch. Biochem. Biophys.* **106,** 467 (1964).
3. AKABORI, S., IKENAKA, T., AND HAGIHARA, B., *J. Biochem.* **41,** 557 (1954).
4. AMANO, T., ISOJIMA, S., AND FUJINO, H., *Med. J. Osaka Univ.* **5,** 333 (1954).
5. SUZUKI, K., *Rev. Phys. Chem. Japan* **28,** 24 (1958).
6. NOELTING, G., AND BERNFELD, P., *Helv. Chim. Acta* **31**, 286 (1948).
7. MOFFITT, W., *J. Chem. Phys.* **25,** 466 (1956).
8. MOFFITT, W., AND YANG, J. T., *Proc. Natl. Acad. Sci. U. S.* **42,** 596 (1956).
9. STEIN, E. A., JUNGE, J. M., AND FISCHER, E. H., *J. Biol. Chem.* **235,** 371 (1960).
10. DOTY P., *Rev. Modern Phys.* **31,** 107 (1959).
11. ISEMURA, T., AND FUJITA, S., *J. Biochem.* **44,** 443 797 (1951).
12. ISEMURA, T., *Symp. Cytochem.* **3,** 1 (1954).
13. AKABORI, S., IKENAKA, T., HANAFUSA, H., AND OKUDA, Y., *J. Biochem.* **41,** 803 (1954).
14. AKABORI, S., AND IKENAKA, T., *J. Biochem.* **42,** 603 (1955).
15. CHARLEWOOD, P. A., *J. Am. Chem. Soc.* **79,** 776 (1957).
16. AKABORI, S., "Koso Kenkyuho" (Japanese) (Asakurashoten, ed)., Vol. II, p. 112. Tokyo (1956).
17. ISEMURA, T., TAKAGI, T., MAEDA, Y., AND YUTAKI, K., *J. Biochem.* **53**, 155 (1963).
18. TAKAGI, T., AND TODA, H., *J. Biochem.* **52,** 16 (1962).