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Heat Denaturation of Bovine Serum Albumin. I. Analysis by Acrylamide-gel Electrophoresis

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Heat denaturation of bovine serum albumin (BSA) was studied at pH 8.9 in the temperature range 55-85°C. It was shown visually how various denatured-forms of BSA were produced. Depending upon the heating temperature and heating time, the number of discrete zones increased up to 6. The number did not exceed 6, but the next step was the broadening of the zone and the appearance of the tailing. Changes in the percent composition of each component were analyzed as a function of heating temperature and heating time. It was shown that (a) the mechanism of heat denaturation differs at the initial stage and at following stages, and that (b) some fraction of BSA is not denatured. A minor component 1'' was formed under some limited conditions. Effect of anti-denaturant was studied. The denaturation in distilled water was studied; it was found that only the native form was soluble and aggregates were precipitated. Present results were compared with those by Warner and Levy and by Stokrova *et al.* An attempt was made to assign present discrete zones to components postulated by them.

INTRODUCTION

Since the heat denaturation of protein is very popular phenomenon, numerous studies have been made on this subject and many results have been accumulated.[1-3] The serum albumin is one of the proteins of which the denaturation has been studied most extensively. Warner and Levy[4] studied the heat denaturation of bovine serum albumin (BSA) mainly by a sensitive solubility test and proposed a mechanism of heat denaturation, in which the intermediate and aggregates were involved. Stokrova *et al.*[5,6] studied the heat denaturation of human serum albumin by the Tiselius electrophoresis and by the ultracentrifuge. Sedlacek[7] studied it by the light scattering. These workers proposed their own mechanism of heat denaturation. Recently the BSA solution exposed to high pressure was analyzed by the acrylamide-gel electrophoresis[8]. It was suggested that the dimer of BSA was formed under high pressure and that the high pressure lowered the temperature at which the heat denaturation began to occur. This time the heat denaturation of BSA has been studied in more detail. The criterion of the denaturation was the appearance of the dimer in the gel pattern.

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EXPERIMENTAL

Armour's BSA was used. Lot no.'s were: M37088, M37551, B70411, D71209, D71002. A 1 % solution was prepared with 0.0414 *M* tris-0.0035 *M* EDTA-0.0127 *M* H_3BO_3 buffer at pH 8.9. Taking 1.0 ml of the solution into a test tube with a stopper, this was dipped in a water bath at a desired temperature. After a desired time, the test tube was dipped into a water at 0°C to stop the heat denaturation. The study was made in the temperature range 55-85°C. Gel electrophoresis was conducted at pH 8.9.[8] After the electrophoresis, the gel was stained by Amido-black 10 B. All the procedures of electrophoresis, staining, and densitometry were the same as were followed previously.[8] The densitometer used was Ozumer 82 of the Asuka Ind. Co. It was found by the measurement with a thermistor that it took about 60 sec. for 1 ml of the BSA solution to attain exactly to the temperature of thermostat, when the temperature was 65°C.

RESULTS AND DISCUSSION

Pattern of gel electrophoresis

When the temperature was below 55°C, there was no appreciable change in the gel pattern of BSA. At 58°C, however, new zones appeared. With increase in temperature and in heating time, the number of zones increased up to 5 or 6. Above a certain temperature and heating time, there was a tailing in the gel pattern. When BSA solution was heated at 80°C for 30 min., the pattern was stained evenly and strongly and gave no discrete zones. These values of temperature are not absolute, but depend on the Lot no. of BSA. In other words, some BSA gave the zone 2 at 55°C and some BSA did not give a tailing even at 85°C. In general, the BSA denatured at 65°C gave a pattern in which zones were

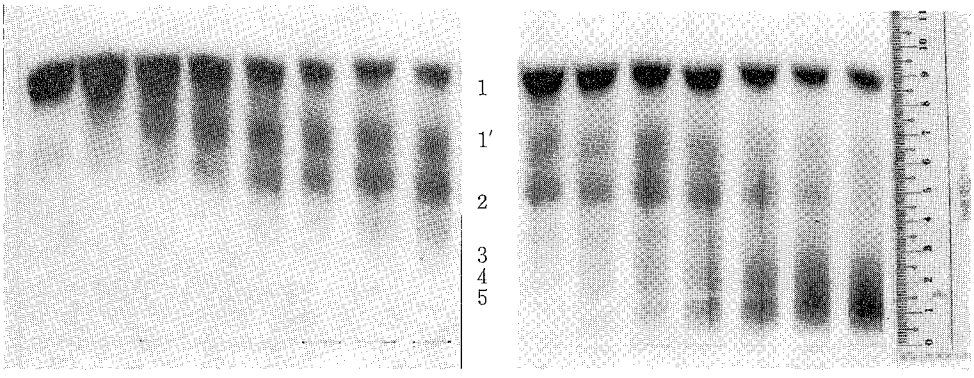


Fig. 1. Patterns of gel electrophoresis of BSA denatured at 65°C. Heating time is as follows.

A: native BSA.	B: 1 min.	C: 2 min.	D: 3 min.	E: 5 min.
F: 7 min.	G: 10 min.	H: 15 min.	I: 20 min.	J: 30 min.
K: 45 min.	L: 60 min.	M: 90 min.	N: 120 min.	O: 210 min.

Gel concentration was 5 %.

separated quite well. In Fig. 1 are given typical examples of gel pattern of BSA heated at 65°C; it is observed how the BSA is denatured as a function of heating time. The zones are numbered 1, 1′, 2, 3, 4 and 5 in the order of migration velocity.

In Fig. 2 is shown how the number of the zone increases with heating temperature and heating time. In area A the pattern was the same as that of the untreated BSA. The new zone 2 began to appear in area B. Zones 3, 4 and 5 began to appear in areas C, D and E, respectively. The pattern had a tailing in area F. It should be noted that the number of the discrete zone did not increase more than 6.

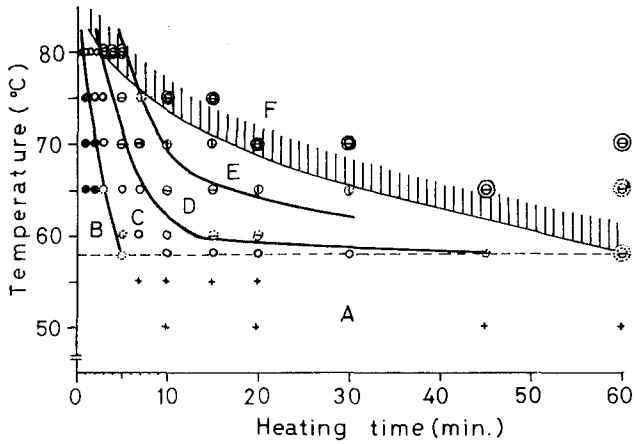


Fig. 2. Number of zones in gel pattern as a function of heating temperature and heating time. Double circle means that the gel pattern had a partial tailing, and dotted circle means that the slowest moving boundary was stained quite slightly.

Solutions of heat denatured BSA were analyzed changing the gel concentration.[9] Taking the migration of the zone 1 as standard, migration ratios of each zone were calculated. Figure 3 was obtained by plotting migration ratios against gel concentration.* As is seen, a horizontal line was obtained for the zone 1′, and sloping lines were obtained for zones 2 and 3. These results indicate that the component in zone 1′ is monomeric albumin and that components in zones 2 and 3 are polymerized or unfolded albumin.

An ultracentrifuge pattern of BSA, for example BSA treated at 70°C for 15 min., gave two boundaries; sedimentation coefficients of them were 3.8 S and 5.4 S. The former value corresponds to that of the native BSA and the latter to a dimerized BSA.[10] Combining results by ultracentrifuge and by gel electrophoresis, followings are concluded. While zones 1 and 1′ are separated in gel electrophoresis, these two components are involved in the slower boundary of the ultracentrifugal pattern. This indicates that the component 1′ is monomeric albumin which differs slightly from the native BSA in conformation. The com-

* Descriptions on the zone 1″ will be given below.

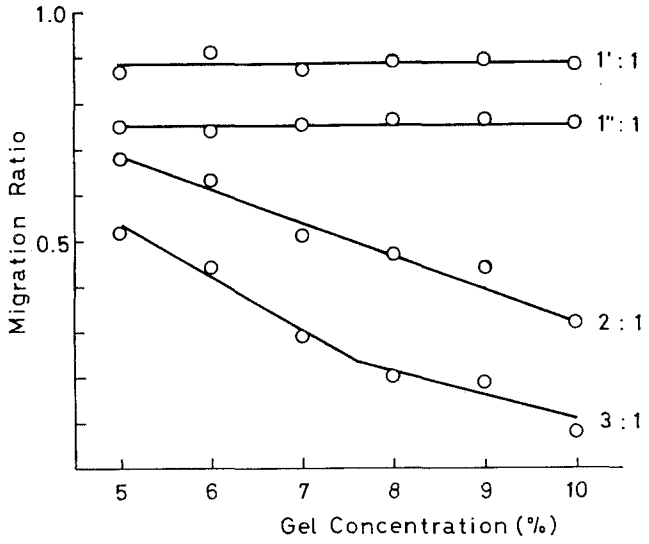


Fig. 3. Migration ratio *vs.* gel concentration. Migration of zone 1 was taken as standard.

ponent 2 is a dimerized albumin, and component 3 might be higher aggregate. After a long period of heating, a gross aggregate was formed, with some unfolding, to give a broad zone near the slot (patterns N to O in Fig. 1), and then a gross unfolding occurred to give a tailing.

Kinetics of denaturation

Figure 4 shows how the percentage of each zone changes with heating time at 65°C. The percentage of the zone 1 decreases monotonously with heating time, the curve for the zone 1′ has a maximum, and the sum of the percentages of zones 2-5 increases with time.* The assumption was made that the percentage of each component is proportional to the area percentage in the densitometer curve or to the staining intensity of each zone. It is observed in Fig. 4 that the slope of each curve changes remarkably at the initial stage of heating, and that it becomes gradual at the following stages. Figure 5 shows the decrease in the amount of the zone 1 with heating time at various temperatures. The higher the temperature, the sharper the decrease. There is a trend that the curve levels off when the heating time is longer enough. These results indicate that (a) the mechanism of heat denaturation differs at the initial stage of heating and at the following stages,** and that (b) some fraction of BSA is not denatured by heat. The amount of BSA, which is resistant to heat denaturation, decreases with increase in temperature. Its amount was found by heating the sample for 90-120 min. at each temperature. It was zero at 80°C, since the zone 1 disappeared at 80°C.

* When the amount of the zone 2 alone was plotted against the heating time, the curve obtained had a maximum locating to the right of the maximum of the curve for the zone 1′.

** A preliminary study indicated that the denaturation at the initial stage followed the second order kinetics.[11]

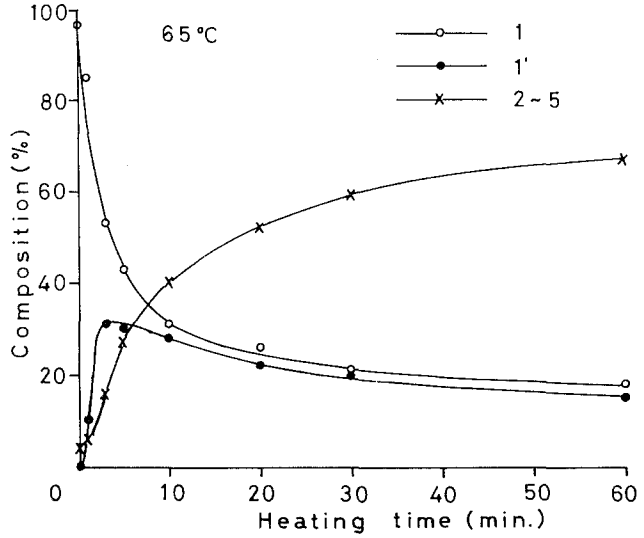


Fig. 4. Percentage of each component as a function of heating time at 65°C.

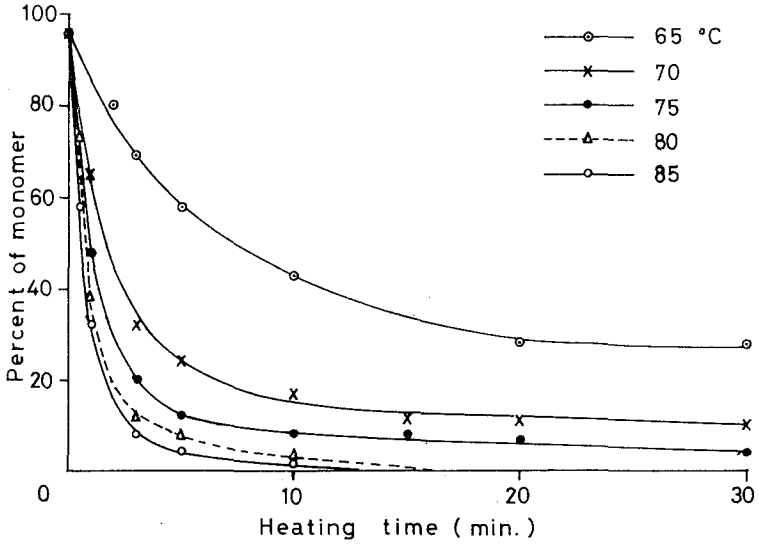


Fig. 5. Decrease of the percentage of zone 1 with heating time.

Effect of additives

A 1% solution of BSA was heated in presence of anti-denaturant, *i. e.*, $AgNO_3$, iodoacetamide, or *p*-chloromercuribenzoate. As was expected, these agents prevented the denaturation, when added in sufficient amount. In other words, there were almost no zones of 1′ and 2, when more than 1 mole of these agents were added to 1 mole of BSA.

The anionic detergent, sodium dodecyl sulfate (SDS), prevented the heat denaturation, when added in the molar ratio SDS/BSA=8–12. When SDS was added to BSA solution while the denaturation was going on, the denaturation

stopped absolutely at that moment. On the other hand, the cationic detergent, dodecyltrimethylammonium bromide, did not prevent the denaturation, or rather promoted it.

Existence of the zone 1″

Careful experiment of the gel electrophoresis revealed the existence of a minor component adjacent to the zone 1. When a 1 % solution of BSA was heated under a very mild condition, for example, 3-7 min. at 55°C or less than 5 min. at 65°C, a zone appeared. The zone is named 1″. Later, it was found that the zone 1″ appeared while the untreated BSA solution was stored in a refrigerator for more than a week (see Fig. 6). The zone was proved to be a monomer by the experiment changing the gel concentration (Fig. 3).

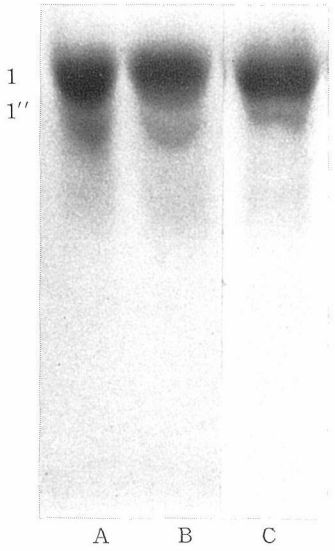


Fig. 6. Existence of zone 1″. A: pattern of untreated BSA after 4 weeks' storage, B: pattern of BSA, heated for 1 min. at 65°C, after 4 weeks' storage, and C: pattern of BSA, to which $AgNO_3$ was added in mole ratio $AgNO_3$/BSA=1.4, heated for 15 min. at 65°C.

The study was made whether or not the gel pattern of heat-denatured BSA would change during the storage in a refrigerator. Hereafter, the study was limited to BSA denatured at 65°C (see Fig. 7).

i) BSA heated for 2-5 min.: The percent composition changed, during storage, in zones 1, 1′, 1″, and 2, but not in 3 and 4. The greater part of the change occurred in the first week of storage, *i. e.*, the amount of form 1* increased, with a formation of form 1″, at the expense of the decrease of zones 1′ and 2. This indicates that forms 1′ and 2 were reversed to form 1 during the storage. There was no change after 4 weeks' storage.

* Form 1 means the component involved in the zone 1. Forms 1′, 1″ and 2 have the same meaning.

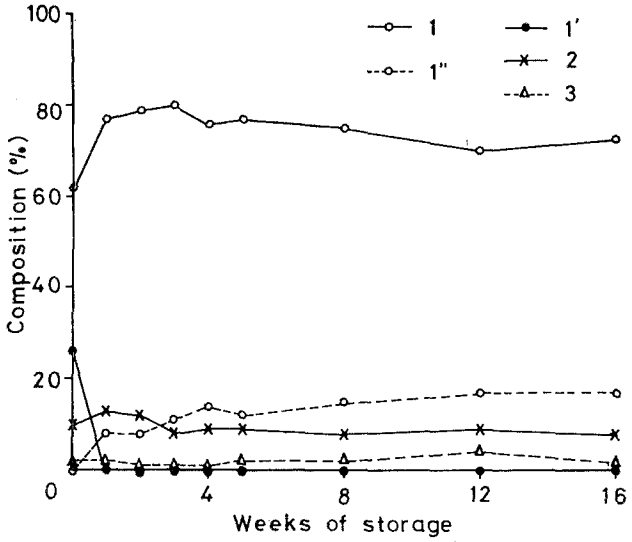


Fig. 7. Changes in the percent of each component of BSA, heated for 1 min. at 65°C, during storage.

ii) BSA heated for 1 min.: Analysis of the gel pattern indicated that two changes were superimposed. One of them was that forms 1′ and 2 were reversed to form 1, with a formation of form 1″; another was that the form 1″ was produced from the form 1. The former change resembles to that occurs, during storage, in BSA heated for 2-5 min., and the latter resembles to that occurs in the untreated BSA after the storage of longer time period.

iii) BSA heated for longer than 5 min.: The pattern had not zone 1″. The zone 1 increased and zones 1′ and 2 decreased during the storage, but the changes were less than those in BSA heated less than 5 min.

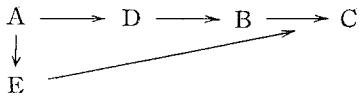
In short, the zone 1″ is produced only at the initial stage of very mild heat-denaturation. Its amount increases while BSA, denatured very gently, is stored in a refrigerator. The untreated BSA solution itself produces the zone 1″ during the storage for more than a week. Another feature is that there might be equilibria $1 \rightleftharpoons 1'' \rightleftharpoons 1'$, within a limit, for BSA denatured very gently.

The BSA solution was heated for 15 min. at 65°C in presence of $AgNO_3$, its mole ratio to BSA being 0.8. The percent composition in gel pattern was: 48 % for zone 1, 29 % for zone 1′ and 23 % for the rest. The BSA heated under the same condition without $AgNO_3$ gave the following composition: 29 % for zone 1, 19 % for zone 1′ and 52 % for the rest. Comparison of these figures indicates that $AgNO_3$ prevented the formation of zones 2-4, but not zone 1′. Then, the mole ratio of $AgNO_3$ to BSA was increased up to 1.6. With increase in the amount of $AgNO_3$, the amount of zones 1′ and 2 continued to decrease. Finally, the zone 1′ disappeared and the zone 2 attained to the same concentration as in the untreated BSA. It is added that the form 1″ appeared when the BSA solution, in which the mole ratio $AgNO_3$/BSA was more than 1.0, was heated for 15 min. at 65°C.

Mechanism

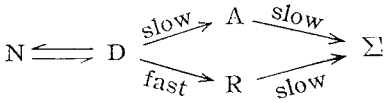
Combining results described above, the mechanism of heat denaturation is given as follows: (i) 1′ (and 1″) is formed, probably with some change in conformation of BSA, (ii) two 1′ are combined to give a dimer probably through sulfhydryl-disulfide exchange reaction, (iii) larger aggregates are formed, and then (iv) large unfolding occurs in BSA.

Warner and Levy[4] proposed a mechanism by which the heat denaturation occurs:

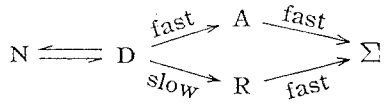


where A is the native BSA, B is the dimer or other small aggregate, C is the aggregated fraction, D is denatured, unaggregated BSA, insoluble at high stopping conditions, and E is unaggregated BSA, soluble at the high salt conditions. Here, A corresponds to the zone 1,* D to the zone 1′ (and zone 1″), B to the zone 2, C to zones 3-5, and E to the BSA which resists to heat denaturation.

Sedlacek (7) presented another mechanism:
for heating at 65°C



for heating at 80°C



where N is the native serum albumin, D is the denatured protein with perturbed tertiary structure, R is the heat denaturation resistant protein. A represents the modified monomer particles and Σ complex aggregates of A and R particles. Here, N corresponds to the zone 1, D to the "isomerized form" or to the zone 1″, A to the zone 1′, R to BSA which resists to heat denaturation, and Σ to zones 2-5.

As was described above, the number of the discrete zone attained to 6. The next step was the gross unfolding instead of the increase in the number of the zone.

Denaturation in distilled water

The 1 % BSA solution in distilled water was heated at 58°C. The white

* Each pattern in Fig. 1 has the zone 1. The zone 1 in Fig. 1, A is of course the native albumin. There is a possibility that the zone 1 in other patterns is not the native BSA, but that in the "isomerized form" by Warner and Levy.[4] If this interpretation is adopted, the assignment of the zone would change a little.

turbidity appeared within 15 sec., and it increased with increase in heating time. After the precipitate was removed by the centrifuge (20 min. at 8000 rpm.), the supernatant was analyzed by the gel electrophoresis. The pattern gave only zones of native albumin and of dimer impurity, indicating that zones 1′, 2, 3 and 4 do not exist in distilled water. In other words, only the native form is soluble and all of the denatured form is precipitated in distilled water; the phenomenon is all-or-none. In this case only, the rate of denaturation is followed by measuring the amount of precipitate.

The authors assumed in a previous paper[8] that the form 1′ was an intermediate of heat denaturation, and that the mechanism of heat denaturation was $1 \longrightarrow 1' \longrightarrow 2$. Now it seems necessary to take into account the form 1″ for consideration of the initial step of heat denaturation. However, it is known nothing about the properties of form 1″ as well as form 1′. It is our next object to isolate separately zones 1′, 1″ and 2, to study their properties and conformations, and to construct an over-all mechanism of heat denaturation.

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