Alkaline denaturation of bovine serum albumin
II. Analysis by isoelectric focusing
in polyacrylamide gels

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SUMMARY

Bovine serum albumins exposed to pH 11.9 for various periods (5 min.-6 hr.) at 20°C were analyzed by isoelectric focusing in polyacrylamide gels. Native BSA** monomers gave, in the electrofocusing pattern, three peaks with pI values 4.84, 5.15, and 5.39. After exposure to alkali, apparent shifts in the isoelectric point of some of the peaks and the appearance of new peaks with pI values 5.48, 5.70, 5.85, and 5.91 were observed.

Components of these peaks are broadly divisible into the following three fractions: 1. The “first fraction” which consists of the main components of native BSA monomer (pI ≤ 5.15). 2. The “second fraction” composed of SS-interchanged isomers (5.15 < pI > 5.70). 3. The “third fraction” consisting of irreversibly unfolded products (pI ≥ 5.70). The alkaline denaturation can be expressed by the following reaction diagram: the “first fraction” → the “second fraction” → the “third fraction”.

When the solution of native BSA monomer is exposed to pH 11.9 at 20°C for 4 hr., new components 1, 1'', 1', 2, and 3 are formed (the “second reaction”). Each of these components was isolated in the pure state by preparative disc gel electrophoresis and analyzed by electrofocusing. The results obtained are as follows: The component 1 and the native BSA monomer are composed of only the “first and second fractions”, but differ from each other in the situation of ionizable groups. Components 1'', 1', 2, and 3 each consists of all the three “fractions”. The components 2 and 3 are similar and 1'' and 1 also resemble each other, both in the state of charged groups. It is postulated that in the “second reaction” fatty acids, the impurity bound to BSA, migrate from the component 1'' to the component 1 in the course of the reaction, 1 → 1''.

Key words: serum albumin, alkaline denaturation, mechanism, electrofocusing.
INTRODUCTION

Isoelectric focusing as developed by Svensson and Vesterberg\textsuperscript{1}) is an effective means for studying conformation of proteins\textsuperscript{2,3}). Investigations of isoelectric heterogeneity of BSA have been performed by this method, and electrofocusing behaviors of commercial\textsuperscript{2,4-9), defatted\textsuperscript{6,8-11), heat-treated BSA\textsuperscript{6,8), and those of BSA in the presence\textsuperscript{2,7,11,12) of urea have been reported.

Recently, the alkaline denaturation of BSA has been studied by polyacrylamide gel electrophoresis, and it was found that at the initial step of the denaturation 8-9 discrete zones corresponding to components 1, 1', 1", 2", 2', 2, 3, 4, and 5 appeared in the gel\textsuperscript{13) (cf. a photograph in ref. 13). Components 1, 1", and 1' are monomers, components 2", 2', and 2 are subsequently produced (the "first reaction"). After the component 2 attained to a reasonable amount, the components 1" and 1' are formed from the component 1 through the intramolecular SH-S-S exchange reaction, leading to the successive formation of components 2, 3, 4, and 5 (the "second reaction")\textsuperscript{13,14}).

The object of this paper is to reconsider the mechanism of the initial step of the alkaline denaturation of BSA by means of isoelectric focusing in polyacrylamide gels. It is well known that the reproducibility of the electrofocusing pattern of serum albumin is affected by the binding of low molecular weight compounds (fatty acids, ampholytes etc.) to the albumin\textsuperscript{6,9,15,16) and by the amount of time for electrofocusing\textsuperscript{7). In order to exclude these effects all the electrofocusing experiments were conducted under identical conditions.

MATERIALS AND METHODS

1. Materials

Armour's crystalline BSA, Lot No. R-73306, was used. The content of dimer impurity was less than 8%. Native BSA monomers were prepared by gel filtration on a Toyopearl HW-55 Superfine column\textsuperscript{18) at pH 7.96, in 0.5 M NaCl-0.1 M Tris buffer. The albumin monomer solution obtained was then desalted and concentrated to 1.5% using Sephadex G-25 column. The concentration of the albumin in aqueous solution was determined with Hitachi's Beckman-type spectrophotometer using \[E_{1cm}^{1%} = 6.67 \text{ at } 279 \text{ nm}\textsuperscript{19). All other chemicals were reagent grade.

2. Procedure of alkali treatment

The procedure of alkali treatment was exactly the same as described previously\textsuperscript{14), and 1 % BSA solution* (1.5ml) of pH 11.9 was prepared in 0.13 M NaCl-NaOH solution at 20°C. After standing for a desired time, 5 min.-6 hr., the solution was dialyzed for 20 hr. at 5°C against 0.01 M phosphate buffer at pH 6.8, to stop the denaturation. The dialyzed solution was concentrated by "collodion bag" (Sartorius-Membranfilter, GmbH) to 1%, and an aliquot of the solution taken out was analyzed by electrofocusing in polyacrylamide gels.

3. Preparative disc gel electrophoresis

Components in alkali-treated BSA solution were isolated with a preparative disc gel electrophoresis apparatus, type CD-50 of Toyokagakusangyo Co. The mechanism of the apparatus was similar in principle to that of Goldon and Louis\textsuperscript{20). The concentration of acrylamide and bisacrylamide used in the resolving gel and stacking gel was 7.7 %×2.6 % and 3.1 %×20 % respectively. The buffer solution for the upper (cathode) vessel was 0.052 M Tris-0.052 M glycine at pH 8.9. The buffer used for elution was identical with that in the lower (anode) vessel which was 0.1 M Tris-0.05 M HCl, pH 8.1. The sample solution containing 120mg protein and 20 %

*1 ml of 1.5% native BSA monomer+0.4 ml of 0.4 M NaCl+0.1 ml of 0.4 M NaOH
sucrose (w/w) was put directly on the stacking gel using a peristaltic pump with a silicone tubing. Experimental conditions were as follows: electrophoretic run 8-9 hr. at 10°C, current density 5 mA/cm², eluted volume per test tube 10 ml, and the elution velocity 1.7-0.9 ml/min*. The eluate was monitored continuously with a UVICON-540M spectrophotometer (Tokyo) at 280 nm. Solutions in several test tubes of the fraction collector were collected to obtain homogeneous fractions. Each of the fractions was dialyzed against 0.01 M phosphate buffer at pH 6.8, concentrated to 1 %, and analyzed by electrofocusing in polyacrylamide gels. Each fraction gave, by gel electrophoresis, one clear zone of component 1, 1", 1', 2 or 3.

4. Isoelectric focusing in polyacrylamide gels

This was carried out according to the method of Wrigley with slight modifications. An apparatus for gel electrofocusing, model E-IES 12-15 of KPI (Japan), was employed. The gel mixture was prepared in twelve glass tubes (15×0.5 cm i.d.) from 16 ml of 15 % acrylamide, 0.75 % N, N'-methylene bisacrylamide, 6 ml of 0.004 % riboflavin, 2.4 ml of 40 % Ampholine**, and 23.6 ml of water. Glass tubes were filled to within 8 mm from the top. After photopolymerization, 40 µl of the sample solution (containing 2 % Ampholine, 30 % glycerol, and 0.5 % protein) was layered on top of the gel, followed by 60 µl of the protecting solution (containing 2 % Ampholine, 15 % glycerol, and 0.8 % glutamic acid). An anode electrolyte (upper vessel) consisted of 0.02 M H₃PO₄, and a cathode electrolyte (lower vessel) was 0.1 M NaOH. A constant current of 1.5 mA per tube was applied for 30 min. by gradually increasing the voltage up to 1060 V. This voltage was maintained for 3.9 hr. till the end of the run, and the current decreased to a final value of 0.4 mA per tube. During the run the temperature of the gel was maintained below 3°C. After electrofocusing the gel was washed with 15 % trichloroacetic acid and stained by amido-black 10B. The stained gel was analyzed with a Shimadzu dual-wavelength TLC scanner, model CS-900. For the determination of the pH gradient, an unstained gel run simultaneously with stained gels was sliced into 8 mm sections. Each of the sections was suspended in 1 ml of water, and the pH was measured at 3°C with a Hitachi-Horiba modal F-7ss glass electrode pH meter.

RESULTS AND DISCUSSION

1. Native BSA monomers

Electrofocusing of the native BSA monomer with 2 % Ampholine, pH range 4-7 under the standard conditions is shown in Fig. 1 (A). Three peaks focused at pH values 4.84, 5.15, and 5.39 were observed. This was highly reproducible.

By isoelectric focusing in a column with a sucrose density gradient of native BSA monomer Rosseneu-Motreff et al. demonstrated three fractions with isoelectric points of 4.89, 5.22, and 5.59, and it was ascertained that bovine albumin complexed with fatty acids was focused near pH 4.9. On the other hand, by electrofocusing of commercial BSA, Wallevik demonstrated that there were at least 6 peaks with pI values ranging from pH 4.58 to 5.45, and that the main components of bovine albumin were focused at pI 4.83 and 5.26. Furthermore, it has been indicated that the peak near pI 4.8 is the bovine albumin combined with fatty acids, and that the albumin subjected to disulfide interchange is focused as a minor peak near pI 5.45.

Judging from the above reports it is estimated that the peaks observed in Fig. 1 (A) correspond to the three isoelectric components shown by Wallevik; viz., the two peaks, pI 4.84 and 5.15, are the main components of native BSA monomer, and the former is the albumin complexed with fatty acids. The peak pI 5.39 is equivalent to the albumin subjected to disulfide interchange.

2. Native BSA monomers exposed to alkali

In Fig. 1(B-H) are given typical gel electrofocusing...
The main components of native BSA monomer

When the solution of native BSA monomer was exposed to pH 11.9 for 5 min, the isoelectric point of the pI 4.84 peak shifted to pH 4.94, and the percentage of the area under the protein peaks with pI values lower than pH 5.15 decreased remarkably with increase in exposure time (see Fig. 1). A similar phenomenon was observed by Ito et al.8) They reported that the amount of isoelectric form pI 4.90, the main component of BSA, decreased by heat treatment of BSA. Hereafter, the main components of native BSA monomer with pI values from 4.84 to 5.15 are called the “first fraction”. The curve a in Fig. 2 shows how the percentage of the “first fraction” decreases as a function of exposure time.

(b) Irreversibly unfolded albumins

As is seen in Fig. 1 (B–H), when the native BSA monomer was exposed for 5 min, 15 min, and 2 hr, new peaks with pI values 5.71, 5.85, and 5.90 appeared, respectively. It is known that focusing of heat-treated BSA gives a new peak pI 5.908), and that in the presence of 6 M urea bovine albumin focuses in 2–4 peaks with pI values near pH 6.11,12) Furthermore, Salaman et al.2) theoretically estimated the pI value of BSA completely unfolded by urea at 6.03.
For these reasons it is believed that the components of the focused peaks in the pH 5.70-5.90 region would be irreversibly denatured products differing in degree of unfolding. These components are named the “third fraction”. The curve c in Fig. 2 shows how the percentage of the “third fraction” increases as a function of exposure time. Next, in Fig. 3 is shown the change in percent composition of each component in the “third fraction” as a function of exposure time; and after exposure for 2 hr. there occurred a marked decrease of pI 5.85 component, being compensated by the appearance of component pI 5.90. These results suggest that the pI 5.90 form is produced from the pI 5.85 component by further unfolding.

(c) SS-interchanged isomers

When the native BSA monomer was exposed to pH 11.9 for 5 min. the isoelectric point of the pI 5.39 peak shifted to pH 5.43, and with increase in exposure time new peaks with pI values 5.52 and 5.48 appeared (see Fig. 1). Hereafter, components of the peaks with isoelectric points ranging from pH 5.39 to 5.54 are named the “second fraction”. The percentage of the area of the “second fraction” reached a maximum (ca. 50 %) when the native BSA monomer was exposed to alkali for 1–3 hr. (see curve b in Fig. 2). Wallevik6) has demonstrated that when bovine albumin was slightly heat-denatured 60% of the protein was focused around pH 5.45. Furthermore, he has concluded that among isoelectric peaks of defatted bovine albumin, the peaks focused at pI 5.39 and 5.45 are irreversibly SS-interchanged albums, and that the former is the “aged” albumin described by Foster and co-workers22,23) and the latter is albumin SS-interchanged to a higher degree than “aged” albumin. From this conclusion it is inferred that the peaks in the “second fraction” are a series of SS-interchanged isomers11,24) caused by the alkali treatment of BSA.

(d) Mechanism of alkaline denaturation

From shapes of curves observed in Fig. 2 the alkaline denaturation of native BSA monomer can be expressed from a viewpoint of isoelectric compo-
component by the following reaction scheme: the "first fraction" → the "second fraction" → the "third fraction".

3. Components isolated from alkali-treated BSA

When the native BSA monomer was exposed to pH 11.9 for 4 hr. at 20°C, 5 components (1, 1", 1', 2, and 3) were formed. As is seen in Fig. 4 these components were isolated by preparative disc gel electrophoresis. Fig. 5 shows that these components were separated in the pure state. Each of these components was analyzed by gel electrofocusing (see Fig. 6).

(a) Distribution of the "second and third fractions"

In Table 1 is shown the distribution in the three "fractions" of each component isolated. The percentage of the area of the "second fraction" was: 23% for the native BSA monomer, 45% for the component 1, and ca. 60% for the other components. It is clear that this increasing percentage of the "second fraction" of the components is indicative of a further degree of SS-interchange.

As is shown in Table 1 the component 1 is composed of the "first and second fractions" but does

Fig. 5. Diagrammatic patterns of polyacrylamide gel electrophoresis.

Gel concentration 5%. Components isolated by preparative disc gel electrophoresis (see Fig. 4) were concentrated to 1%. A, 1% solution of native BSA monomer exposed to pH 11.9 for 4 hr. at 20°C; B, isolated component 1; C, isolated component 1"; D, isolated component 1'; E, isolated component 2; F, isolated component 3.

Fig. 6. Gel electrofocusing patterns of each component isolated from native BSA monomer exposed to pH 11.9 for 4 hr. at 20°C by preparative disc gel electrophoresis.

Ampholine range pH 4-7. Broken line represents pH gradient. X: position along the gel. A, component 1; B, component 1"; C, component 1'; D, component 2; E, component 3.
not contain the "third fraction", the irreversibly denatured products. This fact agrees with our opinion\textsuperscript{13,14} that the component 1 is reversibly alkali-expanded albumin monomers. Next, the percentage of the "third fraction" of components 1" and 1' is ca. 15% and that of components 2 and 3 ca. 33%. These results support the interpretation\textsuperscript{13,14} given previously that the alkaline expansion of components 1" and 1' would be slightly irreversible and that of components 2 and 3 might be more irreversible. The percentage of the "third fraction" may be regarded as a measure of the irreversible unfolding of the components.

(b) Comparison of electrofocusing patterns

As is seen in Fig. 6 there was a resemblance between the two electrofocusing patterns of components 2 and 3. This indicates that these components resemble each other in the state of dissociating groups. The same may be observed in the patterns of components 1" and 1'. Furthermore, almost the same distribution was observed in the three "fractions" of components 2 and 3 (see Table 1). This finding suggests that the difference in the migration velocity of components 2 and 3 in gel electrophoresis depends primarily upon the size and shape of the molecules. Next, there is differences between the two electrofocusing patterns of native BSA monomer and component 1, a monomer of BSA, (see Fig. 1(A) and Fig. 6(A)). This indicates that these monomers are different from each other in charges.

(c) Isoelectric component pI 4.94

It is well known that serum albumin exists as a protein combined with long chain fatty acids\textsuperscript{25}. Brandt and Andersson\textsuperscript{26} reported that when the solution of commercial BSA was heated long chain fatty acids migrated from the aggregating albumin molecules formed to the remaining monomer thereby stabilizing the latter against heat denaturation. Furthermore, Aoki et al.\textsuperscript{27,28} stated that bovine albumin molecules having 6 moles of long chain fatty acid per mole albumin was stable against heat denaturation. Recently, it has been suggested that when the solution of serum albumin was exposed to higher pH some albumin monomer strongly resisted denaturation\textsuperscript{14}.

As is seen in Fig. 6 the pI 4.95 component which must be the albumin combined with fatty acids was observed in the pattern of component 1 alone and not in those of other components. This suggests that when BSA was exposed to alkali fatty acids, attached to BSA molecules, migrate to the component 1 and that the pI 4.95 form observed in the pattern of component 1 is in the form resistant to alkaline denaturation. Under the present conditions of alkali treatment the "second reaction" (1 → 1" → 1' → 2 → 3) takes place\textsuperscript{13,14}; and it is presumed that fatty acid molecules, the impurity bound to BSA, migrate from the component 1" to the component 1 in the course of the reaction, 1 → 1".

REFERENCES

1) Vesterberg, O. and Svensson, H.: Acta
要 旨

露出時間（5分～6時間）を変えて20℃でpH 11.9に曝したBSAを、ゲル等電点電気泳動で分析した。
native BSA monomerでは3つの成分（pI 4.84, 5.15, 5.39）が観察された。アルカリ変性によって、これら成分の等電点ソフトと、5つの新しい成分（pI 5.48, 5.52, 5.70, 5.85, 5.91）が認められた。

これらの諸成分は、(1) native BSA monomerの主成分からなる“first fraction”（pI≦5.15）、(2) SS-交換異性体からなる“second fraction”（5.15<pI≤5.70）及び、(3)不可逆性にunfoldingした生産物からなる“third fraction”（pI≧5.70）の3つに大別される。アルカリ変性は次の反応図式で表わされる。“first fraction”→“second fraction”→“third fraction”

native BSA monomerを20℃で4時間pH11.9に曝すと5つの成分1, 1’, 2, 3, 4が生じる（“second reaction”）。これら5成分を調製用ディスクゲル電気泳動で単離したのち、ゲル等電点電気泳動で分析して次の知見を得た。成分1とnative BSA monomerは何も“first及びsecond fractions”だけからなってきているが、両成分のイオン化できる基の状態は異なっている。成分1, 1’, 2, 3, 4はそれぞれ“first fraction”からなっており、成分2と3及び成分1と1’とはそれぞれ似た電荷の状態を示す。“second reaction”においては、albに結合していた脂肪酸分子は、1→1’sの反応過程で成分1に飛び移るものと推測される。