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Studies on γ-Ray Irradiated Bovine Plasma Albumin Solution*

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Most of the investigation on γ- or x-ray irradiated albumin solution were directed to produce the changes in viscosity, sedimentation, absorption spectrum etc. which were the most destructive process, occuring after irradiation of large dose. The authors used subfractionation technique of bovine plasma albumin (hydroxylapatite column) for the studies on γ-ray irradiated protein solutions and found that the subfractionation technique was very useful in the field of radiation chemistry of proteins. That is, the changes in chromatogram were more radiosensitive than those of viscosity, absorption spectrum and amperometric titration of -SH groups. Therefore, the effects of dose rate, protein concentration, pH and salts on γ-ray irradiation of protein solution were investigated mainly by used column chromatography. It was found that the above stated factors produced the marked effects on γ-ray irradiation of bovine plasma albumin solutions.

INTRODUCTION

The effects of radiation on enzyme or protein solutions have been studied by many investigators by using enzyme activity, absorption spectrum, viscosity, sedimentation, electrophoresis etc. Hardly, any studies using subfractionation technique have been made on effects of ionizing radiation on protein solution except by Rosen1-3. The authors have studied on the effects of various factors, such as protein concentration, pH, salts, temperature, oxygen tension, dose rate etc., on γ-ray irradiated bovine plasma albumin solution by using column chromatography, viscosity, absorption spectrum and amperometric titration1,2,4-6.

EXPERIMENTAL MATERIALS AND METHODS

Armour’s bovine plasma albumin was used. Paper electrophoretic analysis at pH 8.50, I/2 0.045 showed that bovine plasma albumin was almost a single component. However, chromatographic analysis (hydroxylapatite column) showed that bovine plasma albumin had three subfractions eluted by 0.07 M, 0.11 M

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(392)
γ-Ray Irradiated Bovine Plasma Albumin Solution

and 0.40 M of sodium phosphate buffer pH 6.8 and that ratio of three subfractions changed from sample to sample\(^{(16)}\). In the experiment described in the next section (II. Chromatographic Studies), the same sample was used.

Redistilled water was used in these experiments.

Viscosity was measured at 29.9±0.05°C in Ostwald’s viscometer. Absorption spectrum was measured by using Beckman DU spectrophotometer in quartz cell of 10 mm width. Amperometric analysis of -SH groups was carried out by the method of Weissman and Benesch\(^{(9)}\). That is, 1.0 ml of 3 per cent bovine plasma albumin solution was added dropwise to 30 ml of ammoniacal solution (60 per cent ethanol, NH\(_2\)NO\(_3\) : 0.05 M, NH\(_2\)OH : 0.25 M) and titrated with 0.001 N silver nitrate solution.

Chromatography was performed at room temperature by using very coarse grain of hydroxylapatite (1.5 cm×12 cm column) as an adsorbent and 0.02, 0.04 M, 0.07 M, 0.11 M and 0.40 M of sodium phosphate buffer pH 6.8 as eluants\(^{(16)}\). The coarse adsorbent was prepared by authors’ method and this column (1.5 cm×12 cm) could eluted 80~120 ml per hour\(^{(16)}\). However, in these experiments, the elution rate was about 35 ml per hour and the effluent was fractionated into fractions of 3.5 ml each by using a fraction collector. The protein concentration was analysed by the extinction at 280 m\(\mu\), because the extinction of unirradiated protein at 280 m\(\mu\) was nearly equal to that of irradiated one in the authors’ experiment. The adsorbent was poisoned by γ-ray irradiated protein solution\(^{(14)}\). The deactivated adsorbent was washed out with distilled water from 5 chromatographic tubes and suspended in 1 liter of 0.01 M of sodium phosphate buffer pH 6.8. And then, this solution was heated with stirring until 84~87°C. After standing for 1~2 minutes, the heated solution was decanted and 1 liter of 0.01 M sodium phosphate buffer pH 6.8 was added. The same procedure was repeated several times (3~4 times). The deactivated adsorbent was reactivated. By using this reactivation method, the same adsorbent could be used many times\(^{(15)}\).

The irradiation of protein solutions was performed at room temperature in an open pyrex glass tube with γ-rays from Co\(^{60}\) having dose rate of 2.4×10\(^6\) r per hour and 4×10\(^5\) r per hour. After irradiation, pyrex glass tubes were immersed in ice-water and after standing for 24 hours, the samples were analysed.

pH values of protein solution before and after irradiation were measured by glass electrode.

**EXPERIMENTAL RESULTS AND DISCUSSION**

(1) **Comparison of Changes of Chromatogram with those of Viscosity Absorption Spectrum and -SH-groups of Protein**

0.6 per cent bovine plasma albumin solution (0.02 M sodium phosphate buffer pH 6.8) was irradiated with γ-rays having an intensity of 2.4×10\(^6\) r/hr at 25°C. Relative viscosity was measured at 29.9±0.05°C, 24 hours, after irradiation. As reported by Barron\(^{(14)}\), γ-ray irradiation with 9×10\(^4\) r of albumin solution in 0.02 M sodium phosphate buffer pH 6.80 produced a small increase
in viscosity as shown in the left part of Fig. 1. γ-ray irradiation of $9 \times 10^4 \text{r}$ produced marked change in chromatogram. That is, 1st subfraction eluted by 0.07 $M$ was decreased from 77 per cent to 39 per cent as shown in Fig. 1.

Fig. 1. The left diagram shows the relative viscosity of 0.6 per cent bovine plasma albumin solution (0.02 $M$ sodium phosphate buffer pH 6.8) against dose of γ-rays having an intensity of $2.4 \times 10^5 \text{r}$ per hour. The right upper and lower diagram show column chromatogram of unirradiated and irradiated ($9 \times 10^4 \text{r}$) albumin. The abscissa and ordinate show tube numbers of fraction collector and extinction at 280 mμ, respectively.

Amperometric titration of -SH-groups was performed at room temperature in ammoniacal solution (60 per cent ethanol, $\text{NH}_4\text{NO}_3 0.05 M$, $\text{NH}_2\text{OH} 0.25 M$) by titrating with 0.001 $N$ silver nitrate solution (9, 10). 570 mg of bovine plasma
γ-Ray Irradiated Bovine Plasma Albumin Solution

albumin was dissolved in 30 ml of redistilled water. The irradiation of γ-rays was performed at 9°C in an open pyrex tube under the following conditions: dose rate $2.4 \times 10^5$ r per hour, dose $5 \times 10^4$ r, $1.5 \times 10^5$ r, $2.06 \times 10^5$ r, $2.5 \times 10^6$ r, $3 \times 10^6$ r, $5 \times 10^6$ r. γ-ray irradiation with $5 \times 10^5$ r decreased only 17 per cent of -SH groups. On the other hand, in the chromatographic analysis, 1st subfraction eluted by $0.07 M$, was decreased from 65 per cent to 18 per cent. The results were illustrated in Fig. 2. The chromatographic changes of γ-ray irradiated protein were not mainly due to oxidation of -SH groups as in the case of SH-enzyme.$^{11-13}$ However, chromatographic changes were protected by the addition of cysteine.

γ-ray irradiation of 0.033 per cent bovine plasma albumin solution (solvent: redistilled water) was performed at 9°C with γ-rays having dose rate $4 \times 10^3$ r per hour. After irradiation, there was a steady rise of light absorption.$^5$ Therefore, after standing for 24 hours at 0°C, the absorption spectrum was measured at 9°C. Absorption spectrum changes at 240, 251, 297 and 310 mµ of γ-ray irradiated bovine plasma albumin were illustrated in the upper part of Fig. 3. These results were consistent with those of Barron.$^9$ That is, γ-ray irradiation with $4 \times 10^3$ r of 0.033 per cent albumin solutions in water produced a small increase in extinctions at 240 mµ and 251 mµ. On the chromatographic analysis, the irradiation with $4 \times 10^3$ r decreased the 1st subfraction eluted by $0.07 M$ sodium phosphate buffer pH 6.8 from 68 per cent to 28 per cent as shown in Fig. 3.

From comparison of changes of chromatogram with those of viscosity,
absorption spectrum and -SH-groups, it was found that the chromatogram was more sensitive toward radiation than other measurements. Therefore, the authors used chromatographic analysis to study the effects of various factors such as dose rate, temperature, protein concentration, pH, salt, oxygen tension etc., on γ-ray irradiated protein solution.

(II) Chromatographic Studies

By using hydroxylapatite column, the authors studied the effect of γ-ray irradiation on protein solution under the following conditions: dose rate 4×10^3 r per hour and 2.4×10^5 r per hour, protein concentration 3 per cent-0.019 per cent, pH 6.8 and 5.25. The irradiated solutions were immersed in ice-water for 24 hours and chromatographed. As shown in Figs. 4 and 5, only log (percentages of the 1st subfraction) was linear against dose of γ-rays. These results were similar to those on Dow X-2 column. The relation between log (percentages of 1st subfraction) and dose of γ-rays was different from that of heat denaturation of bovine plasma albumin. On the heat denaturation, 1st subfraction was decreased according to reversible 1st order kinetics. And also, on heat denaturation of bovine plasma albumin, percentage of 2nd subfraction was constant for about 100 minute at 60°C and then gradually decreased.

When the concentration was decreased, there was a proportional decrease in 37 per cent dose of the 1st subfraction (D37). That is, D37 of 0.019 per cent, 0.038 per cent and 0.075 per cent were 3×10^3r, 5.1×10^3r, 7.5×10^3r, respectively, at...
γ-Ray Irradiated Bovine Plasma Albumin Solution

16°C, γ-rays having an intensity of $4 \times 10^3$ r per hour. The protein was dissolved in redistilled water on these experiments. Radiosensitivity of albumin solution was also affected by dose rate of γ-rays as reported by Forsberg. D$_{37}$ of 0.019 per cent, 0.038 per cent and 0.075 per cent were $1.4 \times 10^4$ r, $1.6 \times 10^4$ r and $2.2 \times 10^4$ r, respectively, at 28°C, γ-rays having an intensity of $2.4 \times 10^5$ r per hour. These results were shown in Fig. 6. Therefore, the results of γ-ray irradiation on protein solution were markedly affected by protein concentration and dose rate. When the concentration of albumin and dose rate of γ-rays are decreased, D$_{37}$ of 1st subfraction will be as low as that of SH-enzyme. Therefore, some sort of indirect action was apparently involved in the chromatographic changes.

The presence of salts (0.01 M, 0.02 M, 0.04 M, 0.07 M sodium phosphate buffer pH 6.8) in the 0.3 per cent bovine plasma albumin solutions had a marked effect on the changes of chromatogram, as shown on irradiation with $7 \times 10^4$ r at 13°C, γ-rays having an intensity $2.4 \times 10^5$ r per hour. As increasing the concentration of sodium phosphate buffer, 1st subfraction eluted by 0.07 M was decreased and 3rd subfraction eluted by 0.40 M was increased. However, 2nd subfraction was constant as shown in Fig. 7. On the other hand, it was reported that presence of salts had a marked protecting effect on the changes of absorption spectum and precipitation of protein on irradiation of x-rays. The mechanism by which the difference appears between changes in chromatogram and absorption spectrum will be not clear until more data are available.

Fig. 6. 37 per cent dose of 1-st subfraction against protein concentration. γ-ray irradiation was performed at 16°C (○) and 26°C (●). γ-rays having intensities of $4 \times 10^3$ r per hour (○) and $2.4 \times 10^5$ r per hour (●).

![Graph showing D$_{37}$ against dose rate and protein concentration](image)

Fig. 7. The effect of salts on chromatographic changes of γ-ray irradiated bovine plasma albumin. ○, ■ and ● show the subfractions eluted by 0.07 M, 0.11 M and 0.40 M sodium phosphate buffer pH 6.8, respectively. The ordinate and abscissa show percentages of three subfractions and moles of sodium phosphate buffer pH 6.8. The lightest circles show three subfraction of unirradiated albumin.
To study the effect of pH changes, bovine plasma albumin was dissolved either in water (pH 5.25), in 0.02 M acetate buffer (pH 4.10, 4.70, 5.78) or in 0.02 M sodium phosphate buffer (pH 6.68, 7.55). γ-ray irradiation with $7 \times 10^4 \text{r}$ of 0.3 per cent albumin solution was performed at 30°C, γ-rays having an intensity $2.4 \times 10^5 \text{r per hour}$. When bovine plasma albumin solution dissolved in distilled water was irradiated with $7 \times 10^4 \text{r}$ at 30°C, small amount of precipitation occurred. This solution was centrifuged at 2,5000 rpm for 15 minutes. The supernatant solution was chromatographed. The greatest changes in light absorption were observed on x-ray irradiation of alkaline solution. However, in chromatographic analysis, the greatest changes was observed on γ-ray irradiation of acid solution as in the case of SH-enzyme. This may be due to high concentration of $\text{H}_2\text{O}_2$ formed at acid solution. On the chromatographic studies of the effect of pH on heat denaturation, the greatest change was observed in alkaline solutions except at the isoelectric region of albumin as shown in Fig. 9.

The changes in chromatogram were more sensitive toward γ-ray irradiation than those of viscosity, absorption spectrum etc which were most destructive process. However, the mechanism by which changes in chromatogram occur will be not clear, until more informations are available on the molecular structure of albumin subfractions. Therefore, the authors made a little discussion on the results.

**Fig. 8.** Effect of pH on chromatographic changes of γ-ray irradiated albumin. γ-ray irradiation with $7 \times 10^4 \text{r}$ was performed at 30°C, γ-rays having an intensity of $2.4 \times 10^5 \text{r per hour}$. ○, • and ○ show three subfractions which were eluted by 0.07 M, 0.11 M and 0.40 M sodium phosphate buffer pH 6.8, respectively. The lightest three circles show percentages of three subfractions of unirradiated albumin solution (pH 6.8). The ordinate and abscissa show percentage and pH, respectively.

**Fig. 9.** Effect of pH on chromatographic changes of heat denatured albumin (0.20 per cent, 60°C for 90 minutes). See Fig. 8 for other conditions.

**SUMMARY**

The authors studied on the γ-ray irradiated bovine plasma albumin solutions
γ-Ray Irradiated Bovine Plasma Albumin Solution

by using hydroxylapatite column, viscosity, absorption spectrum and amperometric titration of -SH-group. The results obtained were as follows:

1. It was found that the changes in chromatograms were more radiosensitive than those of other measurements.
2. Log (percentage of 1st subfraction) was linear against dose of γ-rays.
3. Dose rate of γ-rays had a marked effect on the changes of chromatogram. When the dose rate of γ-rays was decreased, 37 per cent dose of 1st subfraction was decreased.
4. When pH and protein concentration were decreased and salt concentration was increased, there was a proportional increase in the changes of chromatogram.
5. When protein concentration and an dose rate of γ-rays are decreased, 37 per cent dose of 1st subfraction will be as low as that of SH enzyme.

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