Stability of Urokinase in Solutions Containing Sodium Bisulfite[1)]

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The stabilities of urokinase (UK) in aqueous solution were investigated at pH 5.0—8.0 in the presence (1.0—3.0 × 10^{-3} M) and absence of sodium bisulfite (SBS) both under scattered light (1000 lux) and in the dark using the fluorogenic substrate method. Increasing concentrations of SBS tended to increase the inactivation of UK. In the presence of SBS, with the increase in the pH value, UK gained in stability in the pH range of 5.0—8.0. The stability of UK in the presence of SBS in the dark was larger than that under scattered light, especially at pH 5.0. Therefore, it was suggested that the difference in the residual activities of UK between under light and in the dark was due to free radicals formed during the autooxidation of bisulfite under scattered light.

UK was stabilized by glucose in the presence of SBS both under scattered light and in the dark. One reason for this phenomenon was postulated to be the formation of inactive bisulfite–glucose addition compound.

The degradation products of UK during storage in a solution containing SBS were investigated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. UK was revealed to be split into M.W. 36000 form and M.W. 20000 form by SBS.

Keywords sodium bisulfite; urokinase; inactivation; free radical; stabilization by glucose; scattered light

Sodium bisulfite (SBS) used as a stabilizer in injectable preparations is known to degrade various drugs including thiamine,[2)] fursultiamine,[3)] gabexate mesilate,[4)] nafamostat mesilate,[5)] insulin,[6)] and oxytocin.[6)] However, there are no reports on the reactivity of SBS with urokinase (UK), a plasminogen activator, except the irreversible inactivation of UK reported by Kitamura *et al.*[7)] This irreversible inactivation has not yet been studied in full detail.

It has also been reported that the oxidative radicals formed during autooxidation of bisulfite (BS) (BS radicals) induce the oxidation and destruction of amino acid residues.[8,9)] Another suggestion is that BS radicals are generated by scattered light.[10)]

UK is an enzyme derived from urine which functions to activate plasminogen. UK is composed of a light chain (M.W. 20000) and a heavy chain (M.W. 36000) in which the active site including serine residue is located. These two chains are linked by one disulfide bond.[11)] In the present study, the stability of UK was measured in aqueous solution at pH 5.0—8.0 in the presence and absence of SBS both under scattered light and in the dark. Subsequently, in consideration of the formation of inactive BS–glucose addition compound,[3)] the stabilization of UK by glucose was attempted in the presence of SBS both under scattered light and in the dark. The degradation products of UK in the presence and absence of SBS were investigated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Experimental

Materials UK for injection (Urokinase inj.-Green Cross,® lot No. 055GG, M.W. 55000) 60000 IU was supplied by the Green Cross Co., Ltd. Purified UK of M.W. 55000 was obtained from the product, UK inj. lot No. 055GG, by serial column chromatography as reported by Miwa *et al.*[12)] SBS, Glt–Gly–Arg–MCA,[13)] glucose, pararosaniline and the other agents were commercial products of special grade. Buffer solutions, *i.e.*, 0.05 M acetate buffer (pH 5.0), 0.05 M phosphate buffer (pH 6.0 and 7.0) and 0.05 M Tris–HCl buffer (pH 8.0) were adjusted to an ionic strength (μ) 0.5 with sodium chloride.

Samples and Storage Conditions The stability of solubilized UK inj. (120 IU/ml, *ca.* 15.1 nM) was studied at pH 5.0—8.0 and μ=0.5 in the presence (1.0—3.0 × 10^{-3} M) and absence of SBS under scattered light (indoor 1000 lux) and in the dark at 25 °C. Sample solutions were stored in glass vials of 30 ml in inner volume, and these vials were completely filled with the solutions.

To evaluate the influence of glucose on UK in the presence of SBS both under scattered light and in the dark, glucose was added to the solution of UK inj. (120 IU/ml) buffered at pH 5.0—7.0 and μ=0.5 in the presence of SBS (1.0 × 10^{-3} M).

Determination of UK Activity UK activity was measured by the fluorogenic substrate method using Glt–Gly–Arg–MCA.[13)] The fluorescence was monitored using a Shimadzu fluorospectrometer model RF-510.

SDS–Polyacrylamide Gel Electrophoresis The purified UK (200000 IU/ml) in the presence (1.5 × 10^{-3} M) and absence of SBS were subjected to SDS–polyacrylamide gel electrophoresis.

Electrophoresis System PhastSystem™ and PhastGel Gradient 8-25 plates (Pharmacia LKB Biotechnology AB) were used. Electrophoresis was performed according to the manual for the instrument. After the electrophoresis each gel plate was stained with PhastGel Blue R and destained in a mixed solution of methanol–acetic acid–water (3:1:6). Molecular weight was determined by comparing mobility of several reference proteins simultaneously run including phosphorylase A (M.W. 94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400).

Results and Discussion

Inactivation of UK by SBS under Scattered Light or in the Dark The time courses of the residual activity of UK (120 IU/ml) in the presence (1.0—3.0 × 10^{-3} M) and absence of SBS in 0.05 M phosphate buffer (pH 6.0, μ=0.5) at 25 °C under scattered light or in the dark are shown in Figs. 1 and 2. UK was inactivated by SBS concentration dependently under both conditions. The residual activity of UK in the presence of SBS (1.0 × 10^{-3} M) after 4 h both under scattered light and in the dark was 86.6 and 89.0%, respectively.

In contrast, no significant inactivation of UK was observed in the absence of SBS after storage for 6 h under either scattered light or in the dark. Approximately the initial inactivation of UK by SBS under both conditions was observed to obey a pseudo-first-order reaction. As shown in Figs. 1 and 2, UK activity in the solution stored both ways was reduced with time.

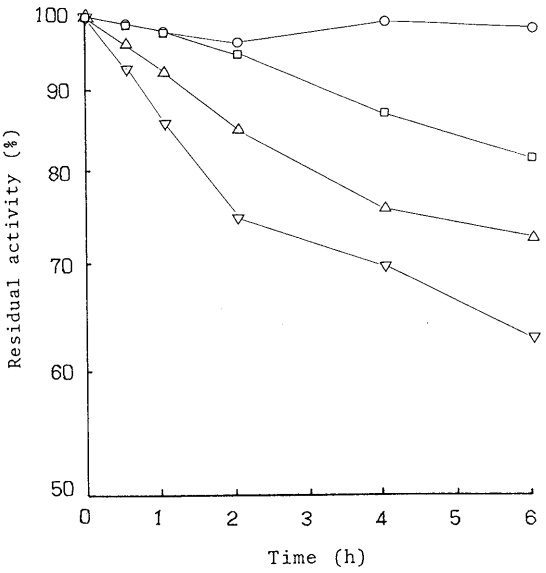


Fig. 1. Semilogarithmic Plots for the Inactivation of UK by SBS in 0.05 M Phosphate Buffer (pH 6.0, $\mu=0.5$) under Scattered Light (1000 lux) at 25 °C

Initial concentration of UK, 120 IU/ml; ○, $[SBS]_{total}=0$ M; □, $[SBS]_{total}=1\times10^{-3}$ M; △, $[SBS]_{total}=2\times10^{-3}$ M; ▽, $[SBS]_{total}=3\times10^{-3}$ M.

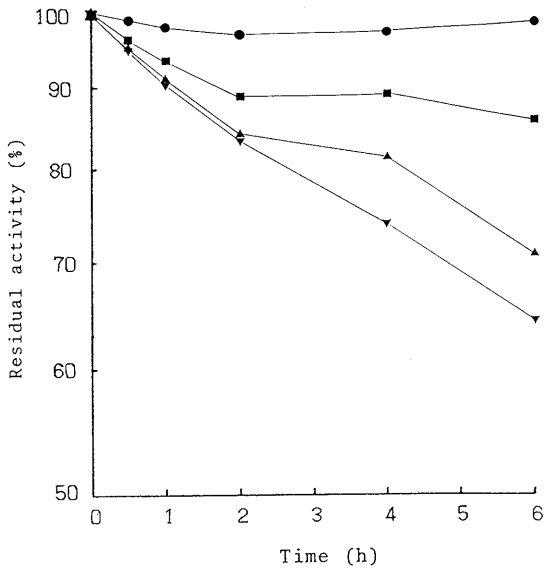


Fig. 2. Semilogarithmic Plots for the Inactivation of UK by SBS in 0.05 M Phosphate Buffer (pH 6.0, $\mu=0.5$) in the Dark at 25 °C

Initial concentration of UK, 120 IU/ml; ●, $[SBS]_{total}=0$ M, ■, $[SBS]_{total}=1\times10^{-3}$ M; ▲, $[SBS]_{total}=2\times10^{-3}$ M; ▼, $[SBS]_{total}=3\times10^{-3}$ M.

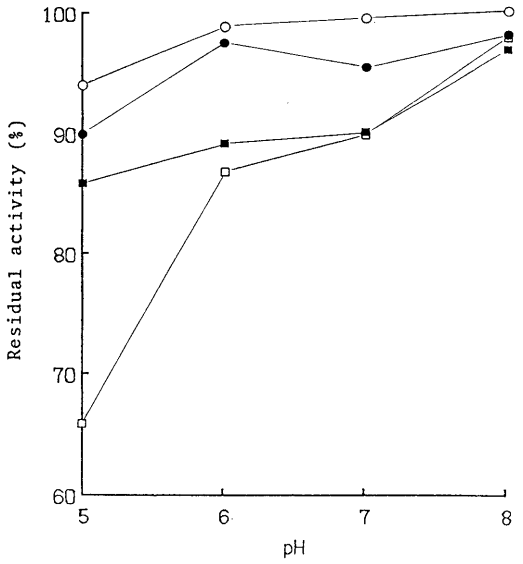


Fig. 3. pH-Stability Curve for UK in the Presence and Absence of SBS under Scattered Light (1000 lux) and in the Dark after 4 h Incubation at 25 °C and $\mu=0.5$

Initial concentration of UK, 120 IU/ml; ○, $[SBS]_{total}=0$ M under scattered light; □, $[SBS]_{total}=1\times10^{-3}$ M under scattered light; ●, $[SBS]_{total}=0$ M in the dark; ■, $[SBS]_{total}=1\times10^{-3}$ M in the dark.

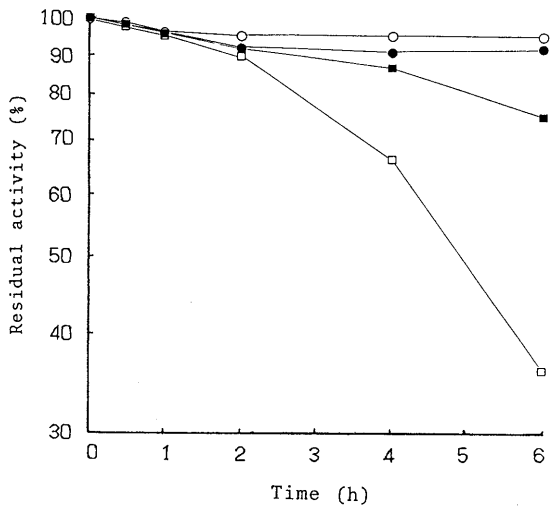


Fig. 4. Semilogarithmic Plots for the Degradation of UK by SBS in 0.05 M Acetate Buffer (pH 5.0, $\mu=0.5$) both under Scattered Light (1000 lux) and in the Dark at 25 °C

Initial concentration of UK, 120 IU/ml; ○, $[SBS]_{total}=0$ M under scattered light; □, $[SBS]_{total}=1\times10^{-3}$ M under scattered light; ●, $[SBS]_{total}=0$ M in the dark; ■, $[SBS]_{total}=1\times10^{-3}$ M in the dark.

Figure 3 shows the effect of pH of the medium on the inactivation of UK by SBS (1.0×10^{-3} M). Both under scattered light and in the dark, the higher the pH value was, the higher the residual activity became in the pH range 5.0—8.0. The activity of UK at pH 5.0 under light was reduced especially markedly by SBS. Therefore, the effect of light on the inactivation of UK at pH 5.0 both in the presence and absence of SBS was investigated.

Figure 4 shows the time course of the logarithmic residual activity of UK at pH 5.0 in the presence and absence of SBS. No significant inactivation of UK was observed in the absence of SBS after storage for 6 h under either light or dark condition. However, in the presence of SBS, the degradation of UK under light was considerably faster than that in the dark. As can be seen in the figure, the UK activity was inactivated more rapidly with elapsed storage time during a 6 h period in the presence of SBS under scattered light.

The difference in the residual activities of UK in the presence of SBS at pH 5.0 between under light and in the dark seemed attributable to the free radicals (BS radicals) formed during the autooxidation of BS caused by scattered light.[10]

The existence of a free radical chain mechanism for the aerobic oxidation of sulfite to sulfate has been well documented.[14] Free radicals such as $\cdot O_2^-$, $\cdot OH$, and $\cdot SO_3H$

are generated during the aerobic oxidation of sulfite and these BS radicals in turn propagate the BS–oxygen chain reaction.[14]

These BS radicals can take part in a number of oxidative reactions including the oxidation of methionine[8] and tryptophan,[9] and can inactivate papain through modification or oxidation of the sulfhydryl (SH) group of a cysteine residue.[15] It is reported that the chain reaction of free radicals such as $\cdot O_2^-$, $\cdot OH$, and $\cdot SO_3H$ is regulated by light and heat.[16]

It is also reported that the activity of lysozyme, an antiphlogistic enzyme, declined markedly at pH below 5 in aqueous solution in the presence of SBS under scattered light.[10] This phenomenon seemed attributable to the BS radicals caused by light.[10]

The equilibrium between BS and sulfite depends on the pH of the medium, and the dissociation constant of BS to sulfite is 6.24×10^{-8} (*ca.* $pK_a = 7.2$).[17]

In consideration of these reports, it was believed that the difference in the residual activity of UK between under light and in the dark was attributable to the BS radicals caused by scattered light.

Effects of Glucose on the Degradation of UK It is generally accepted that aldehydes react with SBS and form relatively inactive BS addition compounds. In the present study, we attempted to stabilize UK using glucose in the presence of SBS in the pH range of 5.0—7.0, in which UK was relatively unstable. The residual activities of UK (initial concentration 120 IU/ml) were 90.3—94.6% after storage for 4 h at 25 °C and $\mu = 0.5$ in the coexistence of SBS (1.0×10^{-3} M) and glucose (5% (w/v), *ca.* 0.28 M) both under scattered light and in the dark. From these findings, the intravenous admixture (mixed infusion) of UK inj. and 5% glucose solution was inferred to be utilizable within 4 h even in the pH range 5.0—7.0.

We subsequently studied the stabilization mechanism of glucose for UK in the presence of SBS at pH 5.0. The residual activities of UK incubated for 6 h at pH 5.0, $\mu = 0.5$ and 25 °C in the coexistence of SBS (1.0×10^{-3} M) and glucose (0—20%) under both light and dark were plotted against the concentration of glucose (Fig. 5). As shown in Fig. 5, UK was more stabilized by glucose concentration dependently. However, a linear relationship was not observed in these plots.

We previously revealed that pseudo-first-order rate constants for the degradation of thiamine and fursultiamine in the coexistence of SBS and glucose depended on the free BS concentration.[3] In addition, approximately the initial inactivation of UK by SBS was considered to follow a pseudo-first-order reaction. For these reasons, the logarithmic residual activities of UK stored for a constant time were assumed to depend mainly on the free BS concentrations. The free BS concentration can be expressed as Eq. 1.

$$[BS]_{free} = \frac{[BS\text{–glucose}]}{K_{B\text{-}G} \cdot [glucose]_{free}} \quad (1)$$

where $K_{B\text{-}G}$ means the equilibrium constant of BS–glucose addition compound, and [ ] expresses the mole concentration of each chemical species. In the present study, 3.65 was utilized for the value of $K_{B\text{-}G}$ at pH 5.0.[3]

The relationship between logarithmic residual activity of UK after incubation for 6 h and the free BS concentration is shown in Fig. 6, and the plots gave straight lines except for the value under light without glucose. The inactivation of UK in the coexistence of SBS and glucose was thus inferred to depend on the free BS concentration.

In Fig. 6, the plots for logarithmic residual activities of UK under scattered light were closely similar to those in the dark except for the condition of 0% glucose, *i.e.* free SBS concentration = 1.0×10^{-3} M. In the absence of glucose, the residual activity of UK under light was markedly reduced by SBS, the influence of the BS radicals caused by the light on UK was therefore considered. However, the influence of light on the stability of UK in the coexistence of SBS and glucose seemed negligible. It was therefore

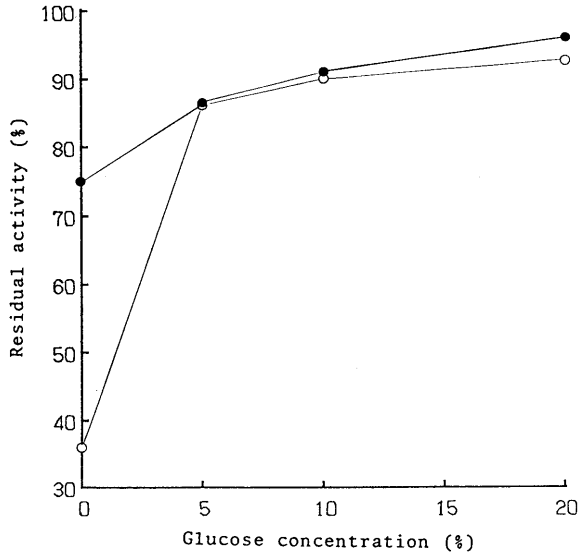


Fig. 5. Relationship between the Glucose Concentration and the Residual Activity of UK in the Presence of SBS Both under Scattered Light (1000 lux) and in the Dark after 6 h Incubation in 0.05 M Acetate Buffer of pH 5.0 with $\mu = 0.5$ at 25 °C

Initial concentration; UK, 120 IU/ml; SBS, 1×10^{-3} M; ○, under scattered light; ●, in the dark.

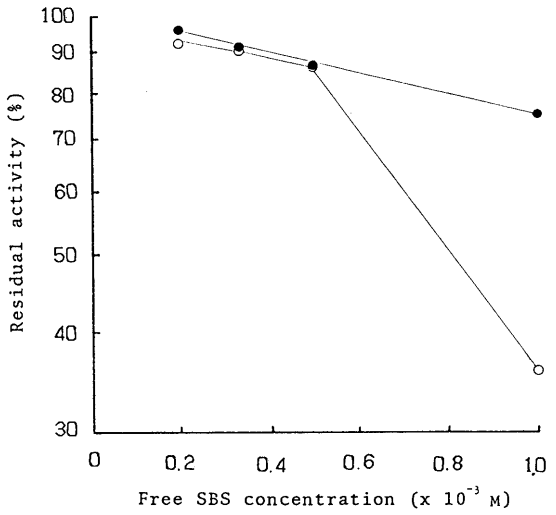


Fig. 6. Relationship between the Free SBS Concentration and the Logarithmic Residual Activity of UK Both under Scattered Light and in the Dark after 6 h Incubation in the Coexistence of Glucose and SBS in 0.05 M Acetate Buffer (pH 5.0, $\mu = 0.5$) at 25 °C

Initial concentration; UK, 120 IU/ml; SBS, 1×10^{-3} M; Glucose, 0, 5, 10, 20%; ○, under scattered light; ●, in the dark.

suggested that glucose suppressed the activity of the BS radicals caused by scattered light; this was inferred to be due to both the decrease of free BS concentration and the effect of glucose as radical scavenger.[18]

It has been reported that OH radical takes part in the chain reaction of the BS radicals,[14] and that glucose acts as the OH radical scavenger.[18] In this situation too, glucose seemed to act as the scavenger in the chain reaction of the BS radicals. From this it was inferred that the inactivation of UK by the BS radicals caused by the scattered light might be largely prevented by glucose.

SDS–Polyacrylamide Gel Electrophoresis The purified UK of M.W. 55000 (200000 IU/ml) obtained by serial column chromatography[12] was incubated in 0.05 M phosphate buffer (pH 6.0, $\mu=0.5$) at 25 °C in the presence (1.5×10^{-3} M) and absence of SBS under scattered light. The molecular weights of the sample species were determined by SDS–polyacrylamide gel electrophoresis (Fig. 7). Purified UK gave a single band in the absence of SBS immediately after the start of incubation. The UK molecule split partially into M.W. 36000 form and M.W. 20000 form during 24 h of incubation. From the report by Miwa *et al.*,[19] the M.W. 36000 form obtained by the incubation of UK in the absence of SBS was inferred to be similar to a functionally-active heavy chain.

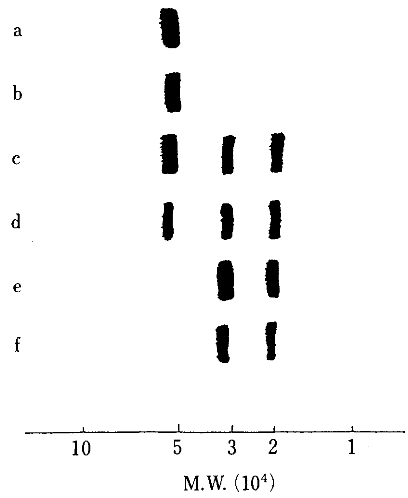
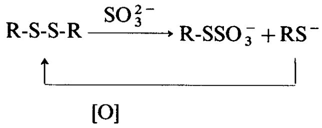


Fig. 7. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoretograms of UK in the Presence of SBS in 0.05 M Phosphate Buffer (pH 6.0, $\mu=0.5$) at 25 °C

Initial concentration; UK, 200000 IU/ml; SBS, 1.5×10^{-3} M; a, UK in the absence of SBS (immediately after the start of incubation); b, UK in the absence of SBS under scattered light (for 3 h); c, UK in the absence of SBS under scattered light (for 24 h); d, UK in the presence of SBS (immediately after the start of incubation); e, UK in the presence of SBS under scattered light (for 3 h); f, UK in the presence of SBS under scattered light (for 24 h).

However, purified UK split partially into M.W. 36000 form and M.W. 20000 form immediately after the start of incubation in the presence of SBS, and the two bands of these forms appeared after the incubations for 3 and 24 h (Fig. 7). Thus the one inter-chain bond of UK was revealed to be cleaved rapidly by SBS.

It is well known that the scission of disulfides by sulfite to form S-sulfonates occurs as follows[9,20]:



Sulfitolysis has frequently been used to cleave disulfide bonds in proteins. Therefore, it appears that sulfitolysis of the disulfide bond of UK is an important reaction responsible for the incorporation of a sulfur group derived from SBS into UK with consequent inactivation of the UK solution.

Referring to the scission of disulfide bond in enzymes by sulfitolysis, the inter-chain disulfide bond generally reacts with SBS more easily than with the intra-chain bond.[6] It was inferred that the inter-chain disulfide bond in UK molecule was also cleaved easily by SBS. This speculation was supported by the finding that only the two bands of M.W. 36000 form (heavy chain) and M.W. 20000 form (light chain) were immediately observed as the degradation products of UK by SBS (Fig. 7).

It was inferred that some of the intra-chain disulfide bonds in the heavy chain reacted with SBS to inactivate the enzymatic activity of the heavy chain. This view was supported by the following findings. The treatment of purified UK with 0.1 M 2-mercaptoethanol immediately resulted in both the complete split of UK molecule to heavy and light chains, and complete inactivation of UK. In contrast, the treatment of UK with a very low concentration of 2-mercaptoethanol resulted only in the complete split of the UK molecule.[21] Therefore, it was considered that UK was not inactivated only by the scission of the inter-chain disulfide bond but that the inactivation by 2-mercaptoethanol came from a change in the three dimensional structure of the heavy chain by reduction of the disulfide bonds.

The treatment of purified UK with 1.5×10^{-3} M SBS at pH 6.0 brought about the partial scission of the inter-chain disulfide bond immediately after incubation and the complete scission of that bond after 3 h incubation (Fig. 7). From the results of Fig. 1, however, the UK activity with 1.5×10^{-3} M SBS was thought to be slightly impaired after 3 h incubation and to be decreased with elapsed incubation time of more than 3 h. Only two bands corresponding to the components of M.W. 36000 and M.W. 20000 were observed even after 24 h incubation of UK with 1.5×10^{-3} M SBS (Fig. 7).

It was therefore suggested that the change of the three dimensional structure by the scission of disulfide bond caused by SBS was, as was that caused by 2-mercaptoethanol, an important factor in UK inactivation and that influence of BS radicals on UK was based on the modification or oxidation of the latter's amino acid residues.[8,9,15]

Since it appears that the change of the three dimensional structure by the scission of disulfide bond caused by SBS is an important factor in the inactivation of UK, such inactivation under scattered light probably involves modification or oxidation of the SH group of UK by the BS radicals.[15]

Consequently, UK was inferred to be inactivated by the scission of its disulfide bonds due to SBS, and the modification or oxidation of its amino acid residues by the BS radicals caused by scattered light. The UK solutions were stabilized by glucose in the presence of SBS both under scattered light and in the dark. One reason for this phenomenon was thought to be the formation of an inactive

BS-added compound, and another reason for it under light was the suppression of the activity of the BS radicals by glucose.

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References and Notes

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