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Inhibitory Effects of Bovine Serum Albumin on Cytotoxicity and Mutagenicity of 6-Sulfooxymethylbenzo[a]pyrene

Young-Sik Cho, Kyung-Joo Cho and An-Sik Chung*

Department of Biological Sciences, Korea Advanced Institue of Science and Technology, Yusung, Taejeon 305-701, South Korea
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ABSTRACT *: A 6-sulfooxymethylbenzo[a]pyrene (SMBP), the ultimate metabolite of methyl-substituted benzo[a]pyrene (BP), has been found to be carcinogenic in mice. These properties may be attributable to its strong reactivity with cellular macromolecules such as DNA. However, serum and its major constituent albumin attenuated significantly the cytotoxicity and mutagenicity of SMBP in bacterial and mammalian cell systems. This inhibitory activity of serum against SMBP-induced cytotoxicity and mutagenicity in Chinese hamster V79 cells appears to be caused by the reduced macromolecular adducts such as DNA and proteins, but serum failed to reduce SMBP binding to naked calf thymus DNA. A number of proteins in the serum could act as nucleophiles that are able to intercept reactive chemicals through covalent binding. Albumin present in the plasma seems to be one of major components responsible for direct binding with SMBP, thereby reducing its reactivity to genetic materials. We here determined which fraction is preferential for SMBP binding through fractionation of SMBP-treated serum with ammonium sulfate. The albumin-containing fraction had slightly more affinity for SMBP than the immunoglobulin-containing fraction. Our results indicate that the covalent modification of plasma proteins may reduce SMBP-induced damage.*

Key Words *: 6-Sulfooxymethylbenzo[a]pyrene, bovine serum albumin, antimutagenicity*

I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a major class of environmental pollutants, which are relatively insoluble and nontoxic in aqueous media. However, they are metabolized to a variety of derivatives through the bioactivation process (Stowers *et al.*, 1985) and some of these ultimate metabolites are highly toxic (Conney, 1982). Methyl-substituted PAHs have been postulated to be metabolized by the hydroxylation of the *meso*-methyl group with subsequent formation of reactive esters containing good leaving groups (e.g. sulfate, phosphate and acetate), which would generate a highly reactive carbonium ion in aqueous media. The carbonium ion reacts with critical cellular nucleophiles to cause DNA damage, leading to cancer by a chain of cellular events (Natarajan *et al.*, 1973). Specifically, 6-sulfooxymethylbenzo[a]pyrene (SMBP) is found to be an ultimate carcinogen capable of inducing a much higher incidence and multiplicity of liver tumors than does its corresponding hydroxymethyl derivative of benzo[a]pyrene as well as benzo[a]pyrene (BP) itself in male B6C3F$_1$ mice (Surh *et al.*, 1990). Moreover, it has a stronger mutagenicity in the bacterial and mammalian systems without further activation than its proximate compound 6-hydroxymethylbenzo[a]pyrene (HMBP) (Cho *et al.*, 1998).

Serum albumin is abundant in the plasma (~10 mg/500 μl serum) and most cells, particularly liver cells (Rothschild, 1977; Peters, 1975). It plays a conspicuous role in maintaining colloid osmotic blood pressure and transportation, and distribution and metabolism of many endogenous and exogenous ligands. In addition, serum proteins intercept reactive DNA-reactive metabolites (Turesky *et al.*, 1987; Okoye *et al.*, 1990; Wild *et al.*, 1990), indicating that they may also have functional groups capable of binding reactive metabolites. In fact, bovine serum albumin (BSA), casein and its pepsin hydrolysis products exhibit protective action against mutagen 4-nitroquinoline 1-oxide (4NQO) in a mammalian cell system

*To whom correspondence should be addressed

(Bosselaers *et al.*, 1994). Here, we investigated the inhibitory effect of BSA against mutagenicity of SMBP on both *S. typhimurium* TA 98 and Chinese hamster V79 cells and the levels of SMBP-mediated macromolecular adducts in V79 cell in the presence of BSA. Also, the adduct levels in SMBP-treated serum fractionated with ammonium sulfate were analyzed on polyacrylamide gel electrophoresis.

II. MATERIALS AND METHODS

1. Chemicals

BSA, proteinase K, DNase-free RNase, calf thymus DNA, 6-thioguanine (6-TG) and Dulbecco's modified Eagle's (DME) medium were purchased from Sigma Chemical Co (St Louis, MO). Fetal bovine serum was obtained from Gibco-BRL (Grand Island, NY). HMBP and SMBP were synthesized as described previously (Natarajan *et al.*, 1973; Surh *et al.*, 1989). Other reagents were of analytical grade.

2. Preparation of rat liver cytosolic fraction

Cytosols from the liver of 4-week old female Sprague-Dawley rats were prepared as described previously (Surh *et al.*, 1989) and stored at -70°C until used. The female liver cytosol was prepared because it contains a large amount of hydroxysteroid sulfotransferase, which was used to activate HMBP with sulfo-group donor PAPS. The protein content of the cytosol was determined by the Lowry assay with BSA a standard (Lowry *et al.*, 1951).

3. Bacterial mutagenicity

The bacterial mutagenicity was performed based on the modification of standard Ames assay using *S. typhimurirum* TA98 which carries the frame shift mutation. In previous report using *S. typhimurium* TA100 and TA98 (Cho *et al.*, 1998), *S. typhimurium* TA98 exhibited dose-dependent response to mutagens. For reference, TA98 and TA100 strains carry the frame shift mutation and base-pair substitution, respectively. A relatively high number ($3\text{-}4 \times 10^9$) were used to increase the sensitivity of assays. A preincubation mixtures containing 100 ml of *S.typhimurium* TA98, mutagens and test agents were kept at 37°C for given times. HMBP (7.2 nmol) was incubated for 60 min at 37°C together with test agents (10 nmol) under investigation in a final volume of 1.1 ml of 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) including liver cytosol (S105) and a PAPS-generating system (5 mM ATP, 0.1 mM EDTA, 5 mM Na_2SO_4, 3 mM $MgCl_2$). The cytosolic fraction and the PAPS-generating system were omitted from the mixture when SMBP (1.0 nmol) dissolved in DMSO was used instead of HMBP. The incubation condition for SMBP was 30 min at 37°C. After incubation, the mixtures were diluted with soft agar, poured onto a hard agar plate and further incubated for 48 hr to allow the growth of His^+ revertant colonies.

4. Cytotoxicity and Mutagenicity assay in V79 cells

Chinese hamster V79 cells do not metabolize chemical procarcinogens such as BP (Katoh *et al.*, 1980). For the determination of cell growth, V79 cells (5×10^5) were plated onto 60-mm tissue culture dishes and continued to incubate for 6 hr. Exponentially growing cells were treated with SMBP (1.5 μM) along with indicated doses of BSA or fetal bovine serum for 30 min and then allowed to grow for 48 hr. The numbers of cells in the BSA-or serum-treated dishes along with SMBP were compared with those in dishes treated with SMBP alone to assess the influence of them on cell growth.

The direct mutagenicity assay in Chinese hamster V79 cells was carried out as described previously with some modifications (Jenssen, 1984; Cho *et al.*, 1996). Mutations at hypoxanthine:guanine phosphoribosyl-transferase (HPRT) locus were measured by the resistance to 6-thioguanine (6-TG). In brief, exponential phase cultures preincubated with a series of BSA or serum were exposed to SMBP (1.5 μM) for 60 min, followed by subcultures. Cloning efficiency was determined by counting the number of colonies 7-8 days after seeding 200-2000 cells per dish while the cells were subcultured to select mutants. Mutation frequencies for 6-TG were expressed as mutants/10^6 survivors and were corrected for cloning efficiency.

As the control, V79 cells in the medium without serum or BSA were treated with DMSO during reac-

tion time. After then, the cells were replaced with complete medium containing 10% serum and allowed to incubate for the measurement of cytotoxicity and mutagenicity. Therefore, the percentage of growth is a value that has already been corrected with the solvent-treated group.

5. Determination of cellular macromolecular adducts in V79 cells

Confluent V79 cells were exposed to SMBP at a concentration of 12.5 μM along with a series of serums for 60 min at 37°C. The cells were harvested by treatment with trypsin, and washed with phosphate-buffered saline (PBS); phenylmethyl sulfonyl fluoride (PMSF) was added to this and all subsequent solutions to a concentration of 0.5 mM. Cell pellets were lysed in 1 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 1 μg DNase-free RNase and 0.5% SDS) for 3 hr at 45°C. The lysates were extracted twice with phenol, then once with chloroform : isoamylalcohol (24 : 1,v/v). Nucleic acid was precipitated from the aqueous phase by the addition of 2.5 vol ethanol at -20°C and protein was obtained from the phenol phase plus interface by the addition of 2 vol. methanol at 4°C as described previously (Huberman *et al.*, 1977). The nucleic acid and protein pellets were rinsed with alcohol, ether and acetone, and then resuspended in 2 ml of Tris-HCl buffer (pH7.4) and 0.5 M NaOH, respectively. The fluorescence emission spectra of macromolecular adducts were recorded between 400 and 450 nm (excitation at 360 nm). For comparison, the amounts of adducts were measured by their fluorescence at 418 and 414 nm (excitation at 360 nm) and expressed in terms of fluorescence intensity per mg DNA or mg protein.

6. Binding of SMBP to calf thymus DNA in the presence of serum or BSA

One nmol of SMBP was added to a final volume of 500 μl of incubation mixture consisting of DNA and various amounts of serum or BSA. After 30 min incubation at 37°C, residual proteins were removed from the mixture by protease digestion and ensuing phenol extraction. The nucleic acid was precipitated by the addition of ethanol in the presence of 0.2 M sodium acetate and then washed with organic solvents as described previously (Watabe *et al.*, 1985). The washed nucleic acid was dried *in vacuo*, dissolved in TE buffer and quantitated by its absorbance at 260 nm. The adducts were measured by their fluorescence at 418 nm (excitation at 360 nm) and expressed in terms of fluorescence intensity/mg DNA (F/mg) for comparison.

7. Identification of SMBP-protein adducts in SMBP-treated serum

SMBP was incubated in a final volume of 500 μl of reaction buffer containing 20% fetal bovine serum at 37°C for 20 min. To identify protein adducts, the SMBP-treated serum was divided into 2 fractions according to the previous method (Sheabar *et al.*, 1993) and their aliquots were subjected to the polyacrylamide gel electrophoresis (PAGE) under nondenaturation condition. The protein and protein adducts were visualized by staining with Coomasie Blue and under UV illumination as fluorescence of BP-chromophore, respectively. The protein amounts in fractionated sample were determined by the Lowry assay and the adduct levels were estimated by their fluorescence at 412 nm while exciting at 360 nm.

III. RESULTS

The inhibition of mutagenic activity of SMBP by either serum or albumin as a function of dose is presented in Fig. 1. Serum had a considerable inhibitory effect on the mutation frequencies of SMBP and HMBP in *S. typhimurium* TA98. The mutagenic activities of SMBP and HMBP were dramatically reduced up to 32% and 25%, respectively, by the treatment with 10% serum. The antimutagenic profiles of BSA were similar to those of serum. Fig. 2 shows the inhibition of SMBP-mediated cytotoxic and mutagenic activity in V79 cells by treatment of serum or albumin. With increasing doses of serum or albumin, there was an enhancement of cell growth and a reduction of mutagenicity. The growth rate was magnified at a dose of 2.5% serum and was more than double at 10% level. Moreover, serum effectively inhibited the SMBP-induced mutagenicity in a dose-dependent manner. The mutation frequency of SMBP was reduc-

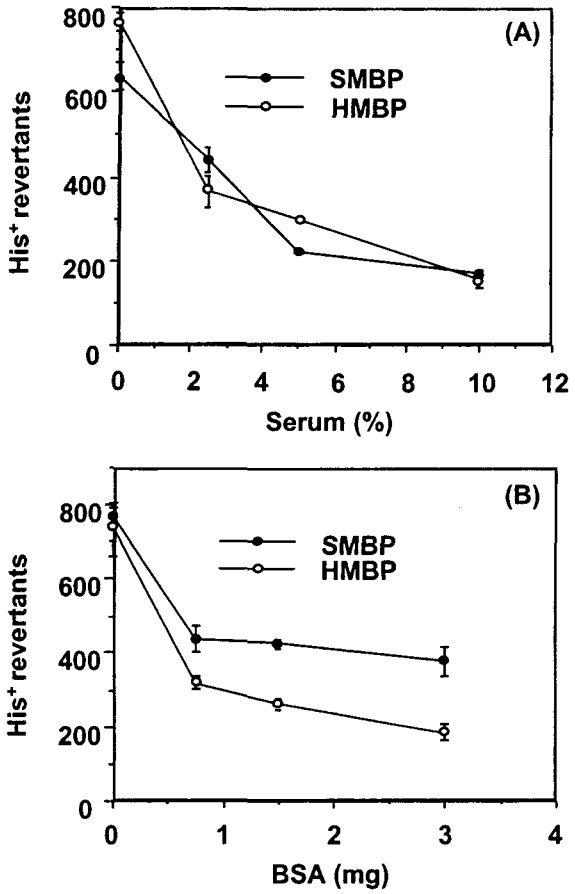

Fig. 1. Inhibitory effects of serum (A) and BSA (B) on mutation frequencies by HMBP and SMBP in *S. typhimurium* TA98. The mutagenic activities of HMBP and SMBP were determined in a final volume of 1.1 ml of 0.1 M phosphate buffer, pH 7.4, containing BSA or serum. HMBP was activated to reactive SMBP in the presence of rat liver cytosol and PAPS-regenerating system as described in Materials and Methods. The doses of HMBP and SMBP were constant at 7.2 and 1.0 nmol, respectively.

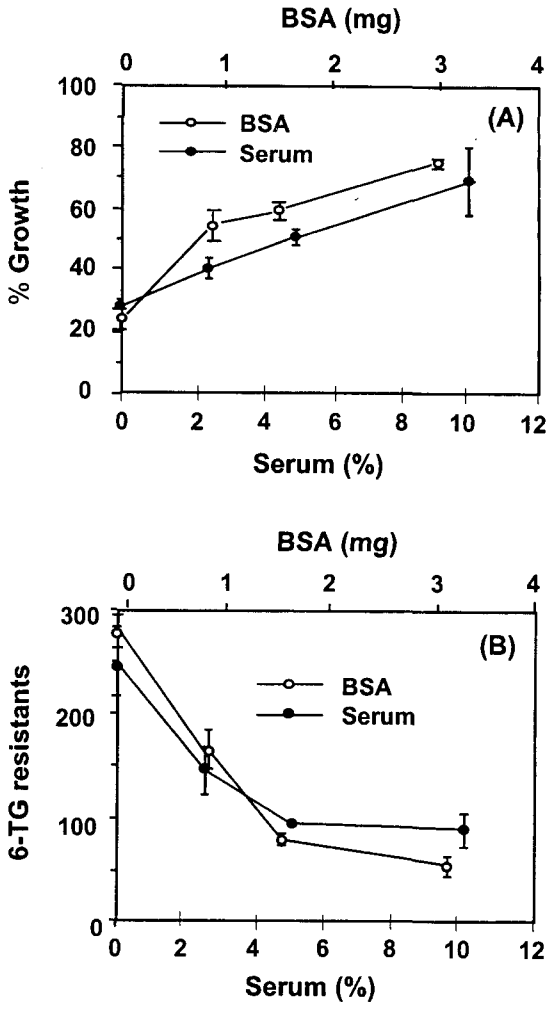

Fig. 2. Effects of serum and albumin on SMBP-induced cytotoxicity (A) and mutagenicity (B) on V79 cells. The cytotoxicity and mutagenicity of SMBP were assayed with various levels of serum or albumin. V79 cells were exposed to 1.5 μM of SMBP for 30 min in the presence of different doses of serum and albumin. The relative growth was calculated as compared to the solvent control. Resistant mutants for 6-thioguanine (6-TG) were determined by counting the colonies, and mutation frequencies were expressed as mutants/10^6 survivors. The values are means of two experiments with SEs.

ed to 38% by the treatment of 5% serum and thereafter leveled off. A similar result was also observed with albumin as determined by the HPRT/V79 point mutation assay.

SMBP produced the covalent adducts with DNA and proteins in V79 cells through the unknown mechanism. The inhibition of SMBP-induced macromolecular adducts by serum is presented in Figure 3a. The adduct level in DNA or protein isolated from the SMBP-treated V79 cells declined by concurrent treatment with serum. Their fluorescence emission spectra were very similar to that of an aqueous solution of HMBP, but somewhat shifted to a longer wavelength (Fig. 3b). The peak maxima of both modified DNA and proteins were located at 418 nm and 414 nm, respectively. To understand their precise antimutagenic potential effects of serum and albumin on the binding of SMBP to naked DNA were investigated (Fig. 4). Neither serum nor albumin altered the binding capacity of SMBP to the calf thymus DNA. Moreover, the covalent binding to DNA occurred rapidly enough to be completed within several minutes (data not shown).

As described previously, SMBP can be bound to DNA or proteins without further activation. The func-

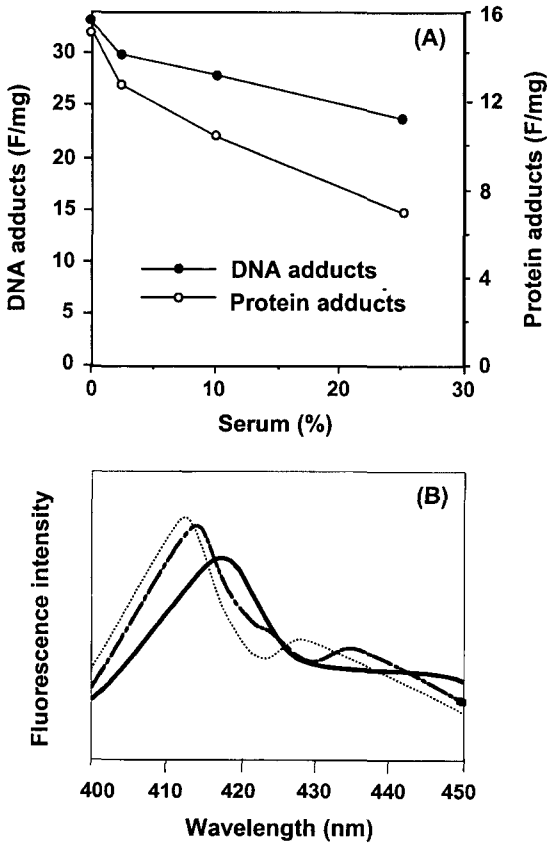

Fig. 3. Inhibition of macromolecular adduct formation in V79 cells treated with SMBP by serum (A) and fluorescence spectra of SMBP-macromolecular adduct in SMBP-treated V79 cells (B). The fluorescence spectra of the adducts were detected from isolated DNA or proteins prepared by phenol extraction as described in Materials and Methods. Emission spectra of isolated DNA (-----) and protein adducts (• - •) are compared with that of HMBP alone (—).

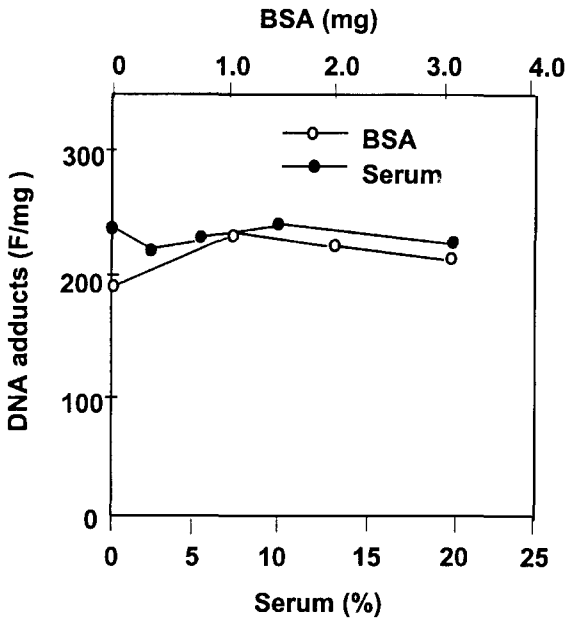

Fig. 4. The effect of serum or BSA on the covalent binding of SMBP to calf thymus DNA. Reaction was initiated immediately after SMBP was added into Tris-HCl buffer (pH 7.4) containing DNA and either increasing amounts of BSA or serum for 30 min-incubation. Adduct forming capacity was expressed by fluorescence intensity per mg DNA purified.

tional groups on serum proteins including albumin may be involved in adduct formation with a carbonium ion, a reactive species derived from SMBP. PAGE results indicated adduct formation of SMBP with subdivided fractions of plasma proteins, judged from identification of protein-associated BP moiety under UV illumination. A fluorescent intensity in the albumin-containing fraction was higher than that in the immunoglobulin-containing fraction, indicating that the covalent adducts between serum proteins and organic electrophiles are formed, and the albumin in serum is especially a major binding protein for SMBP (Table 1 and Fig. 5).

IV. DISCUSSION

Neither serum nor BSA reduced the binding ratio of SMBP to calf thymus DNA although they effectively inhibited the bacterial mutagenicity of SMBP when preincubated with it, suggesting that SMBP reacts much more readily with naked DNA than with proteins. However, since genetic materials are compartmentalized or surrounded by a wide variety of pro-

Table 1. Comparison of protein adducts fractionated with ammonium sulfate from SMBP-treated serum

	Protein Quantity (mg)[a]	Relative Fluorescence[b]	Fluorescence/mg proteins
Fraction containing albumin	1.4	2704	1931.4
Fraction containing imunoglobulin	1.13	1848	1635.4
Total	2.14	3896.7	1920.9
Untreated serum	2.72	79.1	29.1

[a]The protein content was determined by the Lowry assay with bovine serum albumin as standard
[b]Fluorescence intensity was measured at 412 nm (excitation at 360 nm) and its value was arbitrary.

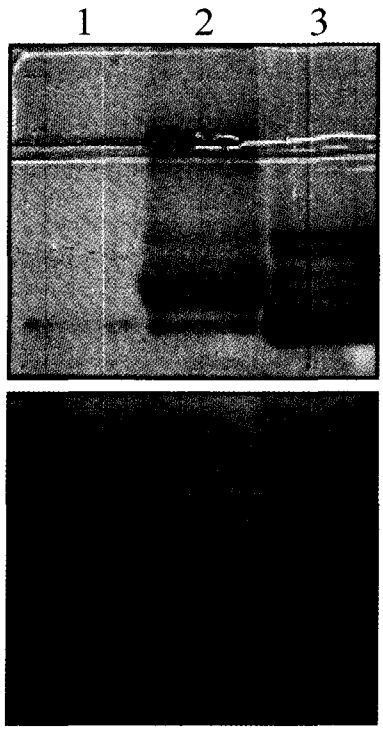

Fig. 5. Analysis of protein adducts from SMBP-treated serum. SMBP was incubated in 0.1 M Tris-HCl buffer containing 20% (v/v) serum at 37°C for 20 min. Reactants were divided into 2 fractions as described in Materials and Methods. Aliquots of each fraction were subjected to electrophoresis under nondenaturation condition. Lane 2 and 3 correspond to albumin and immunoglobulin fractions subdivided from the SMBP-treated serum. For comparison, untreated BSA was loaded into lane 1. Upper panel; fluorescence band in protein adducts under UV illumination, Lower panel: Coomassie blue staining.

teins, the reactive electrophiles do not seem to be directly accessible to DNA. Albumin may serve as a promising interceptor of electrophilic compounds because of its abundance and its role as a carrier of endobiotics and xenobiotics. It has been documented that BSA protects Chinese hamster V79 cells from certain genotoxic compounds (Bosselaers *et al.*, 1994). In our results, the suppression of SMBP-mediated mutagenic activity in V79 cells by the treatment of serum was closely related to a decrease in the level of cellular macromolecular adducts. It seems plausible that the serum proteins act as blocking agents through chemical or physical interaction with several mutagens even if the mechanism of antimutgenicity has not been known. Therefore, the antimutagenic activity of the serum proteins including BSA against SMBP can be partly explained by direct bindings with it.

This kind of protein binding has been known to offer an additional protective mechanism for cells, allowing proteins to encounter and disarm the potentially harmful electrophiles that are also major carcinogens (Heidelberger, 1975; Miller *et al.*, 1977). Carcinogen-binding proteins are broadly classified into two major categories: (a) those that covalently bind carcinogen metabolites and (b) those that participate in a reversible, noncovalent binding (Collin *et al.*, 1984). The covalent modification by some specific proteins serves as a scavenger although it is generally considered to be harmful in the biosystem (Smith *et al.*, 1977). On the other hand, the protein adducts formed in the toxic response process are also applicable to assess the exposure and the risk of potentially harmful substances (Wogan, 1989) since a good correlation between the ability of polycyclic aromatic hydrocarbons to form covalent adducts and their carcinogenic potencies has been documented (Skipper *et al.*, 1990). Especially, albumin adducts have been regarded to be the cause of recent exposure to PAH in occupational environments because of the availability of biological material and a long half-life of the protein, e.g., 4 weeks in the case of albumin (Omland *et al.*, 1994; Santella *et al.*, 1995).

The albumin-containing fraction intercepted the electrophile SMBP more effectively than the immunoglobulin-containing fraction, indicating that the albumin present in the serum appears to display amino acids more readily available for interaction with it. The albumin has a variety of nucleophilic sites capable of reacting with mutagens, but the structure of the major SMBP adduct and its specific site have not been determined yet. Taken together, the accessibility of protein molecules and the presence of nucleophilic binding sites may be decisive factors in determining the antimutagenic properties of proteins. Therefore, the structure of major adducts, requirements of the molecular structure and specific binding sites on BSA will be characterized further to understand their biological significance.

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