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Protein Binding Characteristics of Brazilin and Hematoxylin

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ABSTRACT – In order to investigate the protein binding characteristics of brazilin and hematoxylin to bovine serum albumin (BSA), the fluorescence probe method was adopted. Brazilin and hematoxylin showed strong binding affinity for BSA. It was also confirmed that hematoxylin was bound to BSA stronger than brazilin. The association constants were decreased by the elevation of concentrations of brazilin and hematoxylin. It might be due to the complex formation of the probe and both compounds or the interaction between the probe-protein complex and both compounds. The bindings between both compounds and BSA were dependent on pH and ionic strength. It seems that electrostatic force as well as hydrophobic force is involved in the binding of brazilin and hematoxylin to BSA.

Keywords: Protein binding, Brazilin, Hematoxylin, Bovine serum albumin

Binding of chemicals to plasma protein has been known as an important factor in their availabilities, efficacies and transports in biological system. The unbound chemicals in plasma considered to account for their biological activities. The binding occurs mainly in the albumin fraction of plasma and is reversible.[1] It is analogous to the enzyme-substrate interaction except that the complex does not decompose to yield new products. It is also analogous to most drug receptor complexes unless they involve covalent bond.[2]

The electrostatic and hydrophobic forces in the interaction between small molecules and plasma protein have been considered to be important. Klotz[3] emphasized the contribution of the Van der Waals force to the binding affinity. The hydrophobic alkyl side chains, the aromatic electrons, the hydrogen binding sites and the possible electrostatic interaction may also affect the chemicals binding affinity for plasma protein.[4]

Brazilin and hematoxylin were identified as active principles of *Caesapinia sappan* and *Haematoxylon campechianum*, respectively.[5] Brazilin and hematoxylin have been examined for their biological activities such as effects on capillary resistance,[6,7] histidine decarboxylase function,[8] blood pressure,[9] inflammation,[10-12] chemical heart poisoning[13] and actions of adrenalin on the various isolated organs.[9] Moreover many other articles on the effects of brazilin[14-17] and hematoxylin[7,18-29] were published. Recently brazilin has been further investigated in our laboratory on its hypolipidemic effect,[30] lens aldose reductase inhibitory effect[31] and the protective effect on hepatotoxicity by D-galactosamine and carbon tetrachloride.[32] In this study we investigated the protein binding characteristics of brazilin and hematoxylin with bovine serum albumin as basic data for the further investigations on the bioresponses of both compounds.

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Materials and Methods

Reagents

The chemicals were purchased from following sources: Bovine serum albumin, fraction V. RIA grade (Sigma Co.), 1-Anilinonaphthalene-8-sulfonic acid (Sigma Co.), Brazilin (Fluka AG) and Hema-

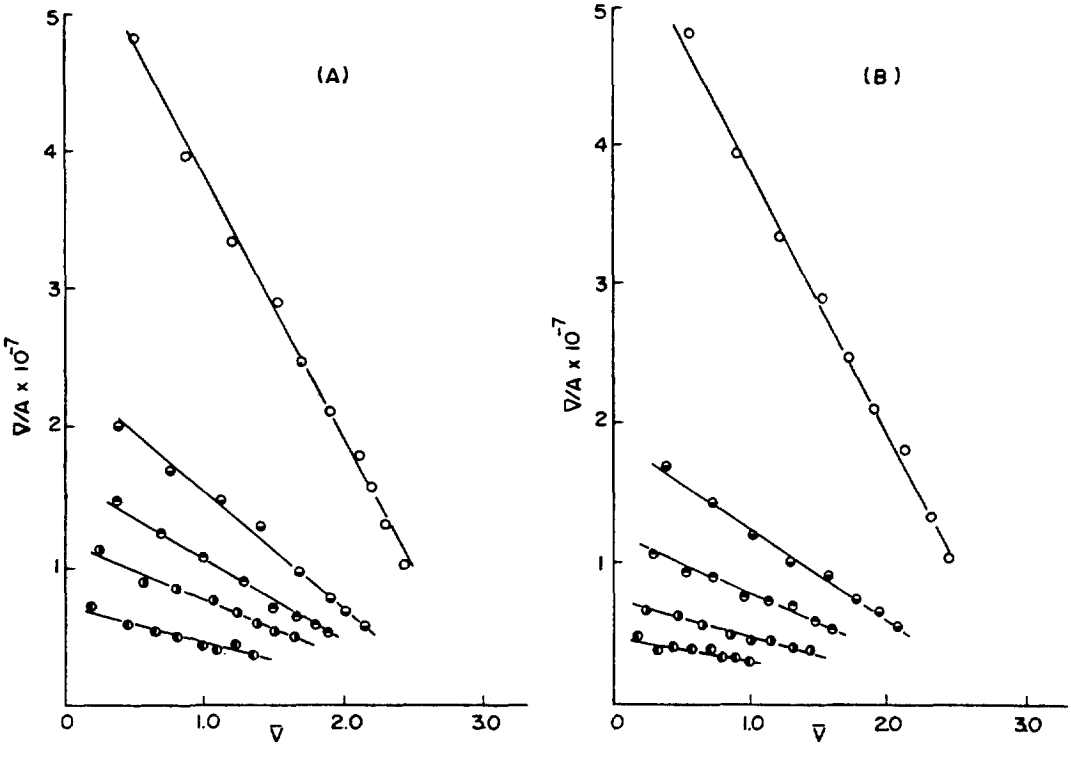

Fig. 1. Scatchard plots of the binding of bovine serum albumin in 1/15 M phosphate buffer, pH 7.4, $\bar{V}$ is the number of moles of bound probe per mole of protein, and A is the concentration of free probe. Key: ○, in the absence of brazilin (A) and hematoxylin (B), ◒, ◓, ◑, ◐, in the presence of 0.10×10^{-4} M, 0.25×10^{-4} M, 0.50×10^{-4} M, 1.00×10^{-4} M brazilin (A) and hematoxylin (B), respectively.

toxylin (E. Merck). All other chemicals used of guaranteed grade.

Binding Assay

Molecular weight of BSA was assumed to be 69,000. The concentrations of albumin in the solutions were determined from the absorbance at 280 nm. The molar concentration was calculated on the basis of $E_{1\%}^{1cm}=6.67$.[33] Fluorescence measurements were made with Baird-Automatic Spectrophotofluorometer Model FC 100 equipped with 150 watts xenon lamp. The entrance slit for the excitation and the exit for the flucorescence emission were 3 and 4 mm, respectively.

Fluorescence titrations of protein solutions with probe in the absence and presence of compounds were carried out manually with microsyringe. Two milliliters of protein solution was titrated with successive addition of 3 μl of 1×10^{-3} M probe solution dissolved in methanol. Methanol did not affect the binding of the probe to BSA in the concentrations used. After each titration, the fluorescence intensities were recorded as a function of probe concentrations. The excitation- and emission-wavelengths were taken to be 375 and 470 nm, respectively. The BSA solution were prepared in pH 7.4 phosphate buffer (1/15 M). To minimize the photodecomposition of the probe, reaction mixtures were exposed to the light only for the short measurement period. Mixtures were exposed to the light only for the short measurement period. To prevent the oxidation of brazilin and hematoxylin, reaction mixtures were deaerated by N_2 gas. The temperature was maintained at 23± 1 ℃ through out all the measuring process. The bindings of probe and compounds to 1.50×10^{-5} M

Table 1. Parameters characterizing the binding of brazilin and hematoxylin to BSA

Compounds	Concentration ($\times 10^{-4}$ M)	n	k (molar $\times 10^{-5}$)	r
Brazilin	0.10	2.8± 0.2	2.4± 0.1	0.995
	0.25	2.8± 0.2	2.1± 0.2	0.995
	0.50	2.8± 0.2	1.9± 0.1	0.980
	1.00	2.8± 0.2	1.7± 0.2	0.996
Hematoxylin	0.10	2.9± 0.3	5.7± 0.2	0.974
	0.25	2.9± 0.3	5.5± 0.2	0.985
	0.50	2.9± 0.3	5.3± 0.2	0.990
	1.00	2.9± 0.3	5.2± 0.1	0.965

Results are the average of five sets of experiments. Experimental condition; pH 7.4 and ionic strength 0.4 t 23℃. ionic strength 0.4 at 23℃. n; the binding sites, k; the association constant, r; correlation coefficients. Values represent mean± S.D.

BSA were determined by the methods of Jun *et al.*[34]

Results and Discussion

Effect of Concentration

Using 1-anilinonaphthalene-8-sulfonic acid, of which flurorescence intensity is thought to be induced by the binding of hydrophobic region of BSA,[35] the protein binding characteristics of brazilin and hematoxylin were determined. The bindings of probe and both compounds to BSA were investigated at four different concentrations of both compounds. The binding parameters of the probe to BSA in the absence and presence of both compounds were calculated using the Scatchard[36] equation (Fig. 1). The competitive bindings of these two compounds to BSA were determined using Klotz *et al.*[37] equation. The results are represented in Fig. 1 and Table 1. Table 1 shows the number of binding sites and the association constants of brazilin and hematoxylin. It was found that the association constants were decreased by the elevation of the concentration of both compounds. The changes of the association constants at different concentrations of both compounds may be due to the complex formation of probe and both compounds or the interaction between probe-BSA complex and both compounds.[37] Under the condition of pH 7.4 and ionic strength 0.4, brazilin and hematoxlyin bind strongly to BSA with association constants of 2.4×10^{-5} M and 5.7×10^{-5} M, respectively.

Effect of pH

Any shift of fluorescence peak from Ex.375 and Em.470 nm was not observed with the alteration of pH. Spectra in the absence and presence of both compounds were measured in the pH region from acidic to alkaline. Fig. 2 shows the Scatchard plots of probe-BSA complex in the absence and presence of both compounds at various pH values. The results are summarized in Table 2. The association constants of brazilin at pH 6.6, 7.4 and 8.0 were 1.3×10^{-5} M, 2.4×10^{-5} M and 3.5×10^{-5} M respectively and those of hematoxylin were 3.5×10^{-5} M, 5.7×10^{-5} M and 7.2×10^{-5} M as association constants. The results indicate that the binding affinities of brazilin and hematoxylin for BSA were increased with elevation of pH values. As the affinity of a small molecule for a protein is influenced by a number of factors, it is rather difficult to interpret the effect of pH on the association constants. For example, a change in pH may affect ionization of the small molecule and/or the protein and affect the number of binding sites exposed and available for binding.[38]

Effect of Ionic Strenth

In order to investigate strength the effect of

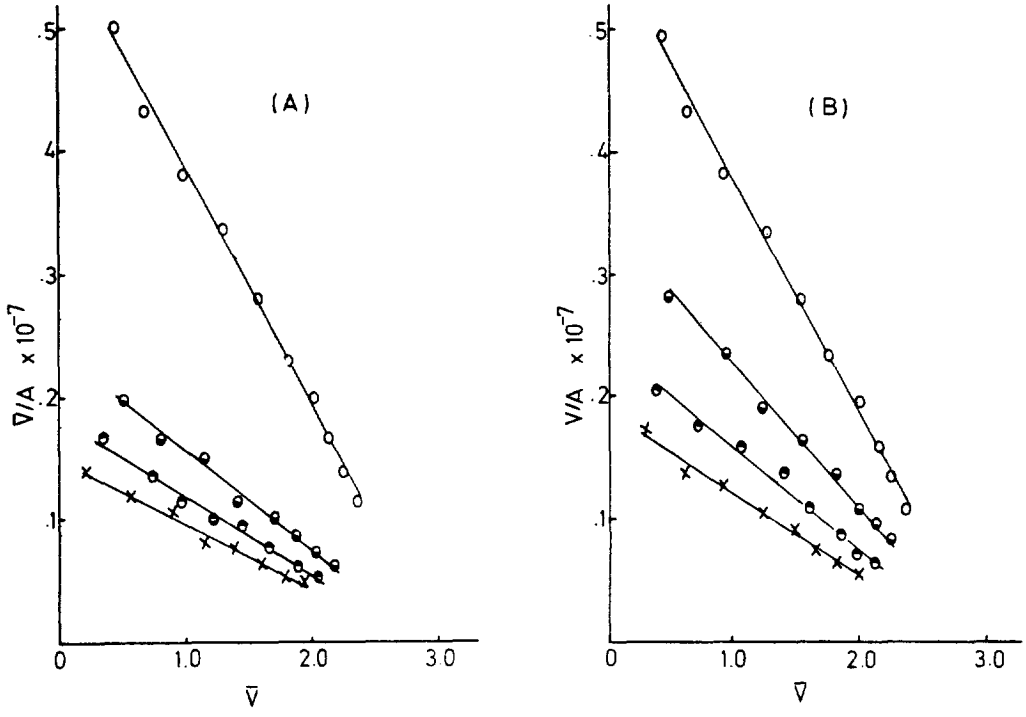

Fig. 2. Scatchard plots of the binding of probe to bovine serum albumin in the presence of hematoxylin (A) and brazilin (B) at various pH. $\bar{V}$ is the number of moles of bound probe per mole of protein, and A is the concentration of free probe. Key: ○, in the absence of both compounds; ◒, pH 6.6; ◓, pH 7.4; X, pH 8.0.

Table 2. Effect of pH on the binding of brazilin and hematoxylin to BSA

Compounds	pH of reaction mixture	n	k (molar×10^{-5})	r
Brazilin	6.6	2.9± 0.2	1.3± 0.1	0.0987
	7.4	2.8± 0.2	2.4± 0.1	0.995
	8.0	2.6± 0.1	3.5± 0.2	0.990
Hematoxylin	6.6	2.8± 0.2	3.5± 0.2	0.998
	7.4	2.9± 0.3	5.7± 0.2	0.974
	8.0	2.7± 0.2	7.2± 0.3	0.965

Results are the average of five sets of experiments. Experimental condition; ionic strength 0.4 at 23℃. n; the binding sites, k; the association constant, r; correlation coefficients.
Values represent mean± S.D.

ionic strength, phosphate buffers of various ionic strength were prepared by adding sodium chloride. The effects of ionic strength on the binding of brazilin and hematoxylin to BSA are shown in Fig. 3 as Scatchard plots. Table 3 resulted from Klotz equation shows that the association affinities of the both compounds for for BSA are increased by the elevation of ionic strength.

The effect of salt concentration on the interaction between both compounds and BSA may be explained in the terms of (a) change in ionic atmosphere of the associating protein molecules, (b)

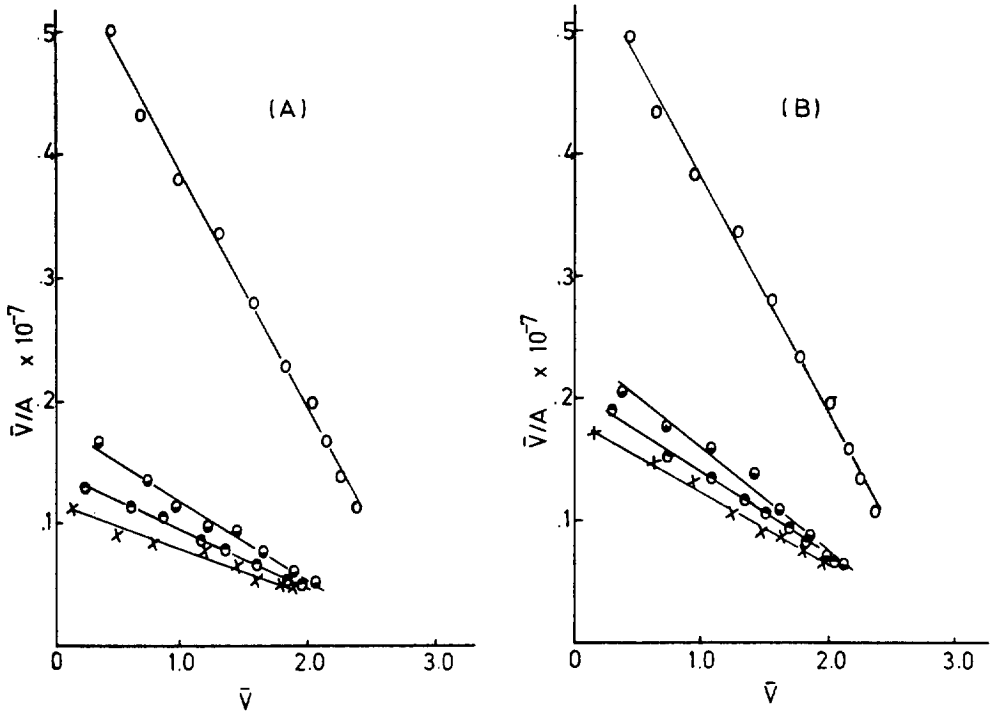

Fig. 3. Scatchard plots of the binding of probe to bovine serum albumin in the presence of hematoxylin (A) and brazilin (B) at various ionic strength. $\bar{V}$ is the number of moles of bound probe per mole of protein, and A is the concentration of free probe. Key: ○, in the absence of both compounds; ◒, ionic strength 0.4; ◓, ionic strength 0.6; and X, ionic strength 0.8.

Table 3. Effect of ionic strength on the binding of brazilin and hematoxylin to BSA

Compounds	ionic strength	n	k (molar×10^{-5})	r
Brazilin	0.4	2.8± 0.2	2.4± 0.1	0.993
	0.6	3.0± 0.2	3.2± 0.3	0.947
	0.8	3.1± 0.2	3.9± 0.3	0.976
Hematoxylin	0.6	3.0± 0.2	3.2± 0.3	0.947
	0.6	3.1± 0.1	6.8± 0.3	0.990
	0.8	3.1± 0.2	7.5± 0.2	0.996

Results are the average of five sets of experiments. Experimental condition; pH 7.4 at 23℃. n; the binding sites, k; the association constant, r; correlation coefficient. Values represent Mean± S.D.

competitive binding by the chloride ions, (c) a salting out effect.[39]

On the basis of the above mentioned experimental results, it is suggested that hydrophobic force plays main role in the binding of brazilin and hematoxylin to BSA and the electrostatic forces are also involved in the binding of both compounds to BSA.

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국문요약

천연색소 Brazilin 및 Hematoxylin의 BSA에 대한 결합 특성을 fluorescence probe법을 이용하여 측정하였다. Brazilin 및 Hematoxylin은 BSA에 대해 강한 결합 친화력을 보였으며, Hematoxylin은 Brazilin 보다 더 강한 결합력을 보였다. Brazilin 및 Hematoxylin의 농도 증가에 따라 결합상수는 감소하였으며, 이는 probe-단백 결합체와 양화합물간의 상호작용 또는 probe와 양화합물간의 결합체 형성에 기인하는 것으로 추정되었다. 양화합물과 BSA의 결합은 pH 및 이온강도에 의존적이었으며, 이 결합에는 electrostatic force 및 hydrophobic force가 관여하는 것으로 추정되었다.

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