Enantioselectivity of bovine serum albumin-bonded columns produced with isolated protein fragments
II. Characterization of protein fragments and chiral binding sites

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Abstract

Enantioselectivity of bovine serum albumin (BSA)-bonded columns produced with isolated protein fragments has been investigated. The BSA fragment, BSA-FG75, was isolated by size exclusion chromatography following peptic digest of BSA. The isolated BSA-FG75 was further fractionated to two fractions, BSA-F1 and BSA-F2, by anion-exchange chromatography. BSA-F1 and BSA-F2 had molecular mass of about 35 000 daltons, estimated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. BSA-F1 has amino acid residues 1–307 estimated by electrospray ionization (ESI) mass spectrometry, while BSA-F2 is an N-terminal half BSA fragment. The BSA, BSA-FG75, BSA-F1 and BSA-F2 proteins were bound to aminopropyl-silica gels activated by N,N'-disuccinimidyl carbonate. The bound amounts of the BSA fragments were 2.2–2.7 times more than that of the intact BSA. Chiral recognition of 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin was obtained with the BSA fragment-bonded columns. The non-enantioselective interactions of benzoin and benzodiazepines except for clorazepate with BSA fragments were increased with protein surface coverages, while those of 2-arylpropionic acid, clorazepate and warfarin were decreased. The BSA fragment columns gave higher enantioselectivity for lorazepam and benzoin, and lower enantioselectivity for other compounds tested, compared with the BSA column. These results might be due to changes in the globular structure of the BSA fragment and/or changes in the local environment around the binding sites.

Keywords: Enantiomer separation; Bovine serum albumin; Proteins

1. Introduction

Many protein-bonded stationary phases have been developed for resolution of enantiomers [1]. Those include albumins such as bovine serum albumin (BSA) [2] and human serum albumin (HSA) [3], glycoproteins such as α_1-acid glycoprotein [4], avidin [5], cellulase [6] and ovoglycoprotein [7], and enzymes such as trypsin [8], α-chymotrypsin [9] and

pepsin [10]. Disadvantages of protein-bonded columns in general have included low capacity, lack of column ruggedness in some cases and a limited understanding of the chiral recognition mechanisms. If a chiral binding site(s) exists on a domain or fragment and if it acts independent of others, chiral columns with the domain or fragment should be able to be made, which could be of higher capacity and be more stable. In a previous paper [11], we isolated a BSA fragment (BSA-FG75) by size exclusion chromatography following peptic digest of BSA,

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which has an average molecular mass of about 35 000 daltons. The intact BSA column gave higher enantioselectivity for the majority of compounds tested than the BSA-FG75 column, while the BSA-FG75 column gave higher enantioselectivity for lorazepam and benzoin than the intact BSA column, and was of higher capacity for benzoin. Also, the BSA-FG75 column was as stable as the intact BSA column for a continuous flow of an eluent.

This paper deals with further isolation of the BSA-FG75 fragment by anion-exchange chromatography, and characterization of the isolated two fragments, BSA-F1 and BSA-F2. The chiral recognition properties of the BSA-F1 and BSA-F2 were compared with those of BSA and BSA-FG75. Further, we discussed chiral binding sites on an N-terminal half of BSA, referred to the work by He and Carter [12], where they clarified ligand binding sites based on the three dimensional structure of HSA.

2. Experimental

2.1. Reagents and materials

BSA was purchased from Nacalai Tesque (Kyoto, Japan). Pepsin from porcine stomach mucosa and N,N'-disuccinimidyl carbonate (DSC) were purchased from Sigma (St. Louis, MO, USA). 1-Propanol of HPLC grade was obtained from Wako (Osaka, Japan). Racemic benzoin was purchased from Nacalai Tesque. Other racemic drugs used were kindly donated from pharmaceutical companies. Silica gels (Ultron-120, 5 μm diameter, 120 Å pore size, 300 m^2/g surface area) used were from Shinwa (Kyoto, Japan). Other solvents and reagents were used without further purification.

Water purified with a Nanopure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the eluent and the sample solution.

2.2. Isolation of BSA fragment

Half-cystinyl BSA was prepared according to the method of King [13]. Briefly, 22.4 mg of L-cystine was dissolved in 0.5 ml of 1 M NaOH solution, and the solution was added to 47 ml of 0.1 M Tris buffer

(pH 7.96). To 34 ml of 0.1 M Tris buffer (pH 7.96), 1.04 g of BSA was dissolved. Both solutions were mixed and reacted at 25°C for 17 h. The reacted solution was dialyzed against distilled water, and lyophilized.

Peptic digestion of half-cystinyl BSA was performed according to the method of King and Spencer [14]. To 8 ml of 0.1 M ammonium formate buffer (pH 3.7) including 3.2 mM octanoic acid, 0.124 g of half-cystinyl BSA was dissolved and preheated at 37°C. To the same buffer, 0.25 mg of pepsin was dissolved. Both solutions were mixed and reacted at 37°C for 30 min. By addition of 2 M Tris buffer (pH 7.9), the reaction was stopped. After dialyzing against 0.01 M Tris buffer (pH 8.0) including 0.3 M NaCl, the reacted solution was applied to Sephadex G-75 column (5×90 cm) that was equilibrated with 0.01 M Tris buffer (pH 8.0) including 0.3 M NaCl at an average flow-rate of 80 ml/h. The eluant was monitored at 280 nm. The separation was performed at 4°C. The first peak was collected and lyophilized. The lyophilized sample was desalted with a Sephadex G-25 (fine) column (5×20 cm) using 15 mM NH$_4$HCO$_3$ as the buffer with an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. The obtained BSA fragment was termed BSA-FG75.

A weight of 100 mg of the BSA-FG75 was dissolved in 1 ml of water and applied to Superformance Fractgel EMD DEAE-650 column (10×150 mm) (E. Merck, Darmstadt, Germany). The eluents used were as follows: eluent A, 20 mM sodium phosphate buffer (pH 7.0); eluent B, 20 mM sodium phosphate buffer (pH 7.0) including 200 mM sodium sulfate. Two fractions, BSA-F1 and BSA-F2, were collected by stepwise elution using eluents A and B (12.5% of eluent B, 0–30 min; 15% of eluent B, 30–120 min; 25% of eluent B, 120–180 min; 80% of eluent B, 180–200 min). The flow-rate was maintained at 0.5 ml/min. The separation was carried out at ambient temperature.

2.3. Preparation of DSC-activated aminopropyl-silica gels

Silica gels (5 g) were dried in vacuo over P$_2$O$_5$ at 150°C for 6 h and the dry silica gels were added to 120 ml of dry toluene. The mixture was heated to

reflux until all the water had been removed as an azeotrope into a Dean-Stark-type trap. Next, 3-aminopropyltrimethoxysilane, corresponding to 10 μmol/m^2 of the specific surface area, were added and reacted for 8 h. The reaction mixture was cooled to room temperature, filtered and washed with toluene and methanol. The isolated silica gels were dried in vacuo over P_2O_5 at 60°C for 2 h. The obtained aminopropyl-silica gel was used for the activation reaction described below.

A weight of 5 g of the gels were slurried in 70 ml of acetonitrile and reacted with 5 g of DSC for 24 h at 30°C. The reaction mixture was filtered and washed with acetonitrile and methanol. The activated silica gels were dried in vacuo over P_2O_5 at 60°C for 2 h.

2.4. Preparation of BSA- or BSA-fragment-bonded materials

BSA proteins were bound to the DSC-activated aminopropyl-silica gels as follow: 0.28 g of the DSC-activated silica gels were slurried in 4 ml of 20 mM phosphate buffer (pH 6.6). To the mixture, 0.14 g of BSA proteins dissolved in 2 ml of the same buffer was added slowly at room temperature for 1 h and further stirred for 20 h at 30°C. Similarly, the BSA fragment was bound to DSC-activated silica gels as follows: 0.28 g of the DSC-activated silica gels was reacted with 0.072 g of the BSA-FG75, BSA-F1 and BSA-F2 using the same reaction conditions. Then the reaction mixtures were filtered, and washed with water and slurry solvent described below.

The BSA and BSA-fragment materials were packed into a 2.1 mm I.D.×100 mm stainless-steel column by the slurry packing method. The slurry solvents were water–ethanol (95:5, v/v) for the BSA materials and 50 mM phosphate buffer (pH 7.5)–1-propanol (60:40, v/v) for the BSA-fragment materials. The same packing solvents were used.

2.5. Chromatography

For chiral resolution of racemic solutes on the BSA and BSA-fragment columns, the HPLC system used was composed of an LC-10AD pump, an SPD-10A spectrophotometer, a Rheodyne 7125 injector, a

C-R6A integrator and an SCL-10A system controller (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.2 ml/min. Detection was performed at 220 or 254 nm. Capacity factors (k'_1 and k'_2), enantioseparation factor (α), and resolution (R_s) of racemates were calculated. All separations were carried out at 25°C using a water bath. The eluents are prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphosphate and organic modifier. The eluents used were specified in the figure and table legends.

For reversed-phase chromatography of the BSA-fragment protein, the same HPLC system as described above was used. The eluents used are as follows: eluent A, H_2O–CH_3CN (80:20, v/v) including 0.1% trifluoroacetic acid (TFA); eluent B, H_2O–CH_3CN (20:80, v/v) including 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. The column used was Cosmosil 5C18-AR (4.6 mm I.D.×250 mm). Detection was carried out at 280 nm. The flow-rate was 1.0 ml/min. All separations were performed at ambient temperature.

2.6. Elemental analysis

The elemental analysis of the BSA- and BSA-fragment-bonded silica materials was performed as follows. A weight of 20 mg of each material was treated with an oxygen flask method [15]. The products of combustion was adsorbed into 1% aqueous hydrogen peroxide solution and determined as sulfate ion using ion-chromatography. The Bio LC chromatograph (Nihon Dionex, Tokyo, Japan) was used for the detection of sulfate ion. It consisted of a pump, a 50 μl sample loop, an anion-exchange column (Dionex Ion-Pac AS-4, 250×4 mm I.D.), a micromembrane suppressor (Dionex AMMS-II) and a conductivity detector. The eluent and regenerant used were 2 mM sodium hydroxide including 2 mM sodium carbonate and 1.25 mM sulfuric acid. The flow-rates of the eluent and regenerant were 1.0 and 3 ml/min, respectively.

2.7. N-Terminal sequencing

A weight of 70 μg of the BSA fragment was reconstituted with 50 μl of water. A 5 μl portion of

the dilution was spotted on a solid support for N-terminal sequencing analysis using an ABI 473A Protein Sequencer (Applied Biosystems Division, Perkin Elmer Japan, Tokyo, Japan).

2.8. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

The mass spectrometer used was a VISION 2000 reflector-type TOF instrument (ThermoQuest K.K., Tokyo, Japan) equipped with a N_2-laser operating at a wavelength of 337 nm with a pulse duration of 3 ns. The laser beam diameter at the sample surface was 70 μm, laser irradiances were in the low 10^6 W/cm² range, close to the threshold for obtaining ions. The ions generated were accelerated to a potential of 5 kV in the ion source and post accelerated to a potential of 20 kV for detection with a secondary ion multiplier. The MALDI-TOF spectra represent the accumulation of 20–25 single laser shots. They were calibrated externally by a standard sample (BSA, molecular mass of 66 430.2 daltons) that was placed on the same target. The matrix used was 2,5-dihydroxybenzoic acid, dissolved in a 2:1 mixture of 0.1% aqueous TFA and acetonitrile at a concentration of 50 mM. Samples were dissolved in a water at a concentration of 10^{-6} M. A 0.5 μl portion of the sample solution was mixed with an equal volume of the matrix solution on the target, resulting in a used sample amount of 500 fmol. After deposition on the stainless-steel target, the sample was air-dried and introduced into the mass spectrometer.

2.9. Electrospray ionization (ESI) mass spectrometry

A Hewlett Packard HP1050 pumping system (Yokokawa Analytical Systems, Tokyo, Japan) and the Finnigan TSQ 7000 fitted with the Finnigan electrospray source (ThermoQuest K.K., Tokyo, Japan) were used. A TSK gel ODS-120T (Tosoh, Tokyo, Japan) packed into a 2 mm×10 mm stainless steel column was used for the separations. The eluents used are as follows: eluent A, 0.05% trifluoroacetic acid (TFA); eluent B, CH_3CN including 0.05% TFA; linear gradient from 30% eluent B at 0 min to 80% eluent B at 10 min. The flow-rate was

maintained at 0.1 ml/min. Approximately 100 pmol of each sample was injected. Mass spectrometric conditions included spray voltage of 4.5 kV, capillary temperature of 200°C, nitrogen sheath gas pressure of 70 psi and auxiliary gas flow-rate of 10 ml/min. Spectra were obtained scanning from 500 to 2500 amu every 3 s.

2.10. Sample preparation

A known amount of a racemic solute was dissolved in methanol or water and the solution was diluted with the eluent to desired concentration. A 5 μl volume of the sample solution was loaded onto a column. The loaded amount was 0.04–0.1 μg.

3. Results and discussion

3.1. Characterization of BSA fragment

The half-cystinyl BSA digested by pepsin was isolated on a Sephadex G-75 size exclusion column and the isolated fragment was termed BSA-FG75. BSA-FG75 was further isolated by anion-exchange chromatography. Fig. 1 shows an anion-exchange chromatogram of BSA-FG75. The fractions of BSA-F1 and BSA-F2 were isolated. Fig. 2 shows reversed-phase chromatograms of half-cystinyl BSA, BSA-FG75, and a mixture of half-cystinyl BSA and
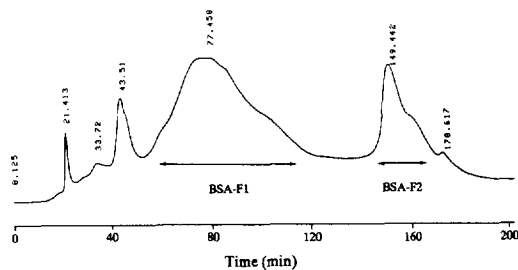

Fig. 1. Chromatogram of BSA-FG75 on an ion-exchange column. Column: Superformance Fractgel EMD DEAE-650 (10×650 mm). Eluent: eluent A, 20 mM sodium phosphate buffer (pH 7.0); eluent B, 20 mM sodium phosphate buffer (pH 7.0) including 200 mM sodium sulfate; stepwise elution using eluents A and B (12.5% of eluent B, 0–30 min; 15% of eluent B, 30–120 min; 25% of eluent B, 120–180 min; 80% of eluent B, 180–200 min). Flow-rate: 0.5 ml/min. Detection: 280 min. The fractions of BSA-F1 and BSA-F2 were collected.
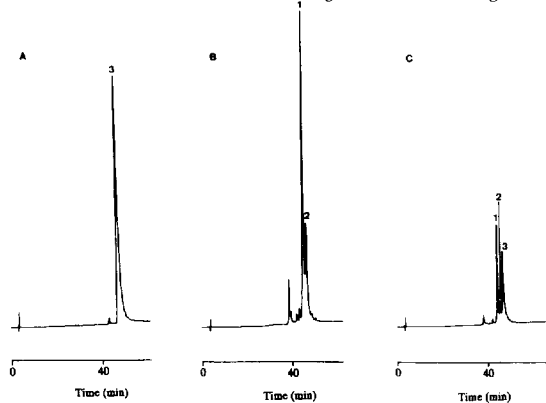
Fig. 2. Chromatogram of half-cystinyl BSA (A), BSA-FG75 (B) and a mixture of half-cystinyl BSA and BSA-FG75 (C) on a reversed-phase column. Column: Cosmosil 5C18-AR (4.6 mm I.D. × 250 mm). Eluents: eluent A, H_2O–CH_3CN (80:20, v/v) including 0.1% TFA; eluent B, H_2O–CH_3CN (20:80, v/v) including 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. Flow-rate: 1.0 ml/min. Detection: 280 nm. Loaded amount: 200 µg. Keys: 1, BSA-F1; 2, BSA-F2; 3, half-cystinyl BSA.

BSA-FG75. Two major peaks of BSA-FG75 corresponded to the isolated BSA-F1 and BSA-F2 proteins. It is found that BSA-FG75 is free from uncleaved BSA, and that BSA-FG75 is a mixture of BSA-F1 and BSA-F2, whose ratio is about 3:1. These fragments were characterized by N-terminal sequencing and mass spectra. With regard to the N-terminal sequencing, the 15 amino acid sequences of BSA-F1 and BSA-F2 were the same with those of uncleaved BSA.

Fig. 3, parts A and B, shows the MALDI-TOF mass spectra of BSA fragments, BSA-F1 and BSA-F2, respectively. The average molecular weights of BSA-F1 and BSA-F2 were estimated to be 35 120±25 and 35 109±35 daltons averaged from M^{2+}, M^+ and $2M^+$ ions by MALDI-TOF mass spectrometry. Fig. 4, parts A and B, shows ESI mass spectra of BSA-F1 and BSA-F2, respectively. The ESI mass spectra of BSA-F1 and BSA-F2 contain ions of multi-charge states. Using these masses the molecular weight of BSA-F1 was calculated to be 35 236±2.4 daltons, which is in good agreement with the predicted molecular weight of the half-cystinyl BSA fragment of the amino acid sequences 1–307, 35 234 daltons. The observed molecular weight is ascribable to amino acid sequences 1–307 having eight disulfide bonds and one half-cystinyl

bond in the 34th. Previously, the amino acid sequence of BSA was determined to consist of 582 amino acid residues [16]. Recently, Hirayama et al. [17] reported that a tyrosine residue in the 156th was lacked in the previous sequence, and that BSA consisted of 583 amino acid sequences. Thus, the cleavage was between 307 and 308 (Asp and Phe), which corresponds to 306 and 307 in the previous sequence.

On the other hand, the observed molecular weight of BSA-F2 was estimated to be 35 149±3.3 daltons by ESI mass spectrometry. However, the amino acid sequences corresponding to the observed molecular weight has not been found. This may be due to that BSA-F2 is less pure than BSA-F1, as shown in Figs. 3 and 4. Two BSA fragments, BSA-F1 and BSA-F2, might have the same amino acid sequence with modification of side chain amino acid(s) and/or with a sulfhydryl group at the 34th position or other position. BSA-F2 is obviously more negative charged and more hydrophobic than BSA-F1 according to the chromatographic behaviors. This difference could be accounted for by differences in degree of denaturation. Further study is required to characterize BSA-F2.

These results revealed that BSA-F1 had amino acid residues 1–307, and that BSA-F2 was N-terminal half of BSA having a similar amino acid sequence with BSA-F1.

3.2. Surface coverages of BSA and BSA-fragment proteins

Table 1 shows the surface coverages of BSA, BSA-FG75, BSA-F1 and BSA-F2 proteins on the protein-bonded materials. Previously [11], we reported that the materials having 120 Å pore sizes showed about 1.5 times higher surface coverages of the BSA proteins than those having 300 Å pore sizes, and that the former materials gave higher enantioselectivity than the latter. Thus, we bound the BSA and BSA fragment by using the silica gels having the 120 Å pore size. As shown in Table 1, the BSA-FG75, BSA-F1 and BSA-F2 materials gave about 2.2–2.7 times higher surface coverages than the intact BSA materials. The higher surface coverages of BSA fragments are ascribable to that the
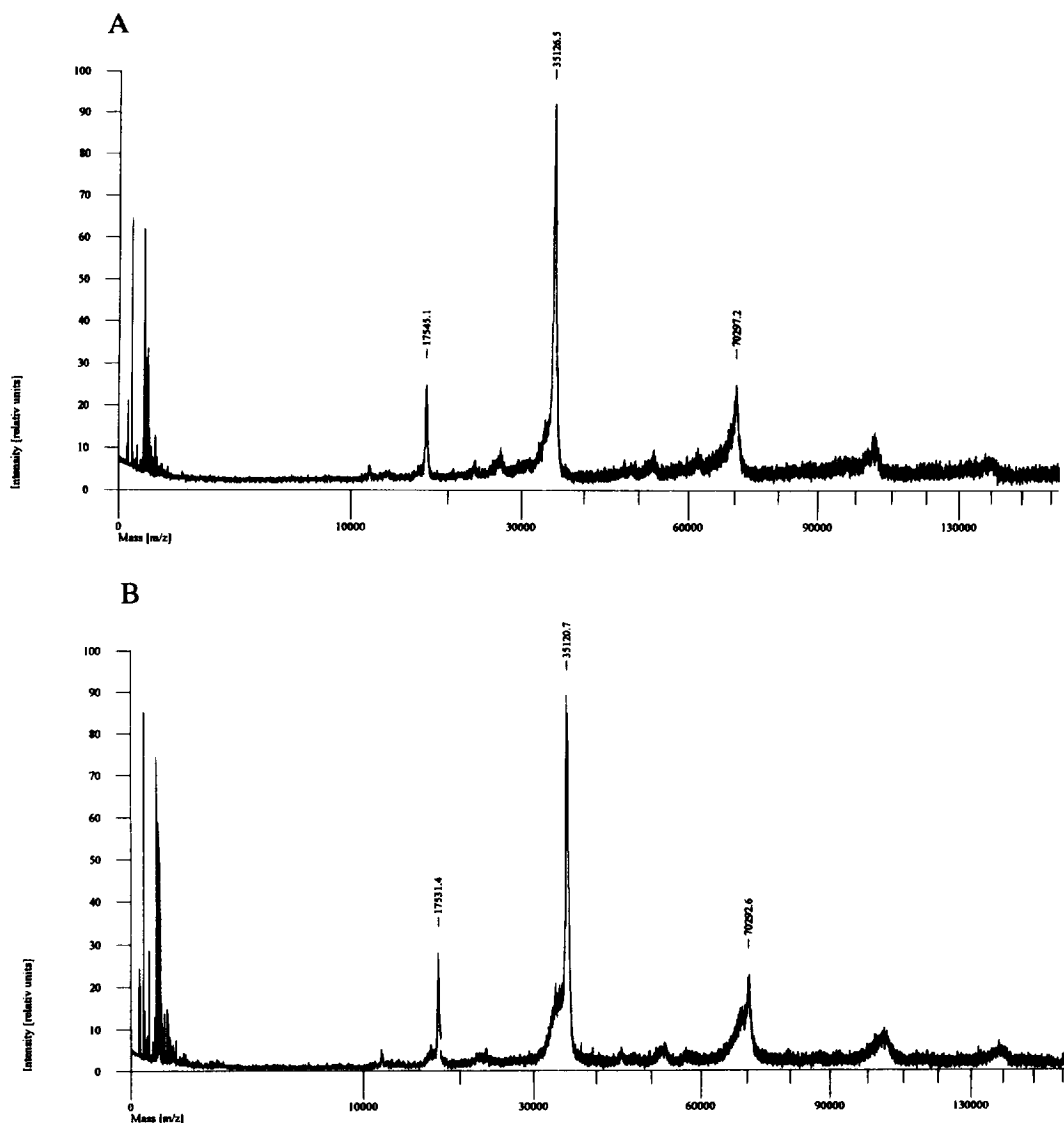
Fig. 3. MALDI-TOF mass-spectra of BSA-F1 (A) and BSA-F2 (B). Other conditions see Section 2.

BSA-fragment proteins should be more accessible to the inner surface of the silica gels than BSA.

3.3. Comparison of retention and enantioselectivity of racemic solutes on BSA and BSA-fragment columns

Table 2 shows comparison of retention and enantioselectivity of benzodiazepines, benzoin and warfarin on the BSA, BSA-FG75, BSA-F1 and BSA-

F2 columns. Table 3 shows comparison of retention and enantioselectivity of 2-arylpropionic acid derivatives on the BSA and BSA-fragment columns. Figs. 5 and 6 show chromatograms of benzoin and warfarin, respectively, on the BSA, BSA-FG75, BSA-F1 and BSA-F2 columns. The BSA-F1 and BSA-F2 columns as well as the BSA-FG75 column gave longer retentions for benzoin and benzodiazepines except for clorazepate than the BSA column, while they gave shorter retentions for 2-arylpropionic acid
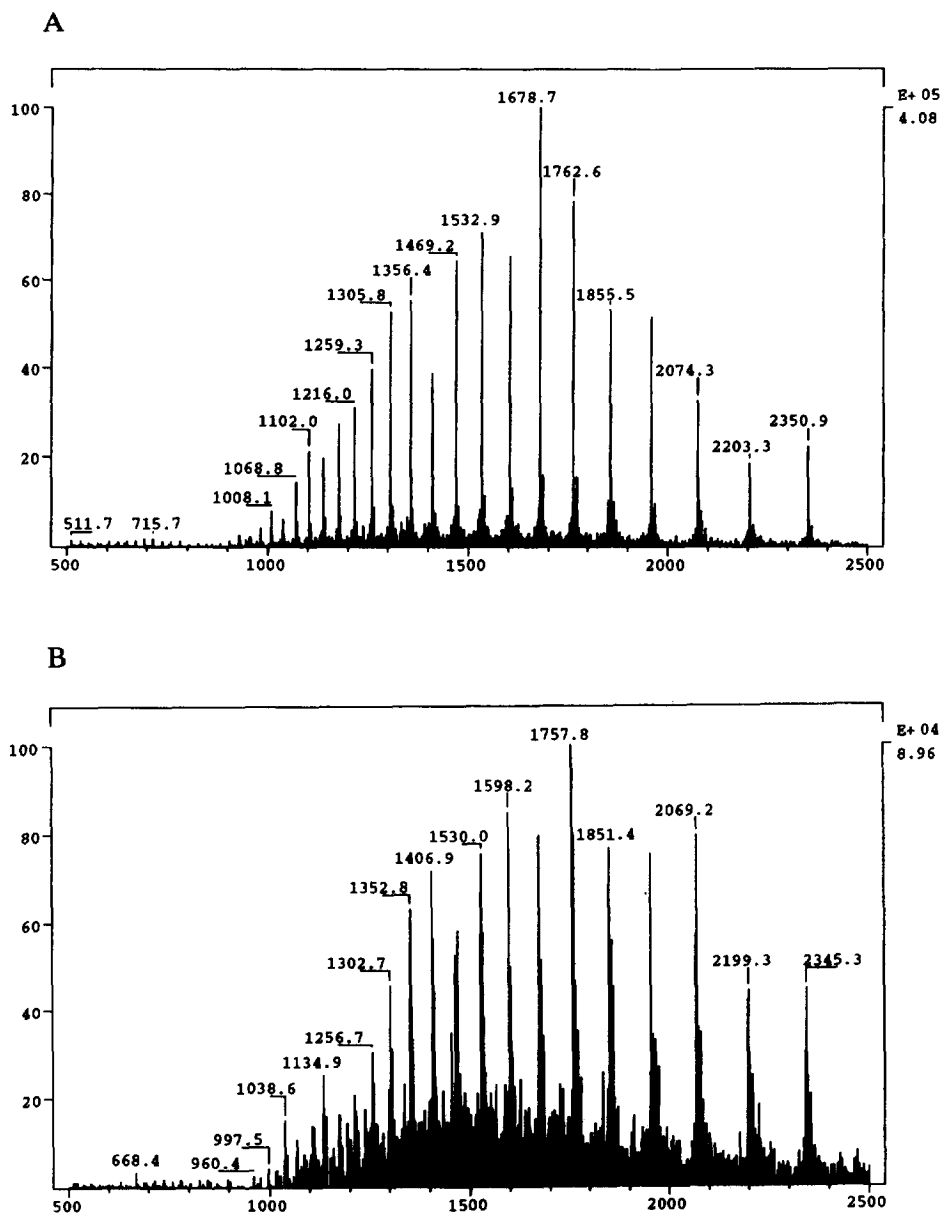
A

B

Fig. 4. ESI mass spectra of BSA-F1 (A) and BSA-F2 (B). Other conditions, see Section 2.

derivatives, clorazepate and warfarin. Though the BSA column gave higher enantioselectivity than the BSA-fragment columns for the majority of compounds tested, the BSA-fragment columns gave higher enantioselectivity for benzoin and lorazepam. The BSA-FG75 column gave shorter retentions for the compounds tested than the BSA-F1 and BSA-F2

columns because BSA-F1, BSA-F2 and other fragment portion were included in BSA-FG75 (see Fig. 1). The BSA-F2 column gave higher enantioselectivity than the BSA-F1 columns in spite of that both fragments were N-terminal half BSAs with similar amino acid sequences.

Recently, He and Carter [12] crystallographically

Table 1

Protein coverages of BSA- and BSA-fragment-bonded columns

Packing	Protein phase sulfur content[a]	Surface coverage (μmol/g)
BSA	0.24	1.9
BSA-FG75	0.34	5.1
BSA-F1	0.33	4.9
BSA-F2	0.28	4.1

[a] Estimated from the elemental analysis data of sulfur.

determined the three dimensional structure of HSA, and tried to locate ligand binding sites to HSA. The primary binding sites of warfarin was an N-terminal half of HSA. Those of 2-arylpropionic acid derivatives and benzodiazepines were on other half of HSA. Though enantioselective drug binding to isolated HSA was intensively studied, only a few reports had been published about comparative aspects of enantioselective drug binding to other albumins. It was reported that despite distinct differences of various serum albumins with respect to sign and degree of enantioselective binding, the differences were usually small [18]. We postulated that ligand binding locations to HSA are the same with those to BSA.

The non-enantioselective interactions of benzoin and benzodiazepines except for clorazepate were increased with protein surface coverages, while those of 2-arylpropionic acid, clorazepate and warfarin were decreased. Benzoin and benzodiazepines could participate in enantioselective binding recognition on an N-terminal half of BSA. On the other hand,
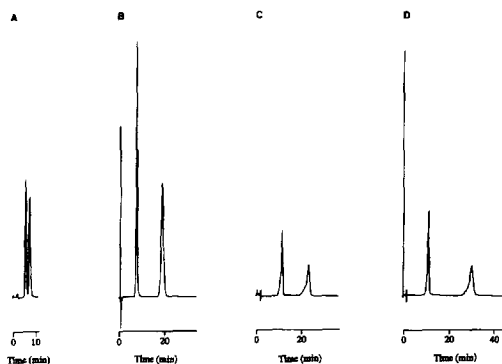

Fig. 5. Chromatograms of benzoin on the BSA (A), BSA-FG75 (B), BSA-F1 (C) and BSA-F2 (D) columns. HPLC conditions: column, 2.1 mm I.D.×100 mm; eluent, 50 mM phosphate buffer (pH 7.5)–1-propanol (96:4, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 254 nm; injected amount, 0.1 μg each.

2-arylpropionic acid derivatives, clorazepate and warfarin could interact with the sites to a lesser extent. However, note that benzodiazepines and 2-arylpropionic acid derivatives participate in enantioselective binding interaction with an N-terminal half of BSA, despite that the primary binding sites of benzodiazepines and 2-arylpropionic acid derivatives are on the other half of BSA.

Warfarin was not resolved on the BSA-fragment column prepared by Andersson et al. [19], but was resolved on the BSA-fragment column prepared by us. The N-terminal half BSA fragment was isolated and bound to aminopropyl-silica gels by Andersson

Table 2

Optical resolution of benzodiazepines, benzoin and warfarin on BSA- and BSA-fragment-bonded columns

Compound	Column							
	BSA		BSA-FG75		BSA-F1		BSA-F2	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Temazepam	2.43	1.83	4.63	1.05	7.88	1.00	5.95	1.12
Lormetazepam	4.98	1.41	9.96	1.13	15.9	1.00	13.7	1.22
Lorazepam	5.30	2.01	13.5	2.34	20.0	2.14	19.9	2.72
Oxazepam	3.13	3.94	6.60	2.53	10.5	2.35	7.78	3.54
Clorazepate	6.93	2.85	3.33	1.33	5.15	1.27	5.33	1.46
Benzoin	1.89	1.46	3.56	2.56	5.31	2.38	5.18	3.17
Warfarin	14.9	1.59	4.44	1.21	8.30	1.29	9.29	1.29

HPLC Conditions: column, 2.1 mm I.D.×100 mm; eluent, 50 mM phosphate buffer (pH 7.5)–1-propanol (96:4, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 254 nm.

Table 3
Optical resolution of 2-arylpropionic acid derivatives on BSA-fragment-bonded columns

Compound	Column							
	BSA		BSA- FG75		BSA- F1		BSA- F2	
	k_1'	α	k_1'	α	k_1'	α	k_1'	α
Ketprofen	9.40	1.14	4.27	1.33	4.48	1.28	6.19	1.34
Ibuprofen	n.e.		8.52	2.09	8.11	1.95	11.5	2.22
Fenoprofen	22.0	1.81	10.3	1.31	10.1	1.27	14.2	1.31
Flurbiprofen	n.e.		12.7	1.59	11.5	1.45	15.9	1.56

HPLC Conditions: column, 2.1 mm I.D.×100 mm; eluent, 50 mM phosphate buffer (pH 6.9)–CH$_3$CN (85:15, v/v) for BSA column and 90:10 (v/v) for BSA-fragment columns; column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 254 or 220 nm. n.e.: not eluted.
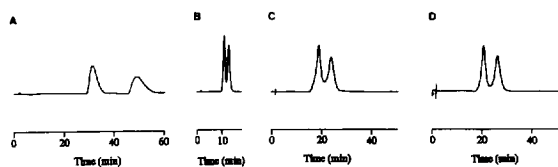

Fig. 6. Chromatograms of warfarin on the BSA (A), BSA-FG75 (B), BSA-F1 (C) and BSA-F2 (D) columns. Other conditions as in Fig. 5.

et al. and us. However, they crosslinked the fragment into aminopropyl-silica gels by glutaraldehyde or adsorbed it onto silica gels, while we bound to aminopropyl-silica gels activated by DSC. These results suggested that the binding sites of warfarin might be lost on isolation and immobilization of the fragment in the case of Andersson et al., because the primary binding sites of warfarin were on an N-terminal half of BSA.

The BSA-fragment columns prepared by us gave higher enantioselectivity for lorazepam and benzoin, and lower enantioselectivity for warfarin, compared with the BSA column. These results might be due to changes in the globular structure of the BSA fragment or changes in the local environment around the binding sites, during the isolation and immobilization.

Acknowledgments

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