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Enantioselectivity of bovine serum albumin-bonded columns produced with isolated protein fragments

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Abstract

The enantioselectivity of bovine serum albumin (BSA)-bonded columns produced with isolated protein fragments was investigated. The BSA fragment BSA-FG75 was isolated by size-exclusion chromatography followed by peptic digestion of BSA. The isolated BSA-FG75 was a mixture of three peptides, and was mainly an N-terminal half peptide(s) with an average molecular mass of about 35 000. The BSA and BSA-FG75 proteins were bound to aminopropylsilica gels activated by N,N'-disuccinimidyl carbonate. The amounts of the proteins bound were about 2 and 5.5 μmol/g for the BSA and BSA-FG75, respectively. Chiral recognition of 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin was obtained with the BSA-FG75-bonded columns, but no chiral recognition of tryptophan or kynurenine was obtained. The intact BSA column gave a higher enantioselectivity than the BSA-FG75 column for most of the compounds tested, whereas the BSA-FG75 column gave a higher enantioselectivity than the intact BSA column for lorazepam and benzoin, and had a higher capacity for benzoin. These results are due to a higher density of chiral recognition site(s) on the BSA-FG75 column. Also, the BSA-FG75 column was as stable as the intact BSA column for a continuous flow of eluent.

1. Introduction

Many protein-bonded stationary phases have been developed for the resolution of enantiomers [1]. These include albumins such as bovine serum albumin [2] and human serum albumin [3], glycoproteins such as α_1-acid glycoprotein [4], ovomucoid [5], avidin [6] and cellulase [7] and enzymes such as trypsin [8], α-chymotrypsin [9] and lysozyme [10]. Disadvantages of protein-bonded columns in general have included low capacity, lack of column ruggedness in some instances 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 independently 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. Recently, it was found that the third domain of turkey ovomucoid is enantioselective to at least two classes of compounds, benzodiazepines and 2-arylpropionic acid derivatives, and the glycosylated group is not needed for chiral recognition of these compounds [11]. Also, a chiral binding site was located and the chiral recognition mechanism was elucidated by using NMR spectroscopy and computational chemistry [11].

Andersson et al. [12] isolated a bovine serum albumin (BSA) fragment, of molecular mass ca. 38 000, by enzymatic cleavage, and cross-linked the fragment into aminopropylsilica materials by

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glutaraldehyde or adsorbed it on silica materials. However, the BSA fragment-bonded column obtained could have less capacity and enantioselectivity, and be less stable than the intact BSA-bonded column. In this study, we modified the methods for the isolation and binding of a BSA fragment. The retention and enantioselective properties of the obtained BSA fragment column were compared with those of the intact BSA column.

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 by pharmaceutical companies. The structures of these racemates are shown in Fig. 1. Silica gels (Ultron-120, particle diameter 5 μm, pore size 120 Å, surface area 300 m^2/g; and Ultron-300, particle diameter 5 μm, pore size 300 Å, surface area 100 m^2/g) were obtained from Shinwa Chemical Industries (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 were dissolved in 0.5 ml of 1 *M* NaOH solution and the mixture was added to 47 ml of 0.1 *M* Tris buffer (pH 7.96). A 1.04-g amount of BSA was dissolved in 34 ml of 0.1 *M* Tris buffer (pH 7.96). The two solutions were mixed and reacted at 25°C for 17 h. The reacted solution

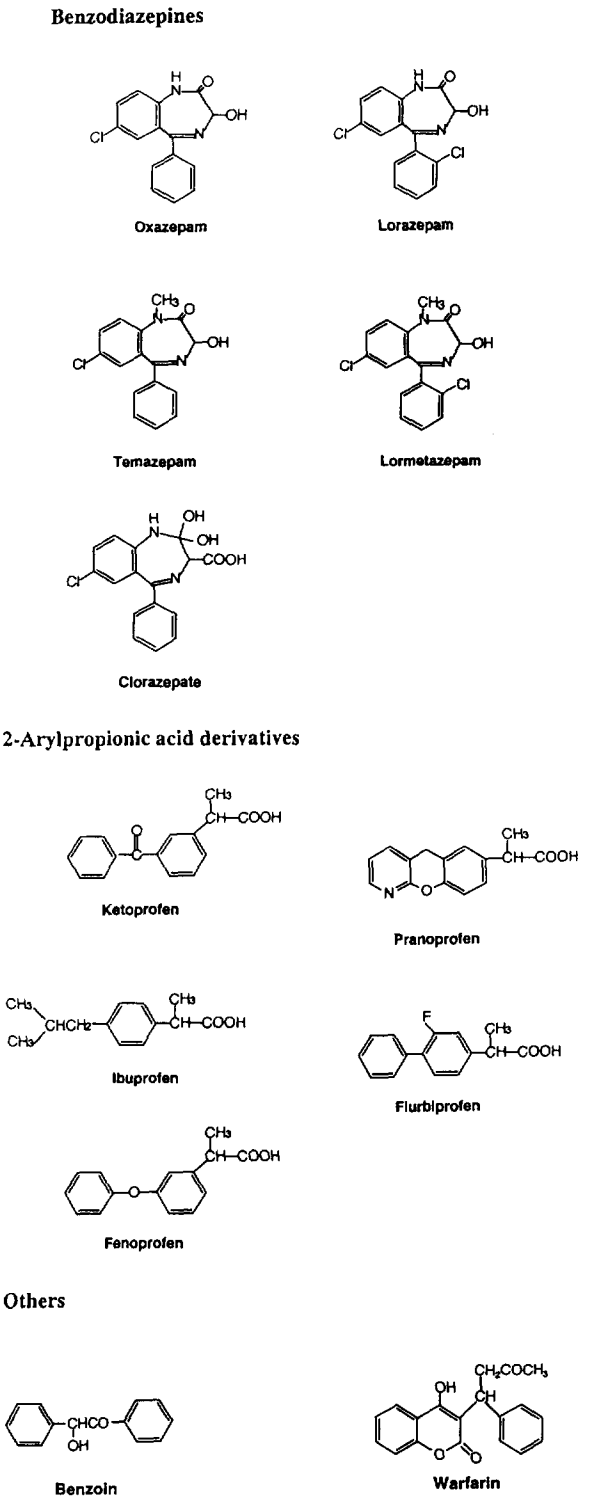

Fig. 1. Structures of the racemic solutes.

was dialysed against distilled water and lyophilized.

Peptic digestion of half-cystinyl BSA was performed according to the method of King and Spencer [14]. A 0.124-g amount of half-cystinyl BSA was dissolved in 8 ml of 0.1 *M* ammonium formate buffer (pH 3.7) containing 3.2 m*M* octanoic acid and preheated at 37°C. A 0.25-mg amount of pepsin was dissolved in the same buffer. The two solutions were mixed and reacted at 37°C for 30 min. The reaction was stopped by addition of 2 *M* Tris buffer (pH 7.9). After dialysing against 0.01 *M* Tris buffer (pH 8.0) containing 0.3 *M* NaCl, the reacted solution was applied to a Sephadex G-75 column (90 × 5 cm I.D.) that was equilibrated with 0.01 *M* Tris buffer (pH 8.0) containing 0.3 *M* NaCl at an average flow-rate of 80 ml/h. The eluate 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 (20 × 5 cm I.D.) using 15 m*M* NH_4HCO_3 as the buffer at an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. The BSA fragment obtained was termed BSA-FG75.

2.3. Preparation of DSC-activated aminopropylsilica gels

Silica gels (5 g) were dried in vacuo over P_2O_5 at 150°C for 6 h and 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, was 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 aminopropylsilica gel obtained was used for the activation reaction described below.

Amounts 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 aminopropylsilica gels as follows: 0.28 g of the DSC-activated silica gels was slurried in 4 ml of 20 m*M* 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 the mixture was 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 using the same reaction conditions. Then both the reaction mixtures were filtered and washed with water and slurry solvent described below.

The BSA and BSA-FG75 materials were packed into a 100 mm × 2.1 mm I.D. stainless-steel column by the slurry packing method. The slurry solvents were 5% ethanol for the BSA materials and 50 m*M* phosphate buffer (pH 7.5)–1-propanol (60:40, v/v) for the BSA-FG75 materials. The same packing solvents were used.

2.5. Chromatography

For chiral resolution of racemic solutes on the BSA and BSA-FG75 columns, the HPLC system used was composed of an LC-10AD pump, an SPD-10A spectrophotometer, a Rheodyne Model 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'), enantioseparation factors (α), and resolutions (R_s) of the racemates were calculated. The asymmetry factor (η) was calculated as reported previously [15]. All separations were carried out at 25°C using a water-bath.

The eluents were prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphos-

phate and organic modifier. The eluents used are specified in the figures and tables.

For reversed-phase chromatography of the BSA-FG75 protein, the same HPLC system as described above was used. The eluents used were as follows: eluent A, 0.1% trifluoroacetic acid (TFA); eluent B, water–acetonitrile (20:80, v/v) containing 0.1% of TFA; linear gradient from 25% B at 0 min to 80% B at 90 min. The column used was Cosmosil 5C18-AR (250 mm × 4.6 mm I.D.). 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 using ion chromatography combined with the oxygen flask method for sulfur.

2.7. N-Terminal sequencing

A 70-μg amount of the BSA fragment was reconstituted with 50 μl of water. A 5-μl portion of the solution was spotted on to 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 (Finnigan MAT, Tokyo, Japan) equipped with a nitrogen 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/cm^2 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) that was placed on the same target. The matrix used was 2,5-dihydroxybenzoic acid, dissolved in 0.1% aqueous TFA–acetonitrile (2:1) 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 sample amount used of 500 fmol. After deposition on the stainless-steel target, the sample was air-dried and introduced into the mass spectrometer.

2.9. Sample preparation

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

3. Results and discussion

3.1. Characterization of BSA fragment

Fig. 2 shows a chromatogram of half-cystinyl BSA digested by pepsin on a Sephadex G-75 size-exclusion column. The fraction of the BSA fragment BSA-FG75 was collected. It was found from reversed-phase chromatography of the BSA-FG75 that the isolated BSA-FG75 fraction was free from uncleaved BSA, and that the BSA-FG75 was a mixture of peptides. Fig. 3 shows the MALDI-TOF mass spectrum of the BSA fragment BSA-FG75. The BSA-FG75 consisted of three peptides, with molecular masses of 35 000, 32 000 and 27 000. The average molecular mass of the main digest peptide was calculated to be 35 113 ± 3, averaged from M^{2+}, M^+ and $2M^+$ ions. With regard to the N-terminal sequencing data, the first sequence of the BSA-FG75 started from Asp, which is the N-terminus of the uncleaved BSA. Also, the second and third sequences started from Phe at the 11th and 49th amino acid positions in BSA were observed, as reported by Erlandsson and Nilsson [16]. These results revealed that the isolated BSA-

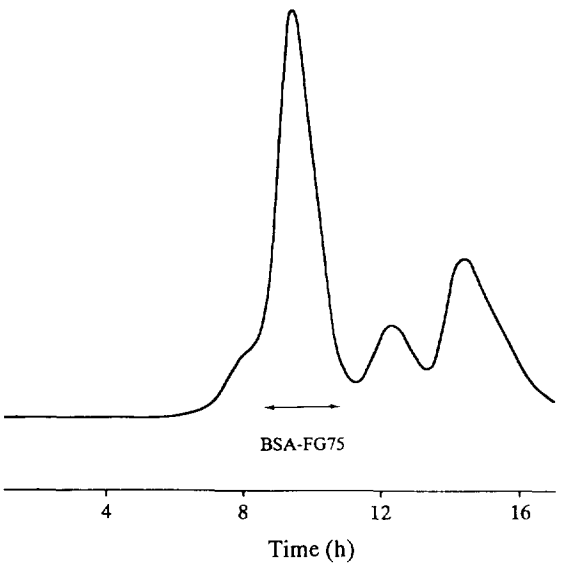

Fig. 2. Chromatogram of peptic digest of BSA on a size-exclusion column. Column, Sephadex G-75 (90 × 5 cm I.D.); eluent, 0.01 *M* Tris buffer (pH 8.0) containing 0.3 *M* NaCl; flow-rate, 80 ml/h; detection wavelength, 280 nm. The fraction of a BSA fragment, BSA-FG75, was collected.

FG75 was mainly an N-terminal half peptide(s) with an average molecular mass of about 35 000.

3.2. Surface coverages of BSA and BSA-FG75 proteins

Table 1 shows the surface coverages of BSA and BSA-FG75 proteins on the protein-bonded materials. With regard to comparison of the base silica gels having 120- and 300-Å pore sizes, the former materials showed about 1.5-fold higher surface coverages of the BSA proteins. Also, the former materials gave higher enantioselectivity than the latter. Therefore, we bound the BSA-FG75 by using silica gels having a 120-Å pore size. As shown in Table 1, the BSA-FG75 materials gave about 3-fold higher surface coverages than the intact BSA materials. The higher surface coverages of the BSA-FG75 are ascribable to the fact that the BSA-FG75 proteins should be more accessible to the inner surface of the silica gels than the BSA proteins because the molecular mass of the BSA-FG75 is about half of

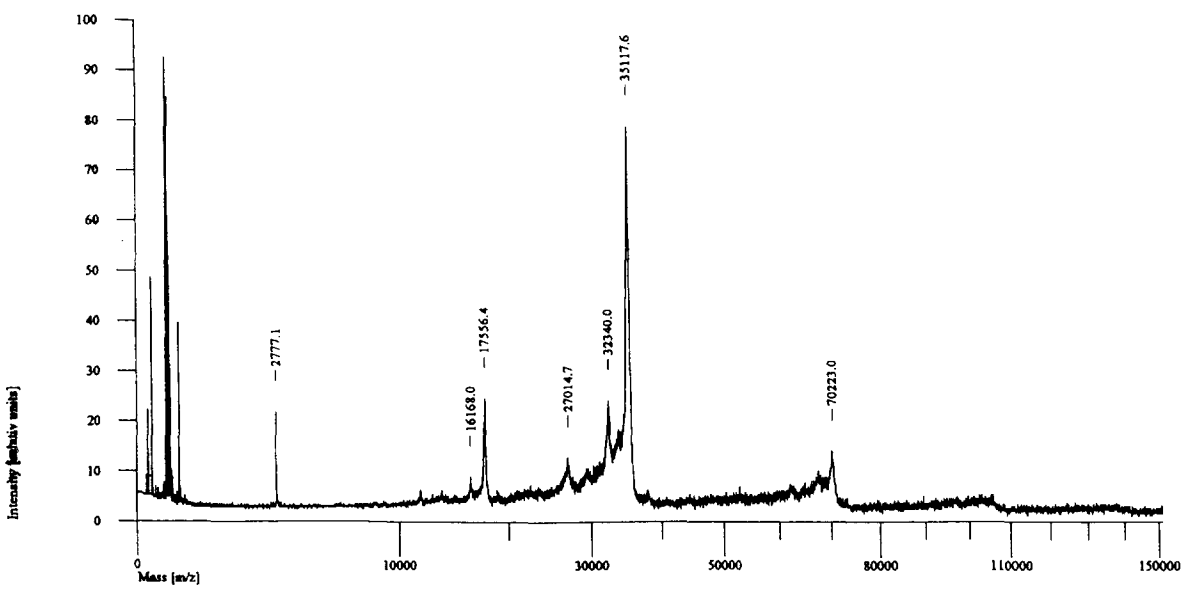

Fig. 3. MALDI-TOF mass spectrum of BSA-FG75.

Table 1
Protein coverages of BSA- and BSA fragment-bonded columns

Packing	Pore size of base silica (Å)	Protein phase sulfur content (%)[a]	Surface coverage (μmol/g)
BSA	120	0.24	1.9
BSA	300	0.16	1.3
BSA-FG75	120	0.34	5.5

[a] Calculated from the elemental analysis data for sulfur.

that of the intact BSA. Also, the effect might be due to the shape of the BSA-FG75 being different from that of the intact BSA.

3.3. Comparison of retention and enantioselectivity of racemic solutes on BSA and BSA-FG75 columns

Tables 2 and 3 compare the retentions and enantioselectivities of racemic solutes on the BSA and BSA-FG75 columns. Benzodiazepine derivatives, benzoin, warfarin and 2-arylpropionic acid derivatives were resolved on the BSA-FG75 column, but tryptophan and kynurenine were not resolved (data not shown). Andersson et al. [12] reported that no chiral recognition of warfarin, tryptophan or kynurenine was obtained on a BSA fragment column. The differences between our and Andersson et al.'s methods are as follows: (1) we isolated the BSA fragment by size-exclusion chromatography, whereas Andersson et al. used anion-exchange chromatography; (2) we bound the BSA fragment to aminopropylsilica gels activated by DSC, whereas they cross-linked it into aminopropylsilica gels by glutaraldehyde or adsorbed it on silica gels; and (3) the surface coverages of the BSA fragments were 5.5 and 4 μmol/g in our and their methods, respectively. However, it is not known why warfarin was separated on our BSA fragment column but not on that of Andersson et al. [12]. Also, our BSA fragment column can resolve 2-arylpropionic acid derivatives, whose separations were not examined by Andersson et al.

With regard to the comparison of the retentions and enantioselectivities of racemates on the BSA and BSA-FG75 columns, the latter column gave longer retentions for benzoin and benzodi-

Table 2
Optical resolution of benzodiazepines, benzoin and warfarin on BSA- and BSA fragment-bonded columns

Compound	Column			
	BSA		BSA-FG75	
	k'_1	α	k'_1	α
Oxazepam	3.13	3.94	6.60	2.53
Lorazepam	5.30	2.01	13.5	2.34
Temazepam	2.43	1.83	4.63	1.05
Lormetazepam	4.98	1.41	9.96	1.13
Clorazepate	3.25	1.94	3.33	1.33
Benzoin	1.89	1.46	3.56	2.56
Warfarin	14.9	1.59	4.44	1.21

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

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

Compound	Column			
	BSA		BSA-FG75	
	k'_1	α	k'_1	α
Ketoprofen	0.68	1.00	0.65	1.00
Ibuprofen	2.30	1.91	1.42	1.40
Fenoprofen	1.81	1.00	1.56	1.16
Pranoprofen	0.57	1.00	0.33	1.00
Flurbiprofen	4.74	1.30	2.00	1.16

HPLC conditions: column, 100 mm × 2.1 mm I.D.; eluent, 50 m*M* phosphate buffer (pH 6.9)–1-propanol (85:15, v/v) containing 4 m*M* octanoic acid; column temperature, 25°C; flow-rate, 0.2 ml/min; detection wavelength, 254 or 220 nm.

azepines, except for clorazepate, and gave higher enantioselectivities for lorazepam and benzoin, as shown in Table 2. Fig. 4A and B show chromatograms of lorazepam on BSA and BSA-FG75 columns, respectively. As shown in Table 3, fenoprofen was resolved on the BSA-FG75 column but was not resolved on the intact BSA column. These results reveal that the binding sites for tryptophan and kynurenine have been lost or otherwise affected, but that the binding site(s) for other compounds tested have been preserved throughout enzymatic cleavage or isolation. The higher enantioselectivities of lorazepam, benzoin and fenoprofen are ascribable to a higher protein density, that is, an increase in the number of chiral recognition site(s) for lorazepam, benzoin and fenoprofen.

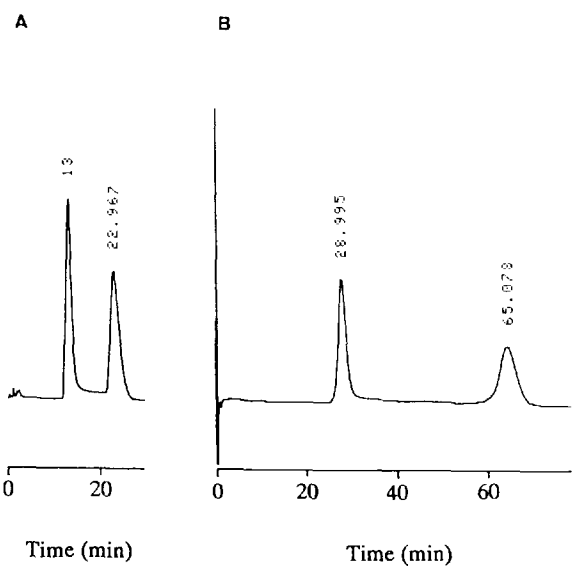

Fig. 4. Chromatograms of lorazepam on the (A) BSA and (B) BSA-FG75 columns. HPLC conditions: column, 100 mm × 2.1 mm I.D.; eluent, 50 m*M* phosphate buffer (pH 7.5)–1-propanol (96:4, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection wavelength, 254 nm; amount injected, 0.1 μg each.

3.4. Comparison of loadability of BSA and BSA-FG75 columns

Table 4 shows the influence of the amounts of benzoin loaded on the capacity factor (k'), peak symmetry (η), enantioselectivity (α) and resolution (R_s) on the BSA and BSA-FG75 columns. Fig. 5 depicts the influence of the amounts of benzoin loaded on peak symmetry (η). The slight decrease in α values was observed as an increase in the amounts loaded for both the BSA and BSA fragment columns, while a marked decrease in the R_s values occurred. The η value of the second-eluted peak on the BSA column was not calculated because of overlapping with the first-eluted peak. The η value for a 1.0-nmol injection on to the BSA column was almost the same with that for a 5.0-nmol injection on to the BSA-FG75 column. Also, the value for a 2.5-nmol injection on to the BSA column was almost the same as that for a 10-nmol injection on to the BSA-FG75 column. Fig. 6A and B show chro-

Table 4
Influence of amount of benzoin loaded on capacity factor (k'), peak symmetry (η), enantioselectivity (α) and resolution (R_s) on BSA- and BSA fragment-bonded columns

Amount injected (nmol)	Packing	k'_1	k'_2	η_1	η_2	α	R_s
0.125	BSA	1.91	2.88	1.03	1.00	1.51	2.30
	BSA-FG75	2.90	6.36	1.03	0.98	2.19	5.98
0.5	BSA	1.90	2.83	1.06	1.22	1.49	1.96
	BSA-FG75	2.90	6.31	1.15	1.02	2.18	5.61
1.0	BSA	1.89	2.80	1.57	1.43	1.48	1.88
	BSA-FG75	2.88	6.22	1.25	1.03	2.16	5.39
2.5	BSA	1.87	2.70	1.60	1.53	1.44	1.67
	BSA-FG75	2.83	5.98	1.42	1.14	2.11	4.16
5.0	BSA	1.83	2.57	1.71	1.95	1.41	1.36
	BSA-FG75	2.74	5.61	1.55	1.32	2.05	3.13
10	BSA	1.79	2.39	2.13	2.00	1.34	1.05
	BSA-FG75	2.68	5.25	1.64	1.66	1.96	2.35
20	BSA	1.73	2.25	2.17	–	1.30	0.78
	BSA-FG75	2.58	4.97	1.66	1.84	1.93	1.94

k'_1 and k'_2 are the capacity factors of the first- and second-eluted benzoin enantiomers, respectively. η_1 and η_2 are the asymmetry factors of the first- and second-eluted benzoin enantiomers, respectively. HPLC conditions: column, 100 mm × 2.1 mm I.D.; eluent, 50 m*M* phosphate buffer (pH 7.5)–1-propanol (96:4, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection wavelength, 254 nm.

matograms of benzoin for 10-nmol injections on to the BSA and BSA-FG75 columns, respectively. These results reveal that the BSA-FG75 column has a higher capacity for benzoin. This is due to an increase in the number of chiral recognition site(s) for benzoin on the BSA-FG75 column compared with the intact BSA column. Andersson et al. [12] reported that the BSA fragment column showed a lower loadability for

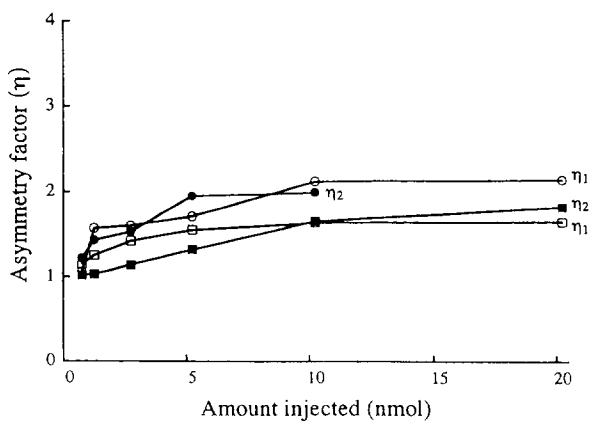

Fig. 5. Influence of the amounts of benzoin loaded on peak symmetry (η). ● = BSA; ■ = BSA-FG75. HPLC conditions as in Table 4.

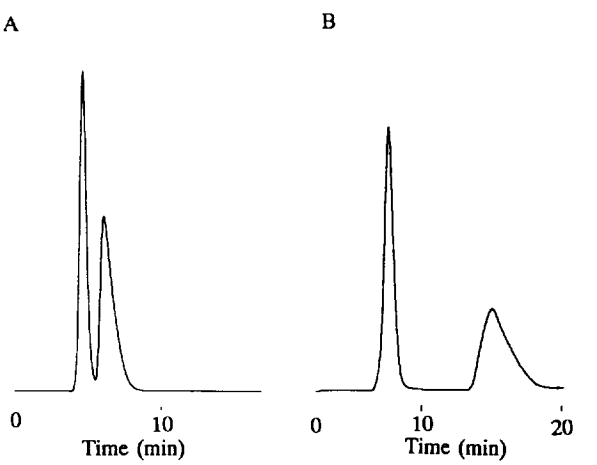

Fig. 6. Chromatograms of benzoin for 10-nmol injections on to (A) the BSA and (B) the BSA-FG75 columns. HPLC conditions as in Table 4.

Table 5
Changes in capacity factor (k'_1), enantioselectivity (α) and resolution (R_s) of racemic solutes with continuous flow of eluent

Solute	Parameter	BSA column		BSA-FG75 column	
		Before	After 200 h	Before	After 200 h
Lorazepam	k'_1	5.30	6.14	13.5	13.5
	α	2.01	1.73	2.34	2.28
	R_s	3.39	3.07	7.04	6.76
Oxazepam	k'_1	3.13	3.58	6.60	6.79
	α	3.94	3.22	2.53	2.37
	R_s	6.69	5.29	6.73	6.44
Clorazepate	k'_1	6.93	6.17	3.33	2.85
	α	2.85	2.88	1.33	1.39
	R_s	4.36	5.46	1.75	1.72
Benzoin	k'_1	1.89	1.99	3.57	3.52
	α	1.46	1.59	2.56	2.51
	R_s	1.40	1.88	6.44	5.23

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

benzoin than the intact BSA column. As described above, there are many differences between our and Andersson et al.'s methods. It is not known why the BSA-FG75 column gave a higher loadability, which is different from the result of Andersson et al. [12]. However, we can only say that the increased number of chiral recognition site(s) for benzoin was preserved on the BSA-FG75 column.

3.5. Stability of BSA fragment column

Table 5 shows the changes in capacity factor (k'_1), enantioselectivity (α) and resolution (R_s) of racemic solutes with a continuous flow of eluent, where 50 m*M* phosphate buffer (pH 7.5)–1-propanol (96:4, v/v) was used. There were slight changes in the k'_1, α and R_s values for both columns before and after a continuous flow of the eluent. It was found that the BSA-FG75 column is stable to the continuous flow of the eluent. Andersson et al. [12] reported that the performance of the BSA fragment column deteriorated significantly after the use of buffers with a high alkanol content. However, our results suggest that the BSA-FG75 column should be as stable as the intact BSA column.

4. Conclusion

A BSA fragment (BSA-FG75), isolated by size-exclusion chromatography followed by peptic digestion of BSA, had an average molecular mass of about 35 000. The intact BSA column gave higher enantioselectivities for most of the compounds tested than the BSA-FG75 column, whereas the BSA-FG75 column gave higher enantioselectivities for lorazepam and benzoin than the intact BSA column, and had a higher capacity for benzoin. These results are ascribable to the higher density of chiral recognition site(s) on the BSA-FG75 column. Also, the BSA-FG75 column was as stable as the intact BSA column for a continuous flow of eluent.

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