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USE OF A FRAGMENT OF BOVINE SERUM ALBUMIN AS A CHIRAL STATIONARY PHASE IN LIQUID CHROMATOGRAPHY

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SUMMARY

Bovine serum albumin (BSA) was cleaved into fragments by proteolytic degradation. A fragment consisting of the N-terminal half of BSA was isolated and immobilized on a silica column. Compared with BSA-based columns, this column showed a retained stereoselective resolving capability for the enantiomers of oxazepam, benzoin and morpholep, whereas the enantiomers of, *e.g.*, tryptophan and warfarin were not resolved.

INTRODUCTION

A large number of chiral selectors have been used for the direct resolution of enantiomers. Owing to their generality and often high enantioselectivity, a number of protein-based phases have been utilized as chiral selectors in liquid chromatography for the direct resolution of enantiomers[1,2]. A large number of basic drugs have, for instance, been resolved on columns containing α_1-acid glycoprotein (α_1-AGP, orosmucoid)[3]. Bovine serum albumin (BSA) has been studied by Stewart and Doherty[4] and later by Allenmark *et al.*[5] as chiral selector. Allenmark and co-workers managed to immobilize BSA covalently on silica[6], and also to immobilize BSA by cross-linking it on silica[7]. These materials have been used successfully as chiral stationary phases to resolve a variety of compounds. Human serum albumin has been adopted as a chiral additive in the mobile phase by Sébille and Thuaud[8] and by Pettersson *et al.*[9]. Both α_1-AGP- and BSA-based columns are commercially available (α_1-AGP-based columns, EnantioPac from Pharmacia, Uppsala, Sweden, and Chiral-AGP from ChromTech, Stockholm, Sweden; BSA-based column; Resolvosil from Macherey, Nagel & Co., Düren, F.R.G.). Some other proteins, *e.g.*, immunoglobulin G (IgG)[10], ovomucoid[11] and α-chymotrypsin[12], have also been used as chiral selectors in liquid

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chromatography. However, columns based on α_1-AGP and BSA dominate the reported applications. Major drawbacks of proteins as chiral selectors in liquid chromatographic systems are low efficiency, which causes low sensitivity in analytical applications, and low loadability, making these phases less useful in preparative work[13]. The main reason for the low loadability seems to be that for each analyte there is only one or a few stereoselective sites on the large protein molecule, leading to a low concentration of stereoselective sites in the column. Owing to the physical size of some proteins, supports with large pores, *e.g.*, 300 Å silica, are necessary. Such silicas have a lower specific surface area, *ca.* 100 m^2/g for a 300 Å pore size compared with a 300 m^2/g for the "standard" pore size of 100 Å. This limits the amount of chiral selector that can be immobilized per unit amount of silica and consequently also the loadability. A low efficiency may be due to the time needed for conformational changes of the protein in the binding and releasing steps. A smaller mass of the protein would require a shorter time to make the necessary conformational motions. It is also difficult to predict chiral recognition on these phases because the mechanism of interaction is not known on a molecular level.

To the best of our knowledge, only intact protein molecules have been used as chiral selectors in liquid chromatography. However, different immobilization techniques affect the properties of the stationary phase[14], and consequently also the condition of the protein. Different pathways are accessible for modifying the proteins and hopefully for improving their chromatographic performance, such as (a) chemical modifications, *e.g.*, altering the side-chains of the amino acids residues to change charge distribution, hydrophilicy and bulkiness, etc., (b) using biosynthetic analogues, which involves a change in the primary amino acid sequence, *e.g.*, by substituting, inserting or deleting amino acids; this may be done by changing the DNA sequence by genetic engineering or by using the species-dependent variance in the amino acid sequence; (c) selecting specified parts of the protein by chemical or enzymatic cleavage to eliminate parts which do not contribute to chiral recognition; or (d) by peptide synthesis. If sufficiently small proteolytic peptides can be obtained which still show stereoselectivity, synthetic analogues could be produced. Another possibility for producing stereoselective proteins is to carry out "*in vivo* printing", which results in polyclonal or monoclonal antibodies directed towards the chiral substrate[10,15].

The primary aim of this project was to study the mechanism of the chiral selectivity of serum albumins and locate stereospecific binding sites on the protein. This was studied by cleavage of albumin into smaller peptide fragments followed by isolation, characterization and use of these fragments as chiral selectors. Also, owing to the decreased size of the chiral selector, the number of selectors immobilized in a column would be increased, leading to a higher loadability. If albumin fragments that consist of one domain (see Fig. 1) or part of a domain show retained stereoselectivity, these are hopefully sufficiently rigid to allow the determination of the three-dimensional structure by X-ray crystallography. This would provide the possibility of performing molecular modelling of the stereoselective docking of the chiral substrate to serum albumin by computer graphics.

BSA was chosen because it had been used as a chiral selector in liquid chromatography, is easily immobilized on silica, inexpensive, readily available and robust and its amino acid sequence is known. The amino acid sequence of BSA has been

reported by Brown[16]. The BSA molecule is estimated to be 141 Å long and 41 Å in diameter, formed like a cigar (see Fig. 1). It consists of a single peptide chain with 581 amino acids stabilized by 17 disulphide bridges and the molecular weight is approximately 66 500. The molecule is organized in three domains which are very flexible with respect to each other but more rigid internally. Owing to the high flexibility of serum albumin, no unambiguous three-dimensional structure from X-ray crystallography has yet been obtained. The isolation of different fragments from serum albumins and their binding to various ligands has also been reported, and a review of serum albumin has been published by Peters[17].

The purpose of this paper is to show that it is possible to cleave BSA into smaller fragments with retained stereoselectivity and that these fragments may be used as chiral stationary phases in liquid chromatography. Some of these results have been reported previously[18].

EXPERIMENTAL

Materials

Bovine serum albumin (BSA) (A-7030), pepsin porcine (P-6887), *rac*-benzoin, N-benzoyl-DL-alanine, N-benzoyl-D-alanine, N-benzoyl-L-alanine, L-cystine and octanoic acid were obtained from Sigma (St. Louis, MO, U.S.A.), D-tryptophan from United States Biochemical (Cleveland, OH, U.S.A.) and DL-tryptophan and L-tryptophan from Merck (Darmstadt, F.R.G.). *rac*-Warfarin was a gift from Ferrosan (Malmö, Sweden), *rac*-oxazepam from Kabi Vitrum (Stockholm, Sweden) and DL-kynurenine sulphate, L-kynurenine sulphate, *rac*-morpholep, 2,4-dinitrophenyl-DL-glycine and *rac*-mandelic acid from Shalini Andersson (University of Linköping, Sweden).

Proteolytic degradation of BSA

The proteolysis was carried out according by the guidelines given in ref. 19, and was optimized to give a reasonable yield of one large peptide under the condition that no or only a very small amount of BSA remained, as estimated from sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography.

In procedure I, 3.0 g of BSA was dissolved in 100 ml of buffer A (0.10 *M* Tris–HCl, pH 7.96). L-Cystine (70 mg) was first dissolved in 1.5 ml of 1 *M* NaOH and

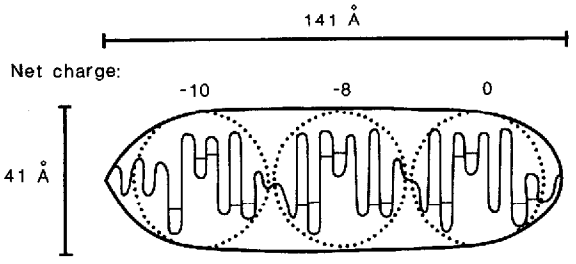


Fig. 1. Model of the serum albumin molecule, based on physical properties. The amino terminus is at the left. The net charges shown above the three domains are for bovine albumin at pH 7. Reprinted from T. Peters, Jr., *Adv. Protein Chem.*, 37 (1985) 161–245, with permission.

then immediately diluted to 140 ml with buffer A. The disulphide bonds of BSA were reduced by mixing the BSA and the cystine solutions and the reaction solution was allowed to stand for 17 h in room temperature (25°C). A 2-ml volume of the BSA–cystine solution was concentrated to approximately 200 μl in a Centricon 30 microconcentrator (Amicon, Danvers, MA, U.S.A.), and the concentrated BSA solution was then diluted with 1.6 ml of buffer B (0.1 *M* ammonium formate in HCl–3.2 m*M* octanoic acid, pH 3.7) and 50 μl of 1 *M* HCl to saturate hydrophobic sites and adjust the pH. A 100-μl volume of 0.5 mg/ml pepsin in buffer B was added and the solution was treated in a ultrasonic bath for 1 min. The reaction vessel was then immersed in a thermostatic water-bath (37°C) with occasional gentle swirling for 30 min. The proteolysis was stopped by adding 300 μl of 2 *M* Tris and 4 ml of water.

Procedure II was identical with procedure I, but all volumes were increased 100-fold and the concentration of the pepsin solution was reduced to 0.2 mg/ml.

Chromatography

Potassium phosphate buffer and ammonium carbonate buffer mobile phases were prepared in Millli-Q grade water obtained by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.). In some instances, 1-propanol, analytical-reagent grade (Merck, Darmstadt, F.R.G.) was used as an organic modifier. All mobile phases were degassed and filtered through a 0.45-μm HV Millipore filter prior to use. Unless stated otherwise, the following chromatographic set-up was used; a Philips PU4003 solvent delivery system (Pye Unicam, Cambridge, U.K.) equipped with a Philips PU4025 UV detector with a 1-μl flow-cell. The detector was connected to a Philips PM8252 strip-chart recorder. Samples were injected with a Rheodyne (Cotati, CA, U.S.A.). Model 7520 valve injector equipped with a 0.5-μl internal loop. Larger volumes were injected with a Rheodyne Model 7125 valve injector equipped with external loops of various sizes (10 μl–30 ml). Eluates from the columns were collected by a Gilson (Villiers-le-Bel, France) Model 201 or 203 fraction collector.

The volumes of unretarded solutes used for calculating the capacity factor (k') and separation factor (α) were determined by injection of sodium nitrite or water or by observing the first baseline disturbance. The resolution (R_s) was calculated according to: $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where t_R = retention time and w = peak width at baseline[20].

Isolation of peptide

Procedure I. The albumin fragment mixture was first concentrated and desalted on Centricon 30 microconcentrators and then subjected to an anion-exchange separation using a Mono-Q HR 5/5 anion-exchange column (Pharmacia, Uppsala, Sweden). A conductivity meter, Type CDM 2d (Radiometer, Copenhagen, Denmark), was employed to monitor the salt gradient. The conductivity cell, consisting of two platinum capillaries separated by a short plastic tube, was connected to the outlet of the UV detector. The experimental conditions are given in Fig. 4. The fractions of interest were concentrated on a Centricon 30 microconcentrator and further purified by size-exclusion chromatography using a Superose 12 HR 10/30 column (Pharmacia) and the conditions given in Fig. 5.

Procedure II. Procedure I was modified to increase the volumes handled. The

concentration steps were carried out using a 200-ml Amicon stirred ultrafiltration cell with Millipore PLGC (10 000 nominal molecular weight limit) regenerated-cellulose low-binding membranes (Millipore) operated at 0.17 MPa (25 p.s.i.). A larger anion exchange column, Mono-Q HR 10/10, and a larger size-exclusion column (Sephadex) were used for the ion-exchange and size-exclusion steps, respectively. Before use, Sephadex G-75 Superfine gel (Pharmacia) was swollen in 0.05 *M* ammonium hydrogencarbonate solution (pH 7.8) to a thick slurry and then packed into a 100 cm × 5 cm I.D. column according to ref. 21. A Gilson Minipuls 2 peristaltic pump (Gilson) with 0.090-in. I.D. pump tube (Elkay Laboratory Products, Basingstoke, U.K.), an LDC Spectromonitor III UV detector using the reference cell (to reduce the pressure drop across the detector cell) and a Servogor SE 120 strip-chart recorder was used in the chromatographic set-up for the size-exclusion step. The conditions are given in Fig. 8.

Electrophoresis

Electrophoresis equipment suitable for use with precast 5 cm × 5 cm PhastSystem gels from Pharmacia was constructed in cooperation with R. Berglund (Department of Technical Analytical Chemistry, University of Lund, Lund, Sweden). SDS-PAGE[22] in the presence of β-mercaptoethanol was performed as described in ref. 23 with PhastGel SDS buffer strips (Pharmacia). The samples were applied using a PhastGel 8/1 sample applicator (Pharmacia) on a PhastGel 10–25 gel gradient (Pharmacia). The gels were stained with Coomassie Brillant Blue (Carl Roth, Karlsruhe, F.R.G.). Pharmacia LMW calibration kit proteins were used for the molecular weight determination (Phosphorylase b 94 000, BSA 67 000, ovalbumin 43 000, carbonic anhydrase 30 000, trypsin inhibitor 20 100, α-lactalbumin 14 400).

Amino acid sequencing

The amino acid sequences were determined at the Institution for Clinical Chemistry, Malmö General Hospital[24]. A Model 470A gas-phase sequencer from Applied Biosystems was used with on-line determination of PTH-amino acids, utilizing a Model 120A PTH analyser from Applied Biosystems.

Preparation of silica columns

The columns were prepared by packing Nucleosil silica (5 μm, 300 Å) obtained from Macherey, Nagel & Co. (Düren, F.R.G.) into stainless-steel 100 mm × 1.6 mm I.D. Nova-HP tubing (Annel, Stockholm, Sweden), using an descending slurry packing technique. A 0.3-g amount of silica was suspended in chloroform–methanol (2:1) and poured into a 75-ml packing bomb. The slurry was packed into the column at 300 bar using methanol as the displacing medium.

Preparative resolution of benzoin

Optically enriched fractions of benzoin, obtained by chromatography on microcrystalline triacetylcellulose (TAC), were used to determine the elution order of the enantiomers. The general preparative procedure is described in ref. 25. A 1-ml volume of a saturated benzoin solution [<10 mg/ml in ethanol–water (95:5)] was injected with a Rheodyne 7126 injector equipped with a 5-ml loop on two 600 mm × 10 mm I.D. columns connected in series. The columns were packed with ConbrioTAC, (15–25 μm) obtained from Perstorp Biolytica (Lund, Sweden) and eluted with etha-

nol–water (95:5) using a Beckman (San Ramon, CA, U.S.A.) 110B pump at 1 ml/min. UV-detection was performed with an LKB Uvicord S 2138 detector (Pharmacia) operated at 224 nm and polarimetric detection with a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 241 MC polarimeter operated at 436 nm.

Immobilization of BSA and peptides

The mixture of peptides obtained by proteolytic degradation of BSA, the purified peptide and BSA was immobilized in 100 mm × 1.6 mm I.D. silica columns. Potassium phosphate and phosphoric acid was added to the solution of peptide or BSA to obtain 0.5 *M* phosphate and pH 5.0. The solution was pumped into the column until a breakthrough detected by the UV detector at 280 nm was observed[13].

RESULTS AND DISCUSSION

Proteolytic degradation

The proteolytic degradation produced several peptides of various sizes, as can be seen from SDS-PAGE (Fig. 2) and size-exclusion chromatography (Fig. 3). It was found that experimental conditions such as concentration of pepsin and BSA, the degree of cystination, time for proteolysis, etc., had a great influence on the cleavage pattern. The volume of the reaction vessel also influenced the proteolysis, making a decrease in the concentration of pepsin necessary to retain the cleavage pattern in the scaled-up procedure.

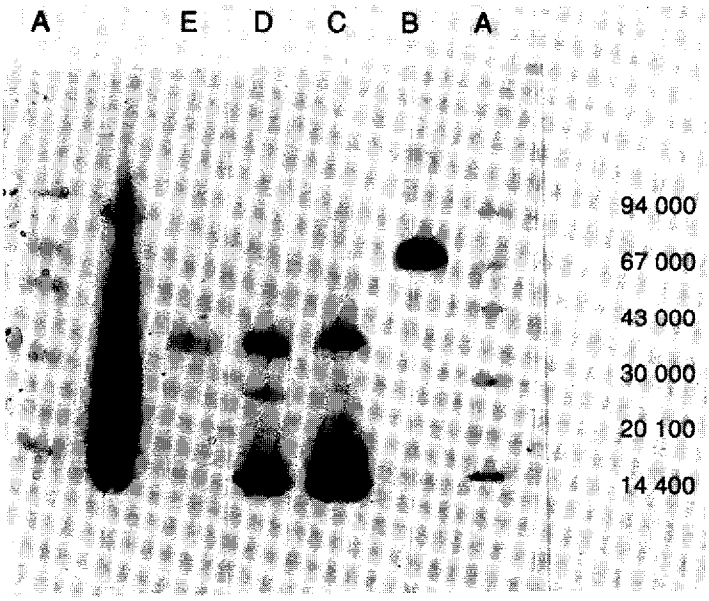


Fig. 2. Electrophoresis separation of samples from different steps of isolation procedure I. Lane A, markers of molecular weight; lane B, intact BSA; lane C, mixture of peptides obtained by proteolysis of BSA (see Fig. 3), these peptides were immobilized on peptide column I; lane D, peptides in the last eluting peak from the anion-exchange column (see Fig. 4); lane E, peptide in the last eluting peak from the size-exclusion step (see Fig. 5), the last peptide was immobilized on peptide column II.

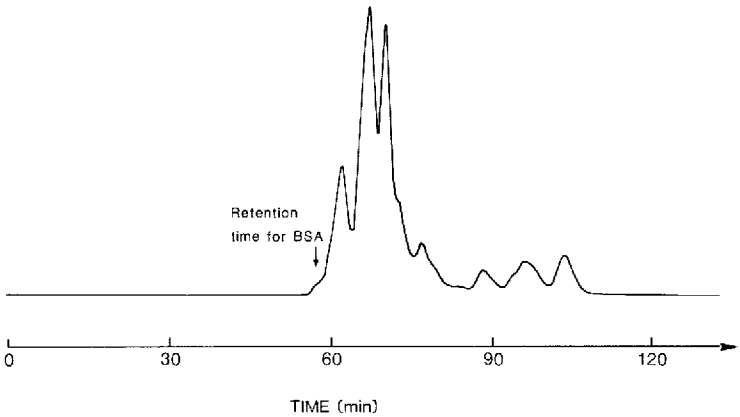


Fig. 3. Size-exclusion chromatography of albumin fragments obtained by proteolytic degradation. Pharmacia Superose 12 HR 10/30 column; eluent, 0.05 *M* NH_4HCO_3 (pH 7.8); flow-rate 0.2 ml/min; UV detection at 280 nm; 0.1 ml injected.

Isolation of peptide

The largest peptide, with an M_r of 38 000, as determined by SDS-PAGE, eluted as the last band during the ion-exchange chromatography according to procedure I (Fig. 4). This indicated that the peptide contained regions with several negative charges. This last band also contained a peptide of M_r 27 000. The larger peptide was further purified by size-exclusion chromatography (Fig. 5), and the results from the

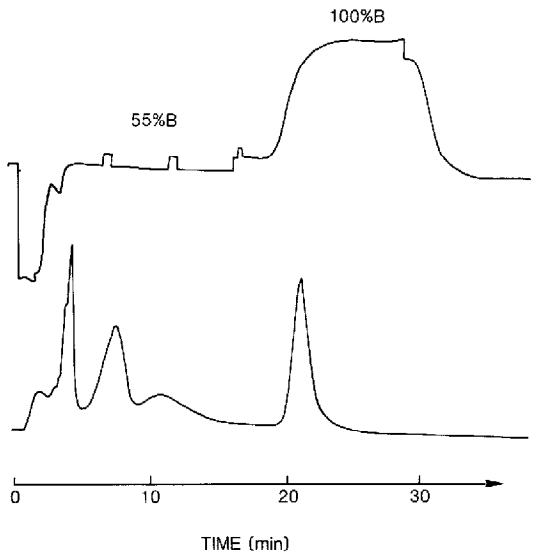


Fig. 4. Ion-exchange separation of albumin fragments according to procedure I. Pharmacia Mono Q HR 5/5 column; eluent A, 0.05 *M* NH_4HCO_3 (pH 7.8); eluent B, 0.05 *M* NH_4HCO_3 (pH 7.8)–0.3 *M* NaCl; flow-rate, 1.0 ml/min; 2 ml injected. The upper trace shows conductimetric detection and the lower trace UV detection at 280 nm.

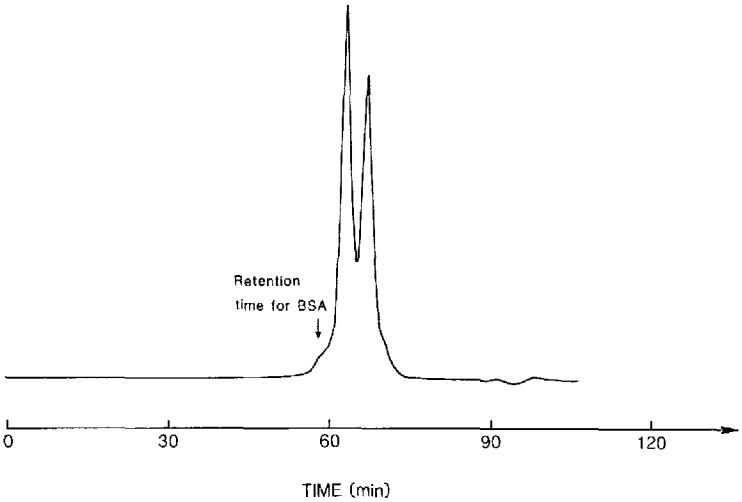


Fig. 5. Size-exclusion chromatography of the last-eluting peak from the ion-exchange separation of albumin fragments according to procedure I (see Fig. 4). Conditions as in Fig. 3. The first peak contained the peptide with M_r 38 000 and the second peak the peptide with M_r 27 000.

amino sequence analysis showed that the peptide consisted of the N-terminal part of BSA starting with amino acid 1 (Asp) (Table I). Owing to the limited accuracy inherent in the electrophoretic analysis, it was not possible to determine exactly the site of cleavage.

The larger anion-exchange and size-exclusion columns used in procedure II showed a lower separation capability (see Fig. 8) than the columns utilized in procedure I. Therefore, the purity of the peptide obtained by procedure II was not as good as that obtained by procedure I.

The amino acid sequence analysis showed that three sequences were present in the peptide solution (Table II). The three peptides are derived from the N-terminal

TABLE I

SEQUENCE ANALYSIS OF THE PEPTIDE SOLUTION OBTAINED BY PROCEDURE I

Amino acid position in BSA	*Identity*	*Yield (nmol)*[a]
1	Asp	0.73
2	Thr	ND
3	His	ND
4	Lys	0.88
5	Ser	ND
6	Glu	0.50
7	Ile	ND
8	Ala	0.76
9	His	0.15
10	Arg	0.15
11	Phe	0.56

[a] ND = Not determined.

TABLE II

SEQUENCE ANALYSIS OF THE PEPTIDES SOLUTION OBTAINED BY PROCEDURE II

Sequence	*Amino acid position in BSA*	*Identity*[a]	*Yield (nmol)*[a]
1	1	Asp	0.20
	2	Thr	0.15
	3	His	0.09
	4	Lys	0.22
	5	Ser	ND
	6	Glu	0.12
	7	Ile	0.13
	8	Ala	0.19
	9	His	0.07
	10	Arg	0.06
	11	Phe	0.12
	12	Lys	0.09
	13	Asp	0.05
	14	Leu	0.08
	15	Gly	0.06
	16	Glu	0.14
2	11	Phe	0.10
	12	Lys	0.03
	13	Asp	0.07
	14	Leu	0.10
3	49	Phe	0.10
	50	Ala	0.16
	51	Lys	0.11
	52	Thr	0.05
	53	ND	ND
	54	Val	0.05
	55	Ala	0.09
	56	Asp	0.04
	57	Glu	0.11
	58	Ser	0.03

[a] ND = Not determined.

half of BSA and contain mainly the same overlapping sequence. Half the amount of the peptides consists of the peptide starting with amino acid 1 (Asp) and the two other peptides starting with amino acid 11 (Phe) and 49 (Phe), representing approximately a quarter each. SDS-PAGE showed that also a small amount (*ca.* 5%) of a larger peptide (M_r 45 000) was present in the peptide solution. The high retention of the peptides on the ion-exchange column is in agreement with the non-uniform distribution of charges of BSA. The calculated net charge at pH 7 decreases from the N-terminal end towards the C-terminal end[17] (see Fig. 1). The isolation of the peptide was very time consuming and difficult to carry out, *e.g.*, owing to time-dependent associations caused by the formation of intermolecular disulphide bonds leading to coelution of different peptides in the size-exclusion step. To increase the loadability of the anion-exchange chromatographic step, attempts were also made to replace the Mono-Q material with cellulose-based ZetaPrep QAE anion exchanger (LKB–Phar-

TABLE III

CHROMATOGRAPHIC DATA FOR PEPTIDE COLUMN I

0.05 *M* phosphate buffers (pH 7.0) were used as mobile phases; 0.05 μg was injected.

Compound	*1-Propanol (%)*	k'_1	k'_2	α	R_s
Tryptophan	0	0.1	0.1	1.0	0
Benzoin	0	0.82	1.6	1.9	1.2
	2	0.61	1.3	2.1	1.5
Oxazepam	2	1.9	3.4	1.8	1.4
Warfarin	2	0.82	0.82	1.0	0

macia). However, the peptides were not as effectively separated as when using the Mono-Q columns. The interactions from the divinylbenzene–polystyrene matrix of the Mono-Q material seemed to promote the selectivity between the peptides.

Evaluation of peptide columns

To confirm that the peptides still had stereoselective properties, the mixture of peptides was immobilized on a silica column (peptide column I), which showed a retained resolving power for benzoin and oxazepam (Table III, Figs. 6, 7). However, the stereoselectivity was lost for tryptophan and warfarin. This showed that one or several peptides had retained stereoselectivity for at least benzoin and oxazepam, and that no intact BSA was responsible for the resolvation of benzoin. The results also indicated that in order to obtain peptides with retained stereoselectivity for, *e.g.*, tryptophan and warfarin, a different cleavage procedure might be necessary, or that such peptides might not have been properly immobilized owing to blockage of the stereoselective sites or not have been immobilized at all on the silica (a small background of UV absorbance was observed during the immobilization procedure).

The peptide obtained by procedure I was immobilized on a silica column (peptide column II). Owing to the limited amount of peptide available, all the peptide injected (*ca.* 10–15 mg) was adsorbed, which means that the column was probably not completely covered. The column showed the same characteristics (Table IV) as that based on the mixture of peptic fragments, *i.e.*, the resolving power for tryptophan and warfarin was lost compared with BSA (see Table V). Benzoin and oxazepam were resolved and showed a better or equal separation (α) compared with intact BSA (Figs. 9, 10). The retention was decreased for all four compounds. The fact that the reten-

TABLE IV

CHROMATOGRAPHIC DATA FOR PEPTIDE COLUMN II

0.05 *M* phosphate buffer (pH 7.0) was used as the mobile phase; 0.05 μg was injected.

Compound	k'_1	k'_2	α	R_s
Tryptophan	0.1	0.1	1.0	0
Benzoin	0.84	2.7	3.1	2.5
Oxazepam	1.6	3.1	1.9	1.2
Warfarin	0.98	0.98	1.0	0

TABLE V

CHROMATOGRAPHIC DATA FOR BSA COLUMN

0.05 *M* phosphate buffers were used as mobile phases; 0.05 μg of benzoin, morpholep, N-benzoylalanine 2,4-dinitrophenylglycine and tryptophan, 0.10 μg of oxazepam, mandelic acid and kynurenine and 2.5 μg of warfarin were injected.

Compound	*pH*	*1-Propanol (%)*	k'_1	k'_2	α	R_s
Benzoin	5.0	0	3.5	6.0	1.7	2.2
	6.0	0	3.0	5.6	1.9	2.4
	7.0	0	2.8	5.7	2.0	2.5
	7.5	0	2.2	3.8	1.7	1.7
	6.0	2	1.2	1.6	1.3	0.9
	7.5	2	1.5	2.1	1.4	1.0
Morpholep	5.0	0	1.0	1.3	1.3	0.8
	6.0	0	0.91	1.1	1.3	0.6
	7.0	0	0.88	1.1	1.2	0.5
	7.5	0	0.54	0.54	1.0	0
	6.0	2	0.46	0.46	1.0	0
	7.5	2	0.47	0.47	1.0	0
Oxazepam	5.0	0	9.0	20.0	2.2	2.1
	6.0	0	8.1	18.7	2.3	2.7
	7.0	0	7.7	15.9	2.0	2.3
	7.5	0	5.3	9.7	1.8	1.9
	6.0	2	3.1	6.4	2.0	2.0
	7.5	2	2.6	5.2	2.0	2.2
N-Benzoylalanine	5.0	0	1.3	2.1	1.6	
	6.0	0	0.63	1.5	2.4	
	7.0	0	0.44	1.7	3.8	
	7.5	0	0.44	1.9	4.4	
	6.0	2	0.53	1.4	2.7	
	7.5	2	0.39	1.3	3.4	
2,4-Dinitrophenylglycine	7.5	0	1.2	4.2	3.6	
	7.5	2	1.1	1.6	1.5	
Mandelic acid	5.0	0	3.0	3.7	1.2	
	6.0	0	0.44	0.62	1.4	
	7.0	0	0.14	0.25	1.8	
	7.5	0	0.12	0.12	1.0	
	6.0	2	0.12	0.12	1.0	
	7.5	2	0.47	0.47	1.0	
Tryptophan	5.0	0	0.29	0.29	1.0	
	6.0	0	0.28	0.32	1.2	
	7.0	0	0.27	0.85	3.2	
	7.5	0	0.32	2.3	7.2	
	6.0	2	0.25	0.31	1.2	
	7.5	2	0.31	1.3	4.3	
Kynurenine	5.0	0	0.24	0.24	1.0	
	6.0	0	0.24	0.32	1.3	
	7.0	0	0.24	1.24	5.2	
	7.5	0	0.29	3.70	13.0	
	6.0	2	0.20	0.20	1.0	
	7.5	2	0.29	2.10	7.3	
Warfarin	5.0	0	51.0	114.0	2.2	
	6.0	0	44.0	60.0	1.4	
	7.0	0	15.0	21.0	1.4	
	6.0	2	3.3	5.0	1.5	
	7.5	2	3.4	5.0	1.5	

TABLE VI

CHROMATOGRAPHIC DATA FOR PEPTIDE COLUMN III

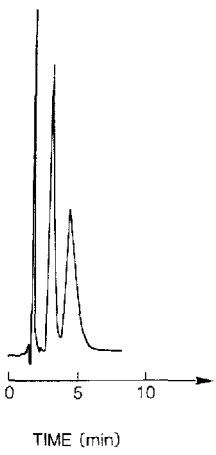
0.05 *M* phosphate buffers were used as mobile phases; 0.05 μg of benzoin, morpholep, N-benzoylalanine, 2,4-dinitrophenylglycine and tryptophan, 0.10 μg of oxazepam, mandelic acid and kynurenine and 2.5 μg of warfarin were injected.

Compound	*pH*	*1-Propanol (%)*	k'_1	k'_2	α	R_s
Benzoin	5.0	0	3.8	6.8	1.8	2.1
	6.0	0	3.0	6.9	2.3	2.3
	7.0	0	2.4	6.3	2.6	2.3
	7.5	0	1.7	4.1	2.4	2.1
	6.0	2	0.66	0.81	1.2	–
	7.5	2	0.82	1.4	1.7	1.0
Morpholep	5.0	0	1.2	1.9	1.6	1.7
	6.0	0	0.75	0.94	1.3	0.4
	7.0	0	0.59	0.59	1.0	0
	7.5	0	0.32	0.32	1.0	0
	6.0	2	0.20	0.20	1.0	0
	7.5	2	0.20	0.20	1.0	0
Oxazepam	5.0	0	10.8	20.4	1.9	1.8
	6.0	0	7.4	5.4	2.1	1.7
	7.0	0	5.7	9.9	1.7	1.2
	7.5	0	3.8	6.0	1.6	0.8
	6.0	2	1.9	2.5	1.3	–
	7.5	2	2.1	3.0	1.5	1.0
N-Benzoylalanine	5.0	0	0.62	0.62	1.0	
	6.0	0	0	0	1.0	
	7.0	0	0	0	1.0	
	7.5	0	0	0	1.0	
	6.0	2	0	0	1.0	
	7.5	2	0	0	1.0	
2,4-Dinitrophenylglycine	7.5	0	0	0	1.0	
	6.0	2	0	0	1.0	
	7.5	2	0	0	1.0	
Mandelic acid	5.0	0	0.41	0.41	1.0	
	6.0	0	0	0	1.0	
	7.0	0	0	0	1.0	
	7.5	2	0	0	1.0	
	6.0	2	0	0	1.0	
Tryptophan	5.0	0	0.35	0.35	1.0	
	6.0	0	0.23	0.23	1.0	
	7.0	0	0.17	0.17	1.0	
	7.5	0	0.17	0.17	1.0	
	7.5	2	0.17	0.17	1.0	
Kynurenine	5.0	0	0.29	0.29	1.0	
	6.0	0	0.24	0.24	1.0	
	7.0	0	0.21	0.21	1.0	
	7.5	0	0.26	0.26	1.0	
Warfarin	5.0	0	30.0	30.0	1.0	
	7.0	0	2.0	2.0	1.0	
	7.5	0	0.71	0.71	1.0	
	7.5	2	0.29	0.29	1.0	
	6.0	2	1.0	1.0	1.0	

tion decreased while the separation was increased or was equal to that with BSA showed that the parts of BSA that gave rise to interactions not contributing to the stereoselectivity had been removed or denatured. It also showed that the proteolytic degradation and the immobilization procedure had not adversely affected the chiral discrimination power for benzoin and oxazepam.

The peptides obtained by procedure II were immobilized on a silica column (peptide column III). The peptide solution contained 0.33 mg/ml of peptide and *ca.* 18 mg were adsorbed. The column showed the same general behaviour as peptide column II (Table VI). The stereoselectivity was lost for all compounds studied except benzoin, oxazepam and morpholep. The separation factors were not as good as those obtained for peptide column II, probably owing to the lower purity of the immobilized peptides.

As a reference, a BSA column was made by immobilizing BSA on a silica column (see Table V, Figs. 11, 12). The BSA solution used contained 0.12 mg/ml of BSA and *ca.* 11 mg of BSA were adsorbed. The elution orders of the benzoin enantiomers on peptide column III and the BSA column were compared by injecting optically enriched fractions of benzoin. The same elution order was observed.



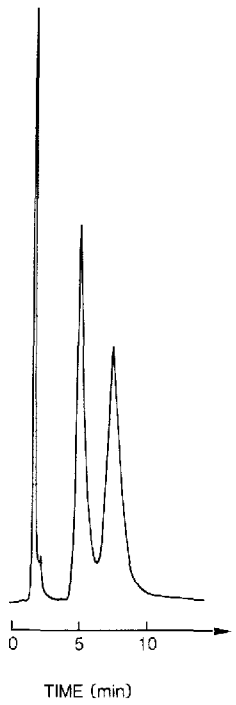


Fig. 6. Resolution of *rac*-benzoin on peptide column I, which contained the mixture of peptides obtained by proteolytic degradation. Column, 100 mm × 1.6 mm I.D.; mobile phase, 0.05 *M* phosphate (pH 7.0); flow-rate 0.1 ml/min; UV detection at 250 nm; 0.05 μg injected in 0.5 μl of methanol. The first peak at the void volume is caused by the injected methanolic solution.

Fig. 7. Resolution of *rac*-oxazepam on peptide column I. Mobile phase, 0.05 *M* phosphate (pH 7.0) containing 2% 1-propanol; flow-rate; 0.1 ml/min; UV detection at 230 nm; 0.05 μg injected in 0.5 μl of methanol.

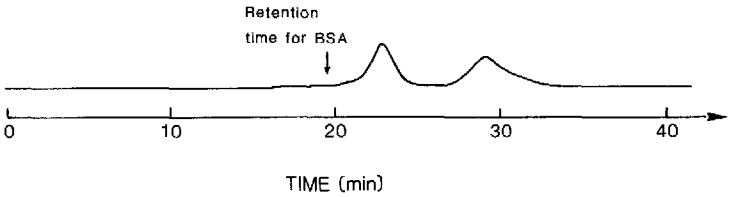
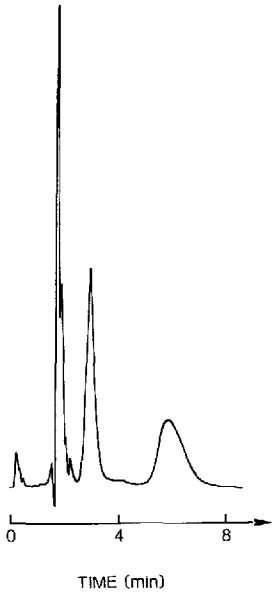


Fig. 8. Size-exclusion chromatography of the last-eluting peak from the ion-exchange separation of albumin fragments according to procedure II. Sephadex G-75 Superfine, 100 × 5 cm column; eluent, 0.05 *M* NH_4HCO_3 (pH 7.8); flow-rate, 0.5 ml/min; UV detection at 280 nm; 12 ml injected. The first peak contained the peptide with M_r 38 000 and the second peak the peptide with M_r 27 000.

A column was also made by immobilizing peptides obtained from procedure II by cross-linking with glutaraldehyde on 3-aminopropylsilica. The results will be reported elsewhere[14].

The binding sites in the immobilized peptide stereoselective to benzoin, oxazepam and morpholep are probably the same as or very similar to those in intact BSA. In theory, although less likely, the cutting of the protein could generate new stereoselective binding sites. The observed loss of steroselectivity for, *e.g.*, tryptophan



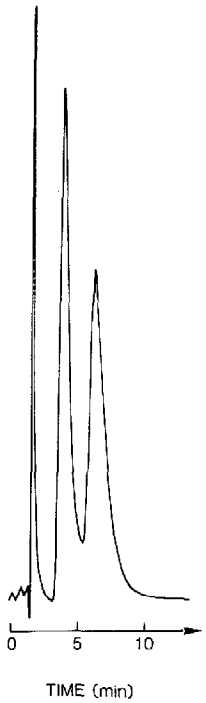


Fig. 9. Resolution of *rac*-benzoin on peptide column II which contained the peptide obtained by isolation procedure I. Conditions as in Fig. 6. A 0.05-μg amount of *rac*-benzoin in 0.5 μl of methanol was injected.

Fig. 10. Resolution of *rac*-oxazepam on peptide column II. Conditions as in Fig. 9. UV detection at 230 nm. A 0.05-μg amount of *rac*-oxazepam in 0.5 μl of methanol was injected.

and warfarin is probably due to removal of the parts of BSA containing the necessary binding sites or due to conformational changes of the immobilized fragment.

The peptide-based columns had a shorter lifetime than the corresponding BSA columns and degraded more rapidly, especially when using 1-propanol as the modifier in the mobile phase. The reason for this instability is not yet known, but may arise from the fact that BSA was cleaved in domain 2, leaving a partial domain which probably disrupts the three-dimensional structure of domain 2 and therefore makes it more susceptible to degradation. Owing to the stability problems encountered, no loadability experiments were performed. The maintained selectivity and decreased retention indicate that a higher productivity should be obtained in preparative resolution of chiral compounds. The time needed to isolate the peptides using procedure I was shorter (weeks) than for procedure II (several months) and the above-mentioned stability problems may have contributed to the lower purity of the peptide obtained by procedure II.

To decrease the amount of peptide needed to cover fully, *e.g.*, a silica column and to reduce the time of the isolation procedure, the use of smaller columns, *e.g.*, packed fused-silica columns with an I.D. of *ca.* 0.2 mm or less, will be used in future work. This will also allow the utilization of small "high-performance" ion-exchange

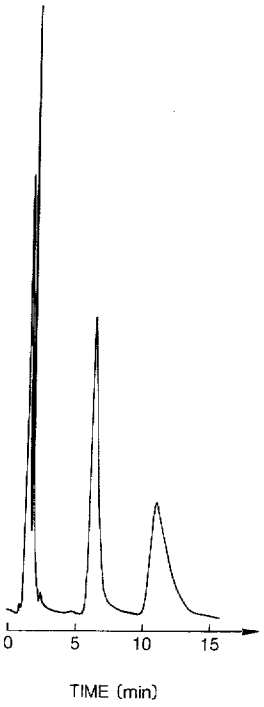


Fig. 11. Resolution of *rac*-benzoin on a 100 mm × 1.6 mm I.D. BSA column. Conditions as in Fig. 6. A 0.05-μg amount of *rac*-benzoin in 0.5 μl of methanol was injected.

Fig. 12. Resolution of *rac*-oxazepam on a 100 mm × 1.6 mm I.D. BSA column. Mobile phase, 0.05 *M* phosphate (pH 7.5); other conditions as in Fig. 10. A 0.10-μg amount of *rac*-oxazepam in 0.5 μl of methanol was injected.

and size-exclusion columns. The preparation of small BSA columns has been suggested[18] and demonstrated by Vindevogel *et al.*[26]. Peptides stereoselective to compounds such as tryptophan and warfarin, which were not successfully resolved by the peptide reported in this paper, may perhaps be isolated from a proteolytic peptide mixture by affinity chromatography. It may also be necessary to cleave the protein in the presence of the ligand in order to protect the binding site during proteolysis.

CONCLUSION

The successful resolution of benzoin, oxazepam and morpholep showed that it is possible to degrade BSA proteolytically into smaller fragments and to immobilize the latter with retained stereoselectivity. Increased or maintained separation factors (α) and decreased capacity factors (k') of the peptide compared with BSA showed that interactions not contributing to the chiral recognition had been removed. It was also concluded that regions stereoselective to benzoin, oxazepam and morpholep should be situated in the N-terminal half of BSA.

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