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Contribution of ionically immobilized bovine serum albumin to the retention of enantiomers

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ABSTRACT

The retention of the enantiomers of mandelic acid and N-benzoylalanine was studied on columns prepared by immobilizing bovine serum albumin (BSA) on an anion exchanger. The amount of BSA fixed on the column is easy to adjust and measure. The adsorption isotherms were determined. For each enantiomer, the isotherm is well accounted for by a bi-Langmuir equation. One term of the isotherm (which is the same for both enantiomers) corresponds to non-selective interactions and the other term to the chiral selective interactions. The column saturation capacity of this second term is 8% larger for the less strongly retained enantiomer. This saturation capacity corresponds approximately to one enantiomer molecule adsorbed for five BSA molecules immobilized. This result is in agreement with the assumption of the hydrophobic cavity of BSA being the chiral selective site.

INTRODUCTION

The study of the behavior of enantiomers in a chiral selective environment has received much attention over the past decade [1,2]. A wide variety of enantiomeric pairs have been resolved on an ever increasing number of chiral stationary phases (CSPs). One class of CSPs is protein immobilized on a solid support, and bovine serum albumin (BSA) is one of these proteins. Three methods of immobilization include covalent bonding to silica [3,4], adsorption on silica [4,5] and ionic immobilization to an anion exchanger [6].

In order to improve the enantioselectivity of a stationary phase, the two options are to increase the density of the enantiomeric discriminator, which improves the selectivity between the antipodes, and to decrease the contribution of the achiral retention which degrades the resolution of a separation without enhancing the selectivity. Usually, these two complement each other, *i.e.*, as the surface density of the discriminator increases, the non-selective interactions decrease. However, an alternative when using chemically bonded silica is end-capping of the residual silanol groups of the support, which has been shown to decrease the total retention while enhancing the resolution [7].

When using an anion exchanger as a support, a varying degree of adsorbed BSA can be obtained via changes in the buffer composition used. With various densities of BSA on the support surface, the retention behavior and the selectivity are altered, allowing the separation of the different contributions toward retention and their study. The system under consideration is the elution of N-benzoyl-DL-alanine and DL-mandelic acid on ionically immobilized BSA.

THEORY

Adsorption isotherm model

The bi-Langmuir isotherm [8,9] is constructed from two Langmuir terms. It has been used previously to describe the adsorption behavior observed when two independent, non-cooperative types of sites coexist on the surface and the solute adsorbs on them with different energies. This model is particularly valid to describe the adsorption of

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enantiomers on a chiral selective stationary phase [10]. The equation of this model is

$$q_x = \frac{q^x_{s,cs} b_{x,cs} C_x}{1 + b_{x,cs} C_x} + \frac{q_{s,ns} b_{ns} C_x}{1 + b_{ns} C_x} \quad (1)$$

The first term of the isotherm (x = L or D) represents the chiral selective interactions responsible for the separation of the enantiomers. The second term accounts for the non-selective interactions affecting the retention of both enantiomers but, as an achiral medium, without favoring either isomer. The chiral selective site has a higher affinity for the enantiomers, which explains the substantial tailing observed at high concentrations.

The saturation capacity, $q_s = a/b$, of the chiral selective site, $q_{s,cs}$, is expected to be similar for the two enantiomers, although steric restrictions due to the geometric configuration could cause slight differences. On the other hand, the saturation capacity of the non-selective sites, $q_{s,ns}$, has to be the same because enantiomers behave identically in achiral environments.

Isotherm determination

The experimental adsorption data to which eqn. 1 is fit for each enantiomer is determined using the method of elution by characteristic point (ECP) [11]. A high-concentration, narrow-plug injection of each enantiomer is eluted from the column and the diffuse, rear boundary of the band profile is monitored. The profile is then converted from detector response to concentration units by direct calibration of the signal. Knowing the retention volume for each mobile phase concentration on the rear boundary of the band profile, the equilibrium concentration of the adsorbed enantiomer is determined from the following differential mass balance equation [12]:

$$q = \int_0^C \frac{V_R - V_0}{V_S} \cdot dC \quad (2)$$

There is substantial agreement between isotherm data derived from the diverse possible methods, and ECP has the advantages of being fast and of requiring a much smaller amount of sample than frontal analysis [12].

EXPERIMENTAL

Equipment

A modular chromatograph was assembled, consisting of a Waters (Milford, MA, USA) Model 510 pump, a Waters gradient controller, a Scientific Systems (State College, PA, USA) LP-21 pulse damper, a Valco (Houston, TX, USA) electric injector with a 50-μl injection loop, a Spectroflow 757 UV detector (Kratos Analytical, Ramsey, NJ, USA) and a Spectra-Physics (San Jose, CA, USA) integrator with a Labnet data acquisition card connected to an IBM AT computer.

Materials

Column. The columns (150 mm × 4.6 mm I.D.) were packed in the laboratory with a quaternary ammonium anion-exchange stationary phase (Vydac, Hesperia, CA, USA) having an average particle size of 10 μm and an average pore diameter of 30 nm.

Chemicals. N-Benzoyl-D-alanine, N-benzoyl-DL-alanine, N-benzoyl-L-alanine, D-mandelic acid, DL-mandelic acid, L-mandelic acid and bovine serum albumin (BSA) (No. A-7638) were purchased from Sigma (St. Louis, MO, USA) and used as received.

Mobile phase. For the pumping of BSA onto the column, the mobile phase was 50 m*M* aqueous phosphate buffer (pH 6.8) for column A and 100 m*M* aqueous phosphate buffer (pH 6.8) for column B. For the elution profiles, the mobile phase was 50 m*M* aqueous phosphate buffer (pH 6.8).

Procedures

After the columns had been packed, BSA was fixed to the anion exchanger by pumping a 2 mg/ml BSA solution onto the column until breakthrough was detected. The mass balance showed that column A had 153.5 mg, column B had 137.8 mg and column C had 0 mg of BSA. Before the elution profiles were measured, the column was equilibrated with *ca.* 100 column volumes of phosphate buffer (50 m*M*, pH 6.8). For the duration of the experiments, the leakage of BSA was negligible. This was checked by measuring the retention volume and the selectivity of the racemic mixtures of mandelic acid and N-benzoylalanine under linear conditions at the start and end of the experiments reported here. No variations of either were observed.

Two sets of elution profiles were obtained. First, the racemic mixtures were injected under linear conditions to determine the capacity factors at infinite dilution, k', and the selectivity, α. Second, the pure enantiomers were injected under overloaded conditions in order to determine their adsorption isotherms by ECP. The elution profiles of the pure enantiomers were converted by direct calibration from the detector response into concentration profiles, the response being the same for two enantiomers. At the wavelengths used, 240 nm for mandelic acid and 280 nm for N-benzoylalanine, the calibration graphs were linear and the regressions were carried out using a standard procedure. A SAS algorithm was used to obtain the best-fit bi-Langmuir parameters by non-linear regression carried out on the University of Tennessee VAX 8800 computer.

RESULTS AND DISCUSSION

Elution profiles

Under linear conditions (Figs. 1 and 2 and Table I), the selectivity of the column for the enantiomers increases with increasing amounts of BSA immobilized in the column. The retention of the less retained enantiomer of each pair, the L-isomer, decreases with increasing amounts of BSA immobilized to the support, while the retention of the more retained enantiomer, the D-isomer, increases. The molecular interaction energy of either L-isomer with the pure anion-exchanger surface (column C) is greater than that of either L-isomer with the BSA molecule (columns A and B). In effect, the BSA molecules are shielding the L-isomer molecules from access to the anion-exchanger surface, causing its retention time and retention factor to decrease (Table I). On the other hand, the D-isomers experience stronger interactions with the BSA molecules than with the anion-exchanger surface. As a consequence, the capacity factors of the D-isomers increase with increasing BSA load (Table I).

Under overloaded conditions (Figs. 3 and 4), a similar behavior is observed. One phenomenon that is not ealisy explained, however, is the crossing of the elution band profiles of the two enantiomers (insets in Figs. 3 and 4). This should be related to

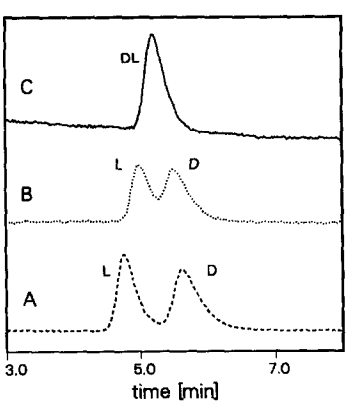

Fig. 1. Chromatograms of DL-mandelic acid resolved under linear conditions for the three columns (A, B and C). For each column, 0.2 nmol of the racemic mixture was injected. Experimental conditions: stationary phase, BSA ionically immobilized on a quaternary ammonium anion exchanger; mobile phase, 50 m*M* phosphate buffer (pH 6.8); flow-rate, 1 ml/min.

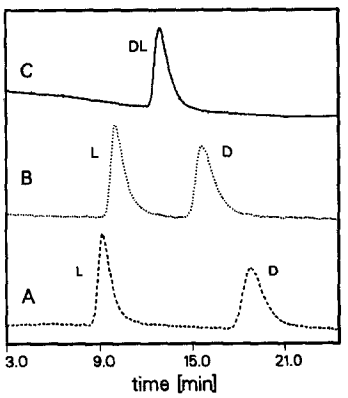

Fig. 2. Chromatograms of N-benzoyl-DL-alanine resolved under linear conditions for the three columns (A, B and C). For each column, 0.2 nmol of the racemic mixture was injected. Experimental conditions as in Fig. 1.

TABLE I

CAPACITY FACTORS AND SELECTIVITIES

Compound	Column	k'_L	k'_D	α
Mandelic acid	A	1.20	1.71	1.43
	B	1.32	1.65	1.25
	C	1.38	1.38	1.00
N-Benzoylalanine	A	3.43	8.08	2.35
	B	3.85	6.53	1.69
	C	4.91	4.91	1.00

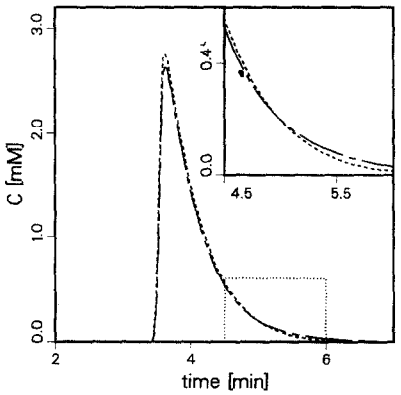

Fig. 3. Overloaded elution profiles of DL-mandelic acid for column B. (– – – –) L-Isomer (amount injected, 1.81 μmol); (– · – · –) D-isomer (amount injected, 1.74 μmol). Experimental conditions as in Fig. 1. Inset: enlargement of dotted region showing the crossing of the band profiles.

the difference observed between the saturation capacities of the selective sites for the two enantiomers and is discussed in the next section.

If non-selective molecular interaction with the surface of the anion exchanger are considered to make the dominant contribution to the enantiomer retention, this contribution should decrease in addition to the column capacity when the density of the BSA immobilized on the surface increases. If we compare the overloaded elution profiles recorded on the three columns (Figs. 5 and 6), it is clear that

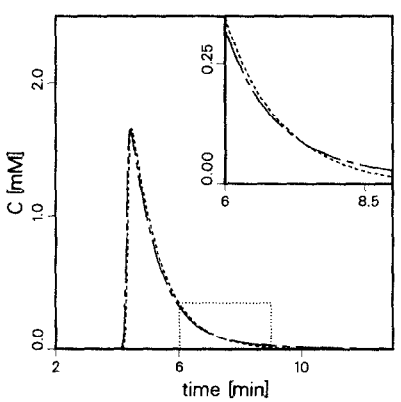

Fig. 4. Overloaded elution profiles of N-benzoyl-DL-alanine for column B. (– – – –) L-Isomer (amount injected, 1.90 μmol); (– · – · –) D-isomer (amount injected, 1.92 μmol). Experimental conditions as in Fig. 1. Inset: enlargement of dotted region showing the crossing of the band profiles.

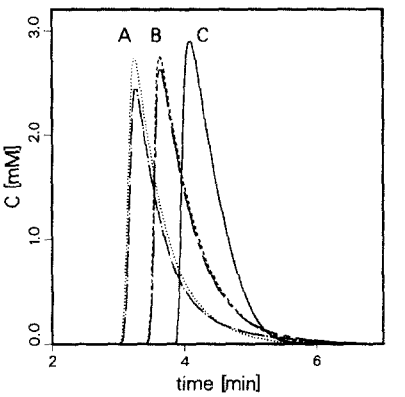

Fig. 5. Overloaded elution profiles of DL-mandelic acid for the three columns. Column A: (· · · · ·) L-isomer (amount injected, 1.69 μmol); (– · – · –) D-isomer (amount injected, 1.52 μmol). Column B: (– – – –) L-isomer (amount injected, 1.81 μmol); (– · – · –)D-isomer (amount injected, 1.74 μmol). Column C: (———) (amount injected, 1.79 μmol). Experimental conditions as in Fig. 1.

this is realized as the fronts of the overloaded peaks of comparable amounts injected elute earlier as the amount of BSA increases in the column.

Adsorption isotherms

The adsorption data (Figs. 7 and 8) for both pairs of enantiomers on the three columns were deter-

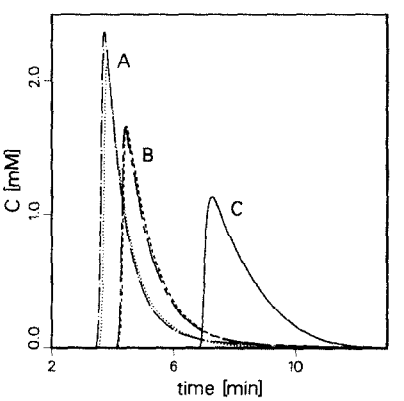

Fig. 6. Overloaded elution profiles of N-benzoyl-DL-alanine for the three columns. Column A: (· · · · ·) L-isomer (amount injected, 2.00 μmol); (– · – · –) D-isomer (amount injected, 2.23 μmol). Column B: (– – –) L-isomer (amount injected, 1.90 μmol); (– · – · –) D-isomer (amount injected, 1.92 μmol). Column C: (———) (amount injected, 1.93 μmol). Experimental conditions as in Fig. 1.

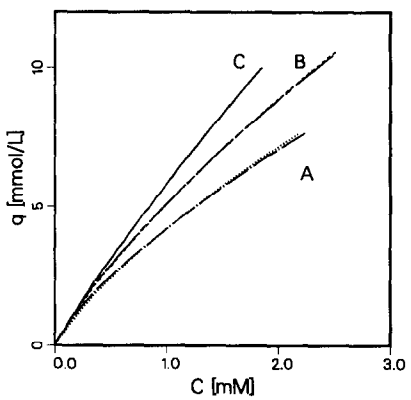

Fig. 7. Adsorption isotherms of DL-mandelic acid determined from the corresponding elution profiles in Fig. 5. Column A: (· · · · ·) L-isomer; (— · — · —) D-isomer. Column B: (— — —) L-isomer; (— - — - —) D-isomer. Column C: (———). For best-fit bi-Langmuir parameters, see Table II.

mined using the ECP method (eqn. 2) and the band profiles in Figs. 5 and 6. The best-fit bi-Langmuir isotherm parameters were calculated from the data for columns A and B, using this isotherm (eqn. 1) as the model. For column C, only a single Langmuir expression was used for the fit of the adsorption data. The values obtained for the parameters are listed in Tables II (mandelic acid) and III (N-benzoylalanine). As the loading of the BSA increases, the initial slope of the selective term of the bi-Langmuir isotherms increases, showing the effect of the protein on the retention of the enantiomers. On the other hand, the initial slope of the non-selective term of the isotherm decreases, demonstrating the shielding of the anion-exchanger surface by the BSA.

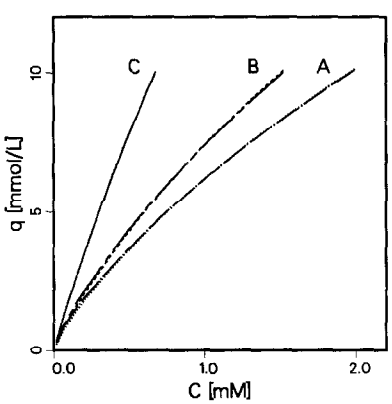

Fig. 8. Adsorption isotherms of N-benzoyl-DL-alanine determined from the corresponding elution profiles in Fig. 6. Lines as in Fig. 7. For best-fit bi-Langmuir parameters, see Table III.

Since we know the amount of BSA that is immobilized on the column (column A = 2.30 μmol; column B = 2.07 μmol) and the volume of the stationary phase (0.52 ml), we can express the saturation capacities of the selective sites (Tables II and II) as the number of molecules of enantiomers adsorbed at saturation per molecule of BSA. For mandelic acid, this ratio is 0.20 for the L-isomer and 0.19 for the D-isomer. For N-benzoylalanine, it is 0.22 for the L-isomer and 0.21 for the D-isomer. These numbers imply that there exists at most one chiral selective site per immobilized BSA molecule. Hence the active site is probably the inside of the hydrophobic cavity of the protein, as suggested previously [13,14]. Unfortunately, with the method of protein immobilization used here, we have no control of the orientation of the protein on the surface of the anion exchanger. It depends largely on the charge distribution on the protein surface. Consequently, it is reasonable to assume that every molecule of protein cannot be accessed by the enantiomers. Only one out of five would be available with our stationary phase. This number should be different, and probably larger, for covalently bonded BSA. As expected from previous work [6,10] and from the numbers just reported, the saturation capacity of the chiral selective sites is low, *e.g.*, *ca.* 40 times smaller than the saturation of the non-selective sites for column A. This explains why the BSA columns are so easily overloaded.

The most intriguing feature of these results is that

TABLE II

BI-LANGMUIR ISOTHERM PARAMETERS FOR MANDELIC ACID

Column	Site	Isomer	a	b (l/mmol)	q_s (mM)
A	Selective	L	2.8	3.2	0.89
		D	5.2	6.6	0.79
	Non-selective		4.1	0.13	31.7
B	Selective	L	2.5	3.1	0.80
		D	3.8	5.1	0.75
	Non-selective		5.2	0.11	46.9
C	Non-selective		6.9	0.13	52.8

TABLE III

B-LANGMUIR ISOTHERM PARAMETERS FOR N-BENZOYLALANINE

Column	Site	Isomer	a	b (l/mmol)	q_s (mM)
A	Selective	L	7.0	7.4	0.95
		D	13.8	15.5	0.89
	Non-selective		6.5	0.19	34.2
B	Selective	L	6.1	6.90	0.89
		D	11.6	14.0	0.83
	Non-selective		7.9	0.21	37.6
C	Non-selective		18.6	0.41	45.3

the isotherms of the two pairs of enantiomers cross for columns A and B where enantioselection occurs. This, of course, is expected following the observation that the elution profiles do cross (inset in Figs. 3 and 4). The assumption made when fitting the adsorption data to the bi-Langmuir isotherm model was that the non-selective site parameters are identical for any pair of enantiomers, but the selective site parameters can vary without constraint (other than when the adsorption behavior is Langmuirian in nature). In all instances, we find that the selective site saturation capacity of the L-isomer is greater than that of the D-isomer by an average of 8% (Tables II and III). This could be explained in one of two different ways.

First, two separate selective sites of adsorption could exist, one for each type of enantiomers. This situation would lead to the profile of the more retained enantiomers passing through the profile of the less retained enantiomers when the column is overloaded. However, under competitive conditions a strong displacement of the less retained enantiomer by the more retained enantiomer is observed [6,10], thus rebutting this possibility. The second possible reason is that the orientation of the enantiomers within the hydrophobic cavity of the protein might impose stronger steric restrictions on the more retained enantiomer, which requires three interactions as opposed to only two for the less retained enantiomer.

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SYMBOLS

a first coefficient of the Langmuir isotherm
b second coefficient of the Langmuir isotherm
C mobile phase concentration of the solute
q equilibrium stationary phase concentration of the solute
q_s saturation capacity
V_0 dead volume
V_R retention volume
V_S stationary phase volume

Subscripts

x D or L, corresponding to the D-isomer and the L-isomer, respectively
cs chiral selective site of adsorption
ns non-selective site of adsorption

REFERENCES

1 D. W. Armstrong and S. M. Han, *CRC Crit. Rev. Anal. Chem.*, 19 (1988) 175.
2 W. H. Pirkle and T. C. Pochapsky, *Chem. Rev.*, 89 (1989) 347.
3 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 237 (1982) 473.
4 S. Andersson, S. Allenmark, P. Erlandsson and S. Nilsson, *J. Chromatogr.*, 498 (1990) 81.
5 P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.*, 370 (1986) 475.
6 S. C. Jacobson and G. Guiochon, *J. Chromatogr.*, 590 (1992) 119.
7 W. H. Pirkle and R. S. Readnour, *Chromatographia*, 31 (1991) 129.
8 D. Graham, *J. Phys. Chem.*, 57 (1953) 665.
9 R. J. Laub, *ACS Symp. Ser.*, 297 (1986) 1.
10 S. Jacobson, S. Golshan-Shirazi and G. Guiochon, *J.Am. Chem. Soc.*, 112 (1990) 6492.
11 E. Cremer and J. R. K. Huber, *Angew. Chem.*, 73 (1961) 461.
12 S. Golshan-Shirazi, S. Ghodbane and G. Guiochon, *Anal. Chem.*, 60 (1988) 2630.
13 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 316 (1984) 617.
14 R. H. McMenamy in V. M. Rosenow, M. Oratz and M. A. Rotschild (Editors), *Albumin, Structure, Function and Uses*, Pergamon Press, Oxford, 1977, p. 143.