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Chiral stationary phases based on intact and fragmented cellobiohydrolase I immobilized on silica*

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ABSTRACT

Cellobiohydrolase I (CBH I) was enzymatically degraded into two fragments which were immobilized on silica. Each fragment was shown to contain at least one enantioselective site for propranolol. The dominating enantioselective site of propranolol and other solutes is located on the main part of the enzyme -the core (C).

CBH I was **immobilized** via its carboxylic groups onto aminopropyl silica and via its amino groups to aldehyde silica. The CBH I-aminopropyl silica separates enantiomers with higher enantioseiectivity than does the CBH I-aldehyde silica, whereas the latter phase promotes high retention. Slow **adsorption–desorption** kinetics dominates the band broadening process of solutes on CBH I-silicas. Addition of dimethyloctylamine to the mobile phase resulted in a significant improvement of the peak efficiency of &blocking agents.

INTRODUCTION

Chiral stationary phases **(CSPs)** based on proteins are frequently and successfully used for separations of enantiomers of various kinds. Proteins commonly utilized as chiral selectors on these **CSPs** are macromolecules with molecular masses ranging from 25 000 to 70 000 and possessing complicated three-dimensional structures, thus displaying a multitude of possible interaction sites, stereospecific as well as **non-stereo**specific. The sites are frequently ligand specific and have different locations in the macromolecule **[1–3]**. The presence of such sites is indicated by the structurally great variety of chiral compounds separated on some of the protein-based materials **[4]**. Some such sites have been localized by means of enzymatic fragmentation of the proteins **[5,6]** and also by **crystallographic** methods **[3]**. One of the bovine serum albumin **(BSA)** fragments tested as chiral selector had lost **enantioselectivity** for some analytes and retained it for others as compared to the intact protein **[5]**. The poor loadability combined with the relatively low column efficiency limit the possibility to use these kinds of **CSPs** for tracing chiral substances, for instance, in biomatrices.

There are several ways to covalently immobilize proteins to a support, as they contain many functionalities. One can expect that **chromato-**

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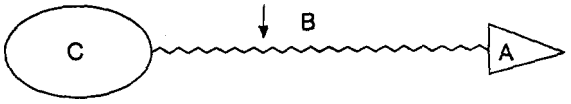

Fig. 1. Structural organisation of CBH I. C = Core, consisting of 425 amino acid residues; B = connecting region, consisting of 35 amino acid residues; A = cellulose-binding domain, consisting of 35 amino acid residues. Papain cleaves at the arrow.

graphic performance depends on the amount of protein as well as on the method used to immobilize it on the support [7]. A BSA-silica CSP obtained by adsorption of BSA on the silica was unable to differentiate between enantiomers of some sulphoxides [8] which were separable on the CSP with covalently immobilized BSA [9,10].

Compared to that for the intact protein, the column efficiency of chiral solutes on a CSP based on a fragment should be improved if the fragmentation strips off non-specific binding sites with high affinity and/or multiple stereospecific sites of the analyte. However, if the dominant stereospecific site shows slow adsorption and desorption kinetics for the analyte, i.e., is rate determining, no significant improvement of the efficiency should be expected. As a consequence of the lower molecular mass of the fragment compared to the intact protein it should be possible to covalently bind a higher molar amount of fragment sites to the support and thus improve the loadability of analytes.

The cellulase, cellobiohydrolase I (CBH I), can easily be cleaved enzymatically into two fragments, as indicated by the arrow in Fig. 1, both of which can be purified by conventional methods. The purpose of this study was to immobilize these fragments on silica, to evaluate the chromatographic performance of the obtained CSPs and to locate stereospecific site(s).

EXPERIMENTAL

Apparatus

The pumps used were a 114 M solvent delivery module in the micro mode (Beckman Instruments, Fullerton, CA, USA) and a Model 2150 dual-piston high-performance liquid chromatographic pump (Pharmacia LKB Biotechnology, Uppsala, Sweden). The injectors were a Model 7520 with a 0.5- or 1-μl loop and a Model 7125 with a 20-μl loop (Rheodyne, Cotati, CA, USA). The variable-wavelength detectors SpectroMonitor 3100, fluid cell volume 1 μl (3 mm path length) and SpectroMonitor D, fluid cell volume 14 μl (10 mm path length) (LDC Analytical, Riviera Beach, FL, USA), were connected to a recorder Model BD 40 (Kipp & Zonen, Delft, Netherlands).The columns were made of stainless steel (Skandinaviska GeneTec, Kungsbacka, Sweden) with PTFE-coated (Svefluor, Uppsala, Sweden) inner surface.

A water bath type 02 PT 923 (Heto, Birkerød, Denmark) was used to thermostat the mobile phase reservoir and the columns at 20.0 ± 0.1°C.

The pH meter was a Model E 623 equipped with a combined pH glass electrode (Metrohm, Herisau, Switzerland).

The spectrophotometer was a Model UV-160 A (Shimadzu Europa, Duisburg, Germany).

Chemicals

Concentrated culture filtrate from the fungus *Trichoderma reesei* chain QM9414 was a kind gift from VTT, the Technical Research Centre of Finland (Espoo, Finland). Papain was supplied by Boehringer Mannheim Scandinavia (Bromma, Sweden). Spherical diol-silica (300 A, 10 μm, 60 m^2/g, 5 $\mu mol/m^2$ of diol) was obtained from Perstorp Biolytica (Lund, Sweden). Spherical aminopropyl modified silica, Nucleosil 100–10NH_2 (100 A, 10 μm, 350 m^2/g of the unmodified silica, 5 $\mu mol/m^2$ of NH,) and Nucleosil 300–7NH_2 (300 A, 7 μm, 100 m^2/g of the unmodified silica, 5 $\mu mol/m^2$ of NH,), was obtained from Macherey-Nagel (Düren, Germany). N-Hydroxymaleimide, triethylamine (TEA), tetrabutylammonium hydrogensulphate (TBA) and 1-hexanesulphonic acid sodium salt monohydrate (HS) were from Fluka (Buchs, Switzerland). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), p-nitrophenyl β-D-lactopyranoside, (*R*)-, (*S*)- and *rac*-propranolol hydrochloride, rat-warfarin,

(D)- and (L)-N-*tert*.-butoxycarbonyl-phenylalanine (N-t-Boc-phenylalanine), and (*R*)- and (*S*)-1-phenylethanol were from Sigma (St. Louis, MO, USA). N,N-Dimethylethylamine (DMEA), N ,N-dimethyloctylamine (DMOA), 1-dodecanesulphonic acid sodium salt (DS), 1-octanesulphonic acid sodium salt monohydrate (OS) and (+)-norephedrin hydrochloride were from Janssen Chimica (Beerse, Belgium). *rac*-Omeprazole , *rac*-oxprenolol hydrochloride, (*R*)- and (S)-alprenolol tartrate monohydrate, *rac*-alprenolol hydrochloride, (*R*)- and (*S*)-metoprolol hydrochloride, *rac*-metoprolol and *rac*-H 125 /72 hydrochloride were kindly supplied by Astra Hassle (Mölndal, Sweden). (*RR*,*SS*)-Labetalol hydrochloride was supplied by Glaxo Group Research (Greenford, UK). (*R*)- and (*S*)-warfarin were kindly supplied by Dr. Istvan Szinai, Central Research Institute for Chemistry of the Hungarian Academy of Sciences (Budapest, Hungary). (*R*)-, (*S*)- and *rac*-prilocaine hydrochloride were kindly supplied by Astra Pain Control (Södertälje, Sweden). The organic solvents as well as the acids and salts used for the preparation of buffers were of analytical grade.

N-Hydroxysulfosuccinimide sodium salt (HSSI) was prepared from N-hydroxymaleimide according to a previously reported method [11].

The solute structures are shown in Fig. 2.

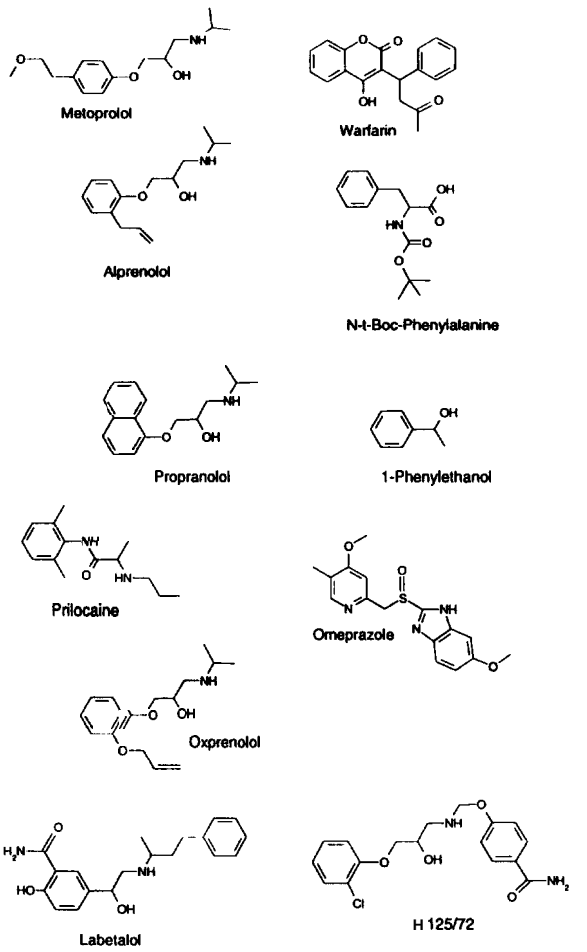

Fig. 2. Solute structures.

Preparation and isolation of intact and fragmented CBH Z

CBH I was isolated from the crude concentrated culture filtrate of *T. reesei* by chromatographic techniques as described previously [12]. Limited proteolysis of CBH I (M_r 65 000) by papain yielded the enzymatically active core protein (M_r 56 000) and a C-terminal protein (M_r 9000) [13] consisting of a part of the connecting region, B, plus the cellulose-binding domain, A [14]. The two fragments, core and BA, were baseline separated and purified by gel filtration according to ref. 13. The purity of the BA fragment was controlled by amino acid analysis [14] and by reversed-phase chromatography on a C_{18} column.

Different batches of crude culture filtrates were used for the preparation of intact CBH I and its fragments.

Preparation of protein solid phases

Immobilization on uldehyde silica. The oxidation of diol-silica to aldehyde silica and the subsequent immobilization of CBH I via the amino groups of the protein have been described previously [12]. In this study 150 mg, 75 mg and 38 mg CBH I were allowed to react per gram of aldehyde silica in order to obtain CBH I silicas with different amounts of protein. The core silica was prepared as done with the CBH I-silica [12] and 150 mg of core fragment were added to the

reaction mixture. Prior to the immobilization reaction, both intact CBH I and the core fragment were stored in an ammonium acetate buffer, pH 5.0.

Carbon and nitrogen elemental analysis of dried CBH I-and core-silica were done by Mikro Kemi (Uppsala, Sweden). The results from the carbon analysis indicated a higher amount of CBH I immobilized to silica than did the nitrogen analysis. This discrepancy was ascribed to the carbohydrate content of the protein. Therefore, the amount of protein immobilized on the silica was calculated from the nitrogen content alone. The amounts of immobilized CBH I were 40.4, 35.8 and 22.1 mg/g silica. The amount of immobilized core fragment was 36.3 mg/g silica.

The CSPs were packed into steel columns as described previously [12].

Immobilization on aminopropyl silica. Intact and fragmented CBH I were immobilized on aminopropyl silica via the carboxylic groups of the proteins [15]. Lyophilized protein was added to aminopropyl silica suspended in 0.1 *M* phosphate buffer (pH 7) and the suspension was treated with an ultrasonic bath for 0.5 min. The resulting slurry was agitated on a rocker table for 24 h, after which EDC and HSSI dissolved in 0.1 *M* phosphate buffer were added and the reaction mixture was rocked again for 24 h. The resulting protein-silica was washed with 0.1 *M* phosphate buffer.

A 155-mg amount of CBH I and 41 mg BA fragment were added separately to l-g samples of 100 A aminopropyl silica, each suspended in 5 ml buffer. A 0.2-g amount of EDC and 0.1 g HSSI dissolved in 0.5 ml buffer were added to effect the coupling reactions.

Two samples of CBH I, 29 and 62 mg, were added to separate 0.5 g 300 Å aminopropyl silica, each suspended in 2.5 ml phosphate buffer. A 2.5-mg amount of EDC and 2.5 mg HSSI dissolved in 0.25 ml buffer were added to the slurry containing 29 mg CBH I. A 5.0-mg amount of EDC and 5.0 mg HSSI dissolved in *0.25* ml buffer were added to 'the slurry containing 62 mg CBH I.

Elemental analysis of the dried solid phases showed that 122 mg CBH I/g silica and 35.7 mg BA fragment/g silica had become immobilized on the 100 A silica. The two 300 Å silica samples contained 22.8 and 53.3 mg CBH I/g silica.

Determination of enzymatic activity

The activity of CBH I immobilized on aminopropyl silica was determined by incubating the solid phase with p-nitrophenyl β-D-lactopyranoside (pNPL) and comparing the amount of p-nitrophenol formed with the amounts formed when CBH I solutions of known concentrations were incubated with the reagent.

A calibration curve was plotted by assaying aliquots of 20 μ*M* CBH I solution with pNPL. To 0-, 5-, 10-, 40- and 100-μl aliquots was added 50 μl 1 *M* acetate buffer pH 5.0 and the volumes were adjusted to 500 μl with Milli-Q purified water. The standards were incubated for 10 min with 0.5 ml 1 mg/ml pNPL. The reaction was stopped by the addition of 1 ml 5 % (w/v) disodium carbonate in Milli-Q purified water. The amount of p-nitrophenol formed was measured spectrophotometrically at 410 nm. The calibration curve for the interval O-2 nmol CBH I was linear with a coefficient of determination, r^2, equal to 0.999.

Two samples, 10 and 100 μl, of a suspension of immobilized CBH I were washed with Milli-Q purified water and centrifuged. The volumes of the samples were adjusted to 450 μl with Milli-Q purified water and 50 μl 1 *M* acetate buffer, pH 5.0, was added to each sample. The assay was performed as above for the standard solutions. The silica content of the CBH I silica suspension was determined by washing the suspension with Milli-Q purified water followed by freeze-drying and weighing. The suspension contained 31 mg silica/ml.

Chromatographic technique

The ionic strength, *I*, was 0.01 unless otherwise stated. The solutes were generally injected in amounts of 1.0-30 pmol dissolved in the mobile phase, corresponding to linear isotherm retention.

Columns with the dimension 100 × 2.1 mm I.D. were used unless stated otherwise. The flow-rate was 0.1 ml/min, equal to a reduced linear flow velocity, *ν*, of 6. When a column with

the dimension 250 x 5.0 mm I.D. was used the flow-rate was 1 ml/min, corresponding to $\nu = 12$.

The capacity factor (k'), enantioselectivity (α), and resolution of completely resolved peaks (R_s) were calculated as described in ref. 16 and the degree of resolution of partially resolved peaks (f/g) was calculated according to ref. 17. The peak asymmetry at the baseline (asf) was calculated as described previously [12]. V_0 was obtained by injection of 50 pmol (+)-norephedrin when the mobile phase contained acetate buffer or by the inflection point of water when the mobile phase contained phosphate buffer.

RESULTS AND DISCUSSION

Structural organisation of CBH Z

CBH I consists of a polypeptide chain made up of 497 amino acid residues, which is stabilized by twelve disulphide bridges and contains about 6% carbohydrate. The CBH I molecule can be divided into three domains (Fig. 1); the core, C, a flexible spacer rich in carbohydrate, B, and a cellulose-binding domain, A [14]. The C-terminal carboxylic group is located in the A domain [18] and the N-terminus of the core domain is a pyroglutamyl group [19]. Furthermore, there are 44 carboxylic groups and 22 amino groups in the side chains of the CBH I molecule. Papain cleaves the protein at a locus shown in Fig. 1 and the BA fragment consists of about 60 amino acid residues. This fragment contains only three charged groups -2 arginines and one histidine, in addition to the N- and C-terminal groups.

Separation of enantiomers on fragments of CBH Z immobilized to silica

Perhaps the most interesting result of this study was the successful separation of *rac*-propranolol at pH 6.8 on intact CBH I as well as on both of its fragments (Table I and Fig. 3). It strongly indicates the presence of different stereospecific sites at different locations in the intact protein. At pH 4.7, the resolution of *rac*-propranolol was lost on the BA fragment due to low retention (Fig. 4 and Table I). The BA fragment was immobilized by use of the C-terminus, see below, whereas this part is free in the CBH I-aldehyde silica. As both the amino group

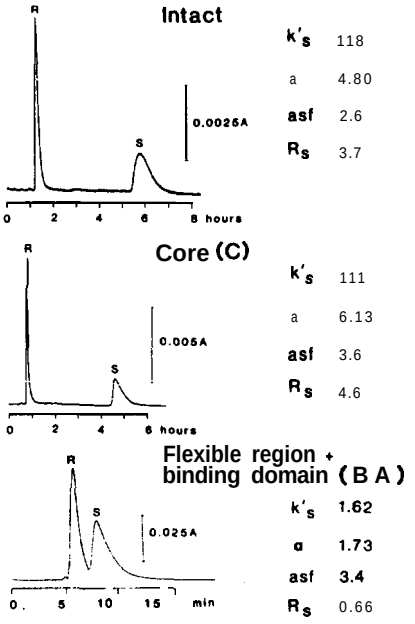

Fig. 3. Separation of the enantiomers of *rac*-propranolol at pH 6.8 on intact and fragmented CBH I immobilized on silica. Mobile phase: 0.065 *M* 2-propanol in phosphate buffer. Sample amount: 1.0 nmol.

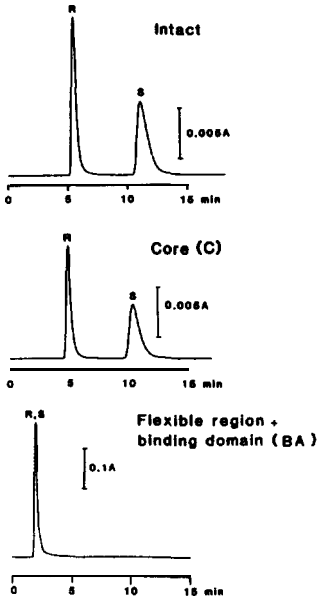

Fig. 4. Separation of the enantiomers of rat-propranolol at pH 4.7 on intact and fragmented CBH I immobilized on silica. Mobile phase: 0.065 *M* 2-propanol in acetate buffer. Sample amount: 1.0 nmol.

TABLE I

INTACT'AND FRAGMENTED CBH I IMMOBILIZED ON SILICA

Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7.

Solute	Parameter	Amount of protein (μmol/g)		
		Core[a] 0.64	BA[b] 4.0	CBH I[a] 0.62
Propranolol	k'_s	3.01	0.21	2.84
	a	3.85	1.8	3.31
	asf	1.3	1.4	1.3
	R_s	4.3	[c]	4.1
Warfarin	k'_R	23.9	22.9	22.1
	asf	1.09	1.00	1.13
	f/g	0.34 2.8	2.2	0.36 2.3
Omeprazole	k'_2	3.20	1.68	3.24
	α	1.17	1.0	1.16
	asf			
	f/g	0.56	1.7	0.50
Prilocaine[d]	k'_R	1.01	0.24	2.05
	asf	1.77	1.0	1.42
	f/g	0.99 1.3	2.1	0.91 1.9

[a] Immobilized on aldehyde silica.
[b] Immobilized on 100 Å aminopropyl silica.
[c] No separation of the racemate observed.
[d] Mobile phase: 0.065 *M* 2-propanol in phosphate buffer pH 6.8.

of the analyte and the N-terminus of the BA fragment are protonated at pH 6.8 it seems unlikely that the N-terminus is involved in the chiral recognition mechanism. Neither should the amide linkage between the support and BA fragment be effected by changing pH in this range. The only group of the fragment that changes charge over the interval from pH 4.7 to 6.8 is histidine that should be close to its pK_a at the latter pH. Thus electrostatic repulsion between the positively charged analyte and the BA fragment seems to have a negative effect on the retention. Over the interval from pH 6.7 to 8.7, where the electrostatic repulsion should decrease, the retention of propranolol increased while the enantioselectivity decreased (Table II). This effect was also observed for amines on CBH I-aldehyde silica [12]. It was suggested that conformational changes of the protein at high pH gave rise to the decrease in the enantioselectivity [12]. Since the pK_a of the analyte is 9.5 [20] it cannot be excluded that the binding affinity of

TABLE II

INFLUENCE OF pH ON CHROMATOGRAPHIC PROPERTIES OF THE BA FRAGMENT

Mobile phase: 0.065 *M* 2-propanol in phosphate buffer.

Solute	pH					
	6.7		7.6		8.7	
	k'_s	*a*	k'_s	*a*	k'_s	α
Propranolol	2.83	1.81	8.76	1.66	33.2	1.37
Prilocaine	0.57	1.0	1.94	1.01	3.77	1.01
Warfarin	2.66	1.00	0.85	1.0	-0.12	1.0
Omeprazole	1.20	1.0	1.06	1.0	0.66	1.0

the uncharged form is different from its protonated form and that it will bind to a different, achiral site on the protein. Minor changes in the conformations of the BA fragment probably occur upon cleavage from the intact CBH I. Further studies will be made to verify the preliminary findings to make sure that the site on the BA fragment is not induced by cleavage and immobilization.

The BA fragment became immobilized in almost ten-fold higher molar amounts than did the core fragment and the intact CBH I (Table I). Despite these differences, the retention of (*R*)- and (*S*)-propranolol was 4 and 14 times higher, respectively, on the core-silica than on the BA fragment when using a mobile phase pH of 4.7 (Table I) and 30 and 70 times higher, respectively, using pH 6.8 (Fig. 3). The retention and enantioselectivity of propranolol were similar on the CBH I-and core-silica under these conditions. One may thus assume that the dominating enantioselective site for propranolol is located in the core domain and that the binding affinity of propranolol to the BA fragment in the CBH I-silica is almost negligible under linear binding isotherm conditions. However, it should be noted that the observed retention and enantioselectivity of propranolol on the core-silica can be due to several different chiral as well as achiral binding sites.

In Table I are given the chromatographic data for some enantiomeric compounds separated on intact CBH I and fragments. The retention order of enantiomers was the same on fragment-and CBH I-silicas. In Fig. 5 is shown the separation of *rac*-prilocaine using the immobilized core domain of CBH I. The enantiomers of proprano-101 and warfarin were more retained by the core and the BA fragment than by the intact protein. The pH dependence of solute retention and enantioselectivity on the fragment phases in the range 4.7 to 6.8 followed the same pattern as on solid phases based on intact CBH I [12]. The retention and enantioselectivity of the amines increased with increasing pH, whereas the opposite was found for the acidic compounds. However, only the enantiomers of propranolol were resolved on the BA-silica phase at pH 6.7 to 8.7 (Tables I and II, Figs. 3 and 4).

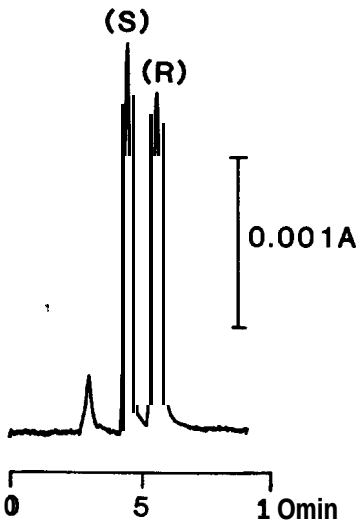

Fig. 5. Separation of the enantiomers of *rac*-prilocaine on CBH I core-silica. Mobile phase: 0.065 *M* 2-propanol in phosphate buffer pH 6.8. Sample amount: 38 pmol.

The loading capacity of the core-silica was investigated at pH 4.7 by injection of (*R*)- and (*S*)-propranolol in amounts ranging from 2.5 pmol to 2.0 nmol. The loading capacity was identical to solid phases prepared from intact CBH I containing the same amount of protein (0.6 μmol/g silica) and immobilized by the same technique, see below. Although the BA fragment was immobilized on silica in almost ten-fold higher molar amount than both the core and intact CBH I, the loading capacity for (*R*)- and (*S*)-propranolol was lower on the BA-silica at pH 6.8. Injection of 5 pmol of each enantiomer on this silica phase resulted in tailing peaks, whereas 0.5 nmol of each was needed to obtain the same overloading on core-or CBH I-silicas.

Covalent immobilization of CBH Z and its fragments to silica matrices and chromatographic performance

Immobilization of CBH I via its carboxylic groups to 300 A amino silica using EDC/HSSI as coupling reagent gave a coupling yield of about 40% (surface density 0.2-0.5 mg/m^2, Table III). Reductive coupling to aldehyde silica via the amino groups of the protein gave a coupling

TABLE III

COUPLING YIELD AND SURFACE DENSITY OF CBH I IMMOBILIZED ON ALDEHYDE AND AMINOPROPYL SILICA

Derivatized silica	Pore diameter (Å)	Ligand density (μmol/g)	Amount of CBH I (mg/g)		Coupling yield (%)	Surface density (mg/m^2)
			In reaction vessel	Immobilized		
Aminopropyl	100	1750	155	122	79	0.35
Aminopropyl	300	500	58	23	39	0.23
Aminopropyl	300	500	124	53	43	0.53
Aldehyde	300	300	38	22	58	0.37
Aldehyde	300	300	75	36	48	0.60
Aldehyde	300	300	150	40	27	0.67

yield that decreased with increasing amount of protein in the reaction vessel. The higher surface area of the amino support, *i.e.*, the higher ligand density, and the higher number of carboxylic groups compared to amino groups on the protein partly explain the more efficient coupling at high protein loading. Differences in pore size distributions between the silica matrices used, in spite of the same mean pore size, might also contribute to the observed differences. The two methods result in different linkages between the protein and the silica spacer; namely amide and amine, respectively. When 100 A amino silica was used the coupling yield was approximately 80% (surface density 0.4 mg/m^2, Table III).

As expected, the small-sized BA fragment could be bound to amino silica with a high surface area in a higher amount than CBH I. The coupling yield was approximately 90% (surface density 0.1 mg/m^2). This immobilization technique means that the C-terminus of the BA domain is linked to the silica spacer, *i.e.*, the N-terminus is free.

Compared to the activity of the free enzyme in solution, immobilization of 53 mg/g CBH I to 300 Å amino silica led to a 30% decline in enzymatic activity. Similar findings have been reported for chymotrypsin [21]. One simple explanation for the loss of activity might be the very narrow pores of the support. In such pores the enzymatically active site might become hidden by deformation of the protein. It can also be assumed that narrow pores would lead to interactions (collisions) between the macromolecules blocking the enzymatically active site. A high ligand density of the silica allows attachment of a large amount of protein at the expense of specific activities of enzymes [22,23]. The enantioselectivity obtained on the 100 Å CBH I phase was lower than on the 300 Å phase (Table IV). No difference in peak efficiency was observed between the two phases. As expected, the retentions were, in general, higher on the 100 Å phase than on the 300 Å phase, due to the high amount of CBH I immobilized to the former silica.

The effect on the chromatographic performance of increasing the CBH I loading on both amino and aldhyde silica phases was studied (Tables IV and V). Irrespective of the matrix used, the retention of the solutes generally increased with higher CBH I loading. However, on the aldehyde silica the retention of (*R*)-propranolol and (R)-alprenolol decreased with increased protein loading. It seems that the (*R*)-enantiomers of the latter compounds have low binding affinity to the protein and are mainly retained by the matrix. Accordingly, inactivation of sites on the matrix should reduce the retention

TABLE IV

IMMOBILIZATION OF CBH I VIA CARBOXYLIC GROUPS ON AMINOPROPYL SILICA

Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7.

Solute	Parameter	100 Å, 122 mg CBH I/g silica	300 Å, 23 mg CBH I/g silica	300 Å, 53 mg CBH I/g silica
Propranolol	k'_S	1.13	0.68	1.56
	asf	2.88	4.65	6.02
	f/g	0.96 1.5	0.97 1.6	2.8" 1.7
Warfarin	k'_R	56.6	6.5	7.24
	a	1.01	1.1	1.17
	asf	2.2		1.7
	f/g		0.07	0.59
Omeprazole	k'_2	1.98	0.85	1.19
	asf	1.0	1.0	1.34
	f/g	1.2	0.79	0.63 1.6
Prilocaine[b]	k'_R	0.36	0.14	0.28
	asf	1.2	1.3	1.6
	f/g	1.5 [c]	2.0 [c]	0.12 1.6

[a] Calculated as R_s.
[b] Mobile phase: 0.065 *M* 2-propanol in phosphate buffer pH 6.8.
[c] No separation of the racemate observed.

(Table V). The retention of (*S*)-propranolol was almost uneffected while the retention of (*S*)-alprenolol increased upon increasing the CBH I loading on aldehyde silica resulting in improved enantioselectivity of propranolol and alprenolol (Table V). A somewhat different chromatographic behaviour of both enantiomers of propranolol was observed at higher protein loadings on 300 Å aminopropyl silica (Table IV). The retention of both enantiomers increased and the enantioselectivity increased at the same time. This may reflect different locations and numbers of attachment points of CBH I to aminopropyl and aldehyde silica as discussed above. The peak symmetry of the solutes on the CBH I-aldehyde silicas was almost uninfluenced by the protein load.

To accurately compare the chromatographic properties of CBH I-aminopropyl and-aldehyde silicas the phases should have contained equal amounts of the protein. Our results show, however, that CBH I-aldehyde silica gives the highest retention, whereas the CBH I-aminopropyl silica gives the highest enantioselectivity (Tables IV and V).

Sample capacity studies on CBH I-aldehyde silicas with different protein loadings using (*R*)- and (*S*)-propranolol as model compounds (Fig. 6a–c) revealed that the enantioselectivity increased with increasing sample amount in the range 1 pmol-0.5 nmol (Table VI). This unusual effect was most pronounced for the stationary phase with the highest CBH I loading. Concentration independent retention and symmetrical peaks (linear isotherm retention) were obtained upon injection of about 1 pmol of each enantiomer on the three phases. Exceeding the pmol range led to reduced retention and increased peak tailing (Fig. 6a–c). The improved enantioselectivity from the pmol to nmol range was due

TABLE V

IMMOBILIZATION OF CBH I VIA AMINO GROUPS ON ALDEHYDE SILICA

Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7.

Solute	Parameter	mg CBH I/g silica		
		22	36	40
Propranolol	k'_S	2.23	2.84	2.87
	a	1.87	2.58	3.20
	asf	1.4	1.5	1.5
	R_s	2.6	3.3	4.1
Alprenolol	k'_S	1.30	1.92	2.17
	α	3.96	5.89	7.36
	asf	1.3	1.4	1.4
	R_s	2.8	4.0	4.4
Prilocaine[a]	k'_R	1.22	3.33	2.05
	asf	1.34	1.18	1.42
	f/g	0.83 1.8	2.0	1.9
			0.85	0.91
Warfarin [b]	k'_R	9.14	14.7	22.0
	asf	1.16	1.17	1.12
	f/g	0.66 1.7	2.4	2.8
			0.69	0.34
N-t-Boc-Phenylalanine	k'_L	1.30	2.15	3.96
	α	1.01	1.00	1.08
	asf	1.5	1.8	1.7
Omeprazole	k'	2.36	2.62	3.24
	f/g	0.13 1.10	1.19	1.16
			0.59	0.50
1-Phenylethanol	k'_S	0.18	0.18	0.24
	α	1.00	1.02	1.01
	asf	1.4	1.4	1.4

[a] Mobile phase: 0.065 M 2-propanol in phosphate buffer pH 6.8.
[b] The increased peak asymmetry of warfarin was due to sample overloading. The analyte was highly retarded on the 36 and 40 mg/g CBH I phases and to be able to detect the solute the column had to be overloaded.

to a higher sample capacity for the (*S*)-enantiomer than of the (*R*)-enantiomer. The sample capacity of the binding site for (*S*)-propranolol at sample loadings of more than 0.1 nmol was lower than the capacity of the binding site for the (R)-enantiomer on all three phases (Fig. 6a–c, *cf.* Fig. 3 in ref. 12).

Influence of flow-rate on *peak efficiency*

(*R*)- and (*S*)-propranolol were used to study the effect of flow-rate on the efficiency of CBH I columns containing different amounts of protein. The enantiomers were injected in amounts corresponding to concentration independent retention at v = 6 (i.e., about 0.1 ml/min). A strong influence of the flow-rate on the efficiency was observed for reduced velocities from 3 to 9 (Fig. 7). This indicates that slow adsorption–desorption kinetics is an important contributor to the

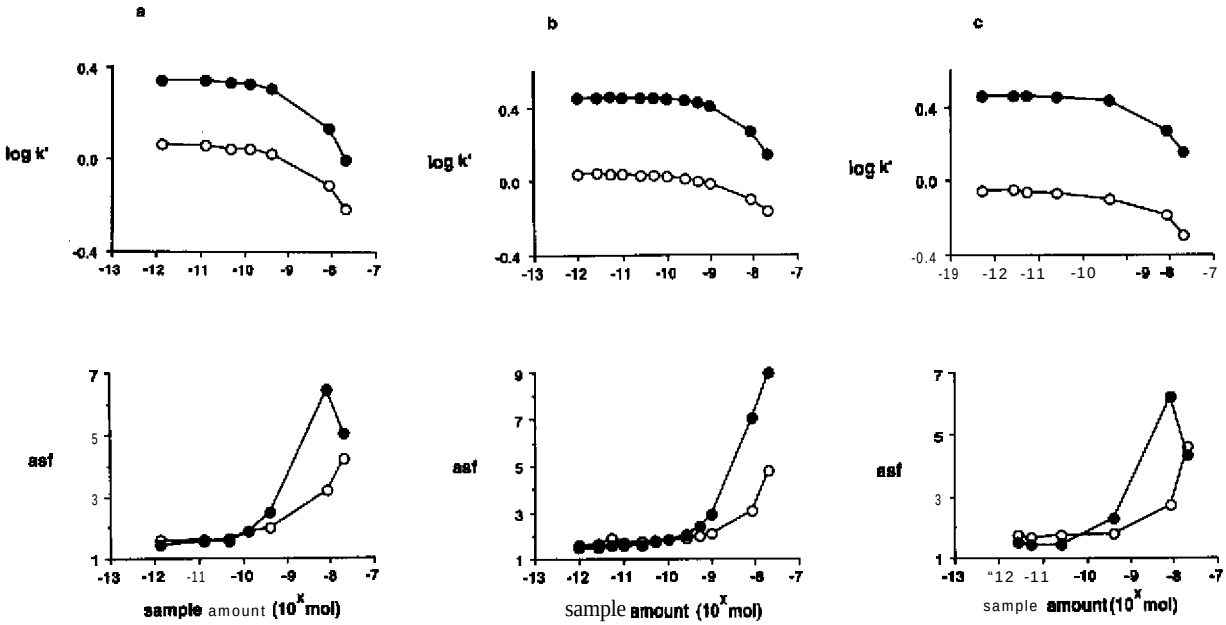

Fig. 6. Loading capacity of CBH I immobilized on aldehyde silica in different amounts. Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7. Symbols: 0 = (*R*)- and ● = (*S*)-propranolol. (a) 22 mg CBH I/g (0.34 μmol/g); (b) 36 mg CBH I/g (0.55 μmol/g); (c) 40 mg CBH I/g (0.62 μmol/g).

plate height of the CBH I phases. The efficiency of the most retained (*S*)-enantiomer was slightly higher than the (*R*)-enantiomer on all three phases. The efficiency and the flow velocity dependence for the propranolol enantiomers were quite similar on phases containing different amounts of CBH I (Fig. 7).

A notable observation was the peak symmetry decline of (*R*)- and (*S*)-propranolol with decreasing flow-rate (Table VII). At the lowest flow-rates the peaks were high and narrow, but showed strong tailing at the bases. With increasing flow-rate the peaks became low and broad and the peak symmetry improved. Corre-

TABLE VI

SAMPLE LOADING AND ENANTIOSELECTIVITY

Solid phases: CBH I-aldehyde silicas. Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7. Solute: (*R*)- *and* (*S*)-propranolol.

Sample amount (mol)	α		
	mg CBH I/g silica		
	22	36	40
$5.0 \cdot 10^{-13}$	1.88	2.60	3.23
$5.0 \cdot 10^{-12}$	1.85	2.61	3.25
$5.0\ 10^{-11}$	1.92	2.64	3.34
$2.5 \cdot 10^{-10}$		2.69	3.37
$5.0' 10^{-10}$	1.93	2.47	3.42
$8.5 \cdot 10^{-9}$	1.78	2.33	2.94
$2.1 \cdot 10^{-8}$	1.63	2.02	2.58

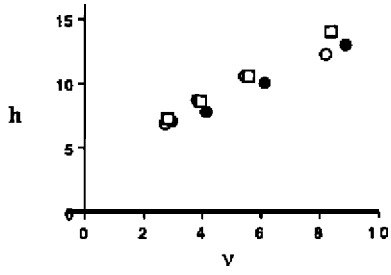

Fig. 7. Influence of flow-rate on efficiency of CBH I immobilized on aldehyde silica in different amounts. Mobile phase as in Fig. 6. Solute: (*S*)-propranolol. Symbols: ● = 22 mg CBH I/g; ○ = 36 mg CBH I/g; □ = 40 mg CBH I/g.

TABLE VII

INFLUENCE OF FLOW-RATE ON PEAK ASYMMETRY

Mobile phase: 0.065 *M* 2-propanol in acetate buffer pH 4.7. Solid phase: 40 mg CBH I/g aldehyde silica. Solute: (*R*)- and (*S*)-propranolol.

Flow-rate (ml/min)	Reduced velocity, ν	*asf*	
		(*R*)	(*S*)
0.010	0.57	1.8	1.7
0.020	1.1	1.7	1.6
0.030	1.8	1.7	1.7
0.049	2.8	1.4	1.4
0.069	3.9	1.5	1.4
0.098	5.6	1.5	1.4
0.147	8.4	1.5	1.4

sponding observations have been reported previously for another kind of chiral system, a chiral counter ion in the mobile phase and porous graphitic carbon as the solid phase [24]. It has been suggested that both kinetic and thermodynamic factors contribute to this kind of peak asymmetry and broadening (Knox and Vasvari [25], Ohkuma and Hara [26]). At low flow velocity the flow-independent, non-linear, partition isotherm is evidenced as a skewed peak [25]. The kinetic effect, which is symmetrical, dominated at high flow velocities [25]. The skewed peak was due to the presence of a small number of secondary tail producing sites characterized by slow adsorption-desorption processes [26]. As the flow-rate increased the retention time was reduced and the number of captured solute molecules at such tail producing sites decreased along with the fractional area of the tail [26]. The peak tailing was suppressed by addition to the mobile phase of an agent which competes with the solute molecules for the tail producing sites [24,26].

Mobile phase additives

As discussed above, protein stationary phases have low adsorption capacities, which means that analyses under non-linear chromatographic conditions sometimes have to be accepted, e.g., sample overloading is often needed when low contents of an enantiomeric impurity have to be determined. The possibility to improve the peak symmetry and efficiency on CBH I-silica by adding charged and uncharged modifiers to the mobile phase was investigated (Tables VIII and IX). To clarify whether the additives would interact differently with the immobilized CBH I at different buffer ion concentrations two ionic strengths (Z = 0.01 and 0.1) were used. Independently of the ionic strength, addition of the hydrophobic additives, e.g., DMOA, OS and 1-pentanol (PE) decreased the retention of the solutes, but only the charged modifiers (DMOA and OS) improved the peak symmetry (Tables VIII and IX). At elevated buffer concentration the chromatographic performance of the β-blocking agents improved (Table VIII and ref. 12). The influence of DMOA on the peak asymmetry is most pronounced at a low ionic strength. The effect of DMOA on the enantioselectivity was dependent of the solute structure and the total ionic strength. A decrease in the enantioselectivity and resolution was observed for warfarin and omeprazole. The enantioselectivity of the P-blockers in the presence of DMOA increased slightly in mobile phases of low ionic strength, but was almost unaffected at a high ionic strength.

The tertiary structure of proteins is stabilized by numerous electrostatic and hydrophobic interactions. Addition of a charged and hydrophobic agent such as DMOA could disturb such interactions and give rise to conformational changes. Competition of DMOA with the enantiomeric solutes for binding sites (specific and non-specific) on the CBH I molecule as well as for non-specific binding sites on the matrix might also be a plausible explanantion for the observed effects. Similar effects of DMOA on chromatographic properties using the Chiral-AGP phase have been observed [27]. The influence of DMEA and TEA on column efficiency and retention of metoprolol and omeprazole was not as pronounced as was found for the hydrophobic DMOA (Tables VIII and IX). The three cationic modifiers gave no significant difference in enantioselectivity for metoprolol at low ionic strength

TABLE VIII

INFLUENCE OF MOBILE PHASE ADDITIVES ON CHROMATOGRAPHIC PERFORMANCE OF SOLUTES UNDER NON-LINEAR CONDITIONS

Mobile phase: 0 or 5.0 mM of the additives 1-octanesulphonate (OS), dimethylethylamine (DMEA), triethylamine (TEA), dimethyloctylamine (DMOA) and 1-pentanol (PE) in acetate buffer pH 5.5 (I = 0.01 or 0.1). Solid phase: CBH I-aldehyde silica. Column dimension: 250 mm x 5.0 mm I.D.

Solute		I = 0.01						I = 0.1			
		–	OS	DMEA	TEA	DMOA	PE	–	OS	DMOA	PE
Metoprolol	k'_s	1.95	1.81	1.54	1.34	0.67	1.62	0.97	0.92	0.46	0.90
	asf	1.24	1.25	1.27	1.30	1.27	1.29	1.41	1.45	1.41	1.43
	f/g	4.7 0.86	4.0 0.85	0.89 3.5	2.5 0.93	0.83 1.4	2.9 0.90	3.3 0.90	2.5 0.93	0.88 1.4	0.82 3.2
Propranolol	k'_s							9.24	8.90	4.90	8.89
	asf							2.30	2.37	2.14	2.27
	f/g							4.3	3.7	2.0	4.7
								0.99	3.5"	4.6"	1.9"
Oxprenolol	k'_2	3.13		2.62	2.27						
	a	1.32		1.36	1.39						
	asf	4.7		3.1	2.8						
	f/g	0.92		0.97	0.98						

[a] Calculated as R_s.

TABLE IX

INFLUENCE OF MOBILE PHASE ADDITIVES ON CHROMATOGRAPHIC PERFORMANCE OF SOLUTES UNDER NON-LINEAR CONDITIONS

Conditions as in Table VIII.

Solute		I = 0.01						I = 0.1			
		–	OS	DMEA	TEA	DMOA	PE	–	OS	DMOA	PE
Warfarin	k'_R	2.77	2.24	3.16	3.19	2.44	2.61	2.85	2.29	2.43	2.69
	a	1.26	1.33	1.20	1.21	1.09	1.28	1.17	1.21	1.09	1.21
	asf										
	f/g	3.6 0.85	2.4 0.91	0.61 3.4	0.63 3.5	0.21 2.6	2.6 0.87	0.68 3.5	2.2 0.80	2.1 0.27	2.6 0.72
Omeprazole	k'_2	4.0	3.4	3.91	3.56	2.3	3.6	3.8	3.2	2.4	3.5
	α	1.09	1.10	1.08	1.09	[a]	1.09	1.07	1.10	1.0	1.09
	f/g	0.32	0.25	0.20	0.26		0.16	0.18	0.29		0.10

[a] A tendency to separation of the enantiomers was observed.

of the mobile phase. In contrast to DMOA, DMEA and TEA increased the retention of warfarin (Table IX). Amine modifiers probably have dual function and acts both as ion-pairing reagents and competitors. Depending on the hydrophobicity and character of the additive as

TABLE X

INFLUENCE OF TRIETI-IYLAMINE (TEA) ON CHROMATOGRAPHIC PERFORMANCE OF AMINO ALCOHOLS

Mobile phase: TEA in acetate buffer pH 5.5 (I = 0.1). Solid phase: CBH I-aldehyde silica. The solutes were injected as racemates.

Solute		[TEA] (mM)			
		0	1	5	10
Propranolol	k'_S	9.56	9.35	8.61	8.00
	a	3.20	3.15	3.10	3.01
	asf	2.1	1.9	2.2	2.1
	R_s	4.1	4.0	4.1	4.1
Alprenolol	k'_2	7.19	7.12	6.50	6.05
	a	6.44	6.49	6.33	6.32
	asf	1.58	1.56	1.49	1.61
	R_s	5.4	5.6	5.7	5.8
Metoprolol	k'_2	0.89	0.86	0.78	
	α	1.8	1.8	1.8	
	asf	1.3	1.3	1.2	
	R_s	1.6	1.5	1.5	
Oxprenolol	k'_2	1.63	1.50	1.40	1.28
	a	2.17	2.16	2.06	2.06
	asf	1.3	1.3	1.3	1.3
	R_s	2.6	2.4	2.4	2.4

well as of the solute one or the other function will dominate.

The chromatographic data from studies of an increased co-ion (TEA) concentration under almost linear binding isotherm conditions on the separation of enantiomeric amino alcohols are presented in Table X. Only a slight reduction in retention and enantioselectivity without improvement in peak symmetry was registered, even at triethylamine concentrations as high as 10 mM.

The possibility to regulate the chiral separation by use of an anionic (alkylsulphonate) or uncharged (PE) modifier was rather limited (Tables VIII, IX and XI). Dodecanesulphonate, a hydrophobic anion, increased the capacity factors for the enantiomeric amino alcohols, probably due to ion-pair retention, but ruined the enantioselectivity (Table XI).

TABLE XI

EFFECT OF SULPHONATES ON CHROMATOGRAPHIC PERFORMANCE OF AMINO ALCOHOLS

Mobile phase: 0.065 M 2-propanol and the additives 1-hexanesulphonate (HS), 1-octanesulphonate (OS) and 1-dodecanesulphonate (DS) in acetate buffer pH 4.7. Solid phase: CBH I-aldehyde silica. Column dimension: 250 x 5.0 mm I.D. Flow-rate: 0.5 ml/min. The solutes were injected as racemates.

Solute		Additive (mM)			
		–	HS (1.0)	OS (5.0)	DS[a] (1.9)
(*RR*,*SS*)-Labetalol	k'_2	*0.40*	*0.44*	0.67	59
	α	2.4	2.2	1.6	1.0
	asf	2.1	2.0	2.6	2.0
H 125/72	k'_2	0.76		0.98	
	α	2.5		1.8	
	asf	1.8		1.4	

[a] The concentration of 1-propanol was 0.13 M.

CONCLUSIONS

Stereospecific sites for the enantiomers of propranolol are located in different domains of the CBH I molecule. The dominant enantioselective binding site of the immobilized protein was found in the core (C) domain. The BA domain gave short retention times and separated only the enantiomers of propranolol at high pH.

The immobilization technique is of utmost importance for the retention and enantioselectivity of the CBH I phase. The enantioselectivity of the CBH I-aminopropyl silica was higher than that of the CBH I-aldehyde silica, whereas the retention was lower. However, further studies on different immobilizing reagents and matrices are needed to optimize the immobilization technique for intact as well as fragmented CBH I.

The efficiency of the CBH I columns is strongly influenced by the flow-rate. No significant differences in peak symmetry were observed between the CSPs based on intact CBH I and its fragments. However, additives in the mobile phase may improve the peak symmetry signifi-

cantly and charged additives seems to be more efficient in this respect than are uncharged ones.

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