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Cellobiohydrolase 58 (P.c. Cel 7D) is complementary to the homologous CBH I (T.r. Cel 7A) in enantioseparations

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Abstract

Cellobiohydrolase 58 (EC 3.2.1.91, P.c. Cel 7D) from *Phanerochaete chrysosporium* was immobilized on silica and the resulting material, CBH 58-silica, was then used as a chiral stationary phase (CSP) in liquid chromatographic separations of enantiomers. The enantioselectivities obtained on CBH 58-silica were compared with those on CBH I-silica (a phase based on a corresponding cellulase from *Trichoderma reesei*). CBH 58-silica displayed higher selectivity than CBH I-silica for the more hydrophilic compounds, such as atenolol and metoprolol, although great similarities in chiral separation of β-adrenergic antagonists were found between the two phases. None of the acidic compounds tested could be resolved on the CBH 58 phase. Moreover, the solutes were retained more on the CBH 58 phase in general, indicating an improved application potential in bioanalysis. Addition of cellobiose or lactose, both of which are inhibitors of cellulases, to the mobile phase impaired the enantioselectivity, indicating an overlap of the enantioselective and catalytic sites. The chiral analytes also functioned as competitive inhibitors and their inhibition constants were determined.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Cellobiohydrolase; Cellobiose; Cellulase; Lactose

1. Introduction

Cellobiohydrolase I (CBH I) (EC 3.2.1.91, T.r. Cel 7A) [1] from *Trichoderma reesei* was the first cellulase successfully used as chiral selector in liquid chromatographic enantioseparations. High selectivity factors were obtained in the separations of basic compounds, especially β-adrenergic blockers [2,3]. The protein shows excellent enantioselectivity when it is coupled to silica [2,3] or to continuous beds [4,5] as well as in capillary electrophoresis [6,7]. As for other protein based CSPs, the retention and enantioselectivity on CBH I-CSP are regulated by the mobile phase parameters, such as pH, ionic strength, organic modifier and type of buffer [3].

Microcalorimetric and chromatographic studies have shown that the binding between the β-blocker

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and CBH I is an entropy driven process, which means that the enantioselectivity increases with temperature [8–10].

Recent studies have revealed that three carboxylic amino acid residues, Glu212, Asp214 and Glu217, in the characteristic tunnel-shaped active site of CBH I are essential in both the catalytic [11,12] and the chiral discriminative mechanisms of CBH I [13]. Site-directed mutagenesis of these three amino acid residues to their corresponding amides individually impaired the catalytic activity [12], and the CSPs based on E212Q and E217Q had practically lost the enantioselectivity which was partly retained in the third mutant, D214N [13].

The X-ray structure of the CBH I core in complex with one of the β-blockers, (*S*)-propranolol, shows that the ligand is positioned so that it can interact electrostatically with Glu212 and Glu217, the hydroxyl group and the ether-oxygen of propranolol forming hydrogen bonds with Gln175 and the naphthyl ring of the ligand stacking on Trp376 [14].

The white rot fungus *Phanerochaete chrysosporium* secretes a complex mixture of cellulases including at least three cellobiohydrolases (CBHs). The dominating cellulase, CBH 58 (EC 3.2.1.91, P.c. Cel 7D), is the counterpart to CBH I from *T. reesei*. Two other CBHs, CBH 62 (also of the CBH I type) and CBH 50, which is homologous to CBH II from *T. reesei*, are produced in much smaller amounts [15,16]. The catalytic domain of CBH 58 shows a fold virtually identical to that of CBH I except for deletions in a few loops covering the tunnel. An extra tyrosine residue is positioned at the entrance to the tunnel. The catalytic trio in CBH I (Glu212, Asp214 and Glu217) and the tryptophans had obvious counterparts in the tunnel of CBH 58 (Glu207, Asp209 and Glu212) in almost the same orientation [17] (Muñoz and Ståhlberg, unpublished).

A recent report [18] states that the relative retention of an enantiomer (*X*) on a protein CSP can be described by the function:

$$k'_x = k'_{\mathrm{ns},x} + \frac{k'_{\mathrm{es},x}}{1 + [\mathrm{competitor}]/K_\mathrm{d}} \qquad (1)$$

where the observed capacity factor (k'_x) is the sum of a non-selective binding capacity factor ($k'_{\mathrm{ns},x}$) and an enantioselective binding capacity factor ($k'_{\mathrm{es},x}$). $k'_{\mathrm{es},x}$ can be completely eliminated by a selective competitor such as cellobiose, whereas $k'_{\mathrm{ns},x}$ is not affected. The dissociation constant (K_d) of the competitor can be calculated from the function (1) as an alternative to classical enzyme kinetics experiments.

The capacity factor ($k'_{x,\mathrm{C}}$) is composed of contributions from both specific and unspecific adsorption [18] where the enantioselective binding sites originate from the protein, whereas the non-selective binding sites may be located both on the silica matrix in the form of silanol groups and on the protein. The non-selective sites may be abundant, but have comparably low intrinsic affinities for a solute. Results supporting this idea have also been reported earlier [10].

The aim of this study was to compare the enantioselectivities of the cellulases, CBH 58 and CBH I, by chromatographic and enzyme kinetics means.

2. Materials and Methods

2.1. Chemicals

(*R*)-, (*S*)- and *rac*-propranolol hydrochloride, *rac*-octopamine, *rac*-metanephrine, *rac*-warfarin, D-(+)-cellobiose and D-(+)-lactose were purchased from Sigma (St. Louis, MO, USA). (*R*)- and (*S*)-alprenolol chloride, *rac*-oxprenolol chloride, *rac*-metoprolol chloride and *rac*-atenolol chloride were supplied by AstraZeneca (Mölndal, Sweden). *rac*-Mexiletine was obtained from Boehringer Mannheim Scandinavia (Bromma, Sweden). *rac*-Ibuprofen was from Läkemedel (Södertälje, Sweden). *Rac*-naproxen was purchased from Syntec Laboratories (CA, USA) and *rac*-chlorthalidone from Ciba-Geigy Läkemedel (Solna, Sweden). *rac*-Norephedrine, *rac*-terbutalin, *rac*-D2250, *rac*-D2253 were kind gifts from the Department of Pharmaceutical Chemistry, Analytical Pharmaceutical Chemistry, Uppsala University. Spherical diol-silica with a particle diameter of 10 μm, pore size 300 Å, area 60 m^2/g and containing 5 $\mu mol/m^2$ of diol was obtained from Perstorp Biolytica (Lund, Sweden). Sodium cyanoborohydride was from Janssen Chemica (Beerse, Belgium). Periodic acid (HIO_4), acetic acid, phosphoric acid, ammonium and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All chemicals

used were of analytical grade. The water used was Milli-Q purified.

2.2. Experimental equipments

The chromatographic system consisted of a LKB 2150 HPLC Pump (LKB-Produkter, Bromma, Sweden), a LDC/Milton Roy Spectromonitor D equipped with a 1-μl cell (LDC/Milton Roy, FL, USA), a Model BD40 recorder (Kipp and Zonen, Holland) and a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop. A pH-meter model E 623 (Metro, Wheres, Switzerland) equipped with a combined pH glass electrode and a spectrophotometer, Shimadzu UV-160A (Shimadzu, Kyoto, Japan) were also used.

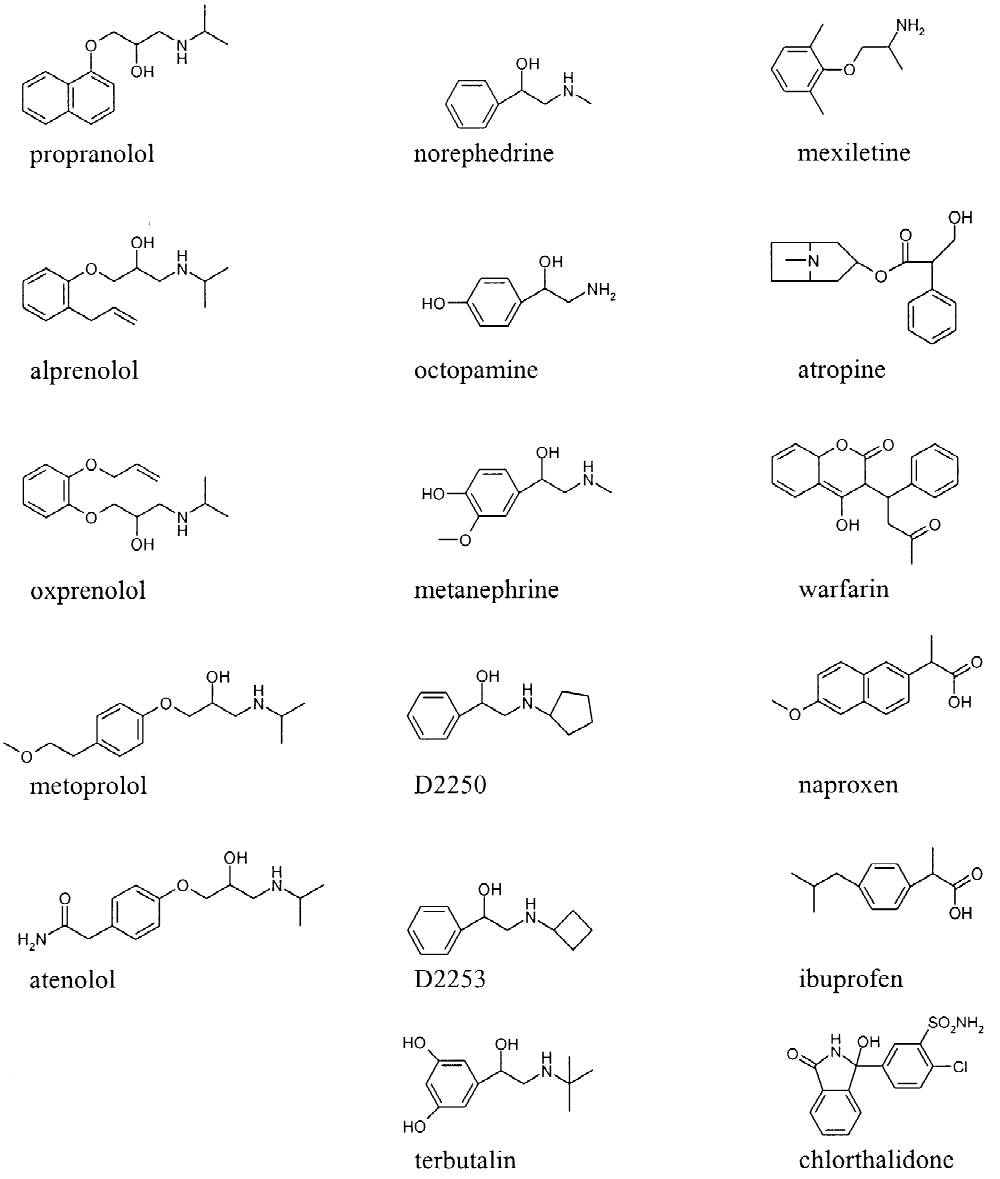

Fig. 1. Chemical structures of the solutes.

2.3. Preparation of the proteins and the columns

CBH I was purified from the concentrated culture filtrate from the fungus *T. reesei* strain QM 9414 obtained from ALKO Research Laboratories (Helsinki, Finland) as described by Bhikhabhai et al. [19]. CBH 58 was purified from the ammonium sulfate precipitates (80% saturation) from fermenter cultivation of *P. chrysosporium* strain K3 [20]. The crude precipitate was first dissolved and transferred into 10 m*M* ammonium acetate buffer, pH 5.6, followed by anion-exchange chromatography on DEAE Sepharose CL-6B using a linear gradient from 10 to 150 m*M* ammonium acetate, pH 4.5. The CBH 58 pool was further purified by anion-exchange chromatography using Hi-Load Q Sepharose 26/10 (Pharmacia, Sweden) with a gradient from 50 to 150 m*M* ammonium acetate, pH 4.5 [15]. The purity of the proteins was verified by SDS–PAGE.

The CBH I- and CBH 58-CSP columns were

Table 1
pH dependence of enantioseparation on CSPs based on CBH I and CBH 58

Solutes	Parameter	pH 3.0		pH 5.0		pH 6.0		pH 6.5		pH 7.0	
		CBH 1	CBH58	CBH1	CBH58	CBH 1	CBH58	CBH 1	CBH58	CBH 1	CBH58
Propranolol	k_1'	0.59	0.85	2.52	14.5	12.1	54.6	24.7	134	50.17	247
	α	1.36	1	3.24	1.68	4.90	2.12	5.18	2.23	5.83	2.28
Alprenolol	k_1'	0.20	0.26	0.96	4.36	5.25	18.6	10.2	40.6	24.0	74.8
	α	1.48	1	5.95	2.43	8.25	3.48	9.16	3.69	9.74	3.78
Oxprenolol	k_1'	0.19	–[a]	0.68	4.20	3.27	20.7	5.61	36.8	13.1	75.1
	α	1		1.86	1.86	2.59	2.09	2.83	2.02	3.19	2.14
Metoprolol	k_1'	0.19	–[a]	0.44	2.07	2.20	7.33	4.18	13.8	9.20	24.9
	α	1		1.61	2.99	2.20	5.13	2.28	5.72	2.43	6.06
Atenolol	k_1'	0.18	–[a]	0.24	1.52	1.05	6.87	1.64	12.8	3.30	27.2
	α	1		1	3.46	1.63	4.92	1.96	5.23	2.11	5.40
Mexiletine	k_1'	–[a]	–[a]	0.61	1.75	3.35	4.46	6.08	7.32	15.5	12.2
	α			1	1	1	1.25	1	1.30	1	1.36
Atropine	k_1'	–[a]	–[a]	0.31	1.36	–[b]	–[b]	–[b]	–[b]	3.30	7.40
	α			1	1					1	1.16
Octopamine	k_1'	–[a]	–[a]	–[a]	–[a]	1.16	1.75	2.06	3.02	4.47	5.60
	α					1.55	1.29	1.74	1.39	1.83	1.47
Metanephrine	k_1'	–[a]	–[a]	–[a]	–[a]	1.09	1.89	1.83	3.28	3.94	6.10
	α					1.17	1	1.26	1.06	1.29	1.10
Norephedrine	k_1'	–[a]	–[a]	0.65	0.66	–[b]	–[b]	–[b]	–[b]	2.12	2.86
	α			1	1					1.11	1
Terbutalin	k_1'	–[a]	–[a]	0.69	0.69	–[b]	–[b]	–[b]	–[b]	2.31	2.97
	α			1	1.49					1.15	4.76
D2250	k_1'	–[a]	–[a]	1.06	0.71	–[b]	–[b]	–[b]	–[b]	3.17	3.74
	α			1	1.56					1	1.36
D2253	k_1'	–[a]	–[a]	0.88	0.80	–[b]	–[b]	–[b]	–[b]	2.82	3.45
	α			1	1					1	1.88
Warfarin	k_1'	4.80	5.5	4.32	4.23	–[b]	–[b]	–[b]	–[b]	0.82	0.80
	α	1.67	1	1.40	1					1	1
Naproxen	k_1'	8.56	10.8	3.09	2.82	–[b]	–[b]	–[b]	–[b]	0.57	0.55
	α	1	1	1	1					1	1
Ibuprofen	k_1'	3.63	5.62	1.50	1.36	–[b]	–[b]	–[b]	–[b]	0.31	0.31
	α	1	1	1	1					1	1
Chlorthalidone	k_1'	1.58	1.86	1.49	1.59	–[b]	–[b]	–[b]	–[b]	1.68	2.13
	α	1	1	1	1					1	1

[a] In the frontal peak.
[b] Not detected.

prepared as described earlier [3,21]. Coupling yields were comparable at 41.6 mg protein/g silica for CBH I and 54 mg protein/g silica for CBH 58.

2.4. HPLC procedure

Buffers of sodium acetate, pH 5.0, and sodium phosphate, pH 3.0, 6.0, 6.5 and 7.0 with an ionic strength of 0.01, unless otherwise indicated, as mobile phases were applied to the CBH columns (with dimension of 100×2.1 mm I.D.) at a constant flow-rate of 0.2 ml/min. Selected concentrations of cellobiose, lactose or 2-propanol were added to the mobile phases individually in different studies and their effects on the chiral separation were analyzed. Milli-Q water was used as a void volume marker. The chromatographic experiments were performed in duplicate at an ambient temperature of ~22°C.

2.5. Enzyme kinetics: inhibition of the enzyme activity by the chiral compounds

The activities of the enzymes were monitored by measuring the release of *p*-nitrophenol (*p*NP) from *para*-nitrophenyl-lactopyranoside (*p*NPL) in the absence or presence of the compounds ((*R*)-, or (*S*)-propranolol, *rac*-atenolol and *rac*-mexiletine) as inhibitors. The experiments were carried out using 2 μ*M* enzyme in 10 m*M* sodium acetate buffer, pH 5.0. The concentration of *p*NPL was varied between 0.125 and 5 m*M*. Inhibitor concentrations were chosen as 0.25 or 0.50 m*M*. The reaction was allowed to proceed for 15 min at 25°C.

The effects of *rac*-warfarin on these enzymes were studied using 2 μ*M* enzyme concentration in 25 m*M* sodium acetate buffer, pH 4.0, on multititer plates at room temperature (23.5°C) with *p*NPL concentrations ranging from 0.125 to 5 m*M*, and warfarin concentration up to 0.62 m*M*. The incubation time was 15 min.

The reaction was, in all cases, terminated by adding an equal volume of 0.5 *M* sodium carbonate (Na_2CO_3) and *p*NP was monitored at 410 nm ($\epsilon =$ 16 590 $M^{-1}cm^{-1}$). Background controls (without enzymes) were treated similarly.

The kinetic parameters K_M, k_{cat}, and K_i (inhibition constant) were determined by non-linear regression analysis using the software 'Simfit' (W.G. Bardsley, Manchester, UK).

3. Results and discussion

3.1. Comparison of the enantioselectivities between CBH 58 and CBH I

The capacity factors and enantioselectivities for a number of chiral solutes including basic and acidic compounds (Fig. 1) on the CBH columns are presented in Table 1. Relatively high selectivities are found for the β-blockers (Fig. 2), with α values in the range of 2–6 at pH 7 on CBH 58-CSP (Table 1). However, the ability of the CBH 58-silica to discriminate between the enantiomers of acidic compounds is poor.

The structural homology (Fig. 3) of the two

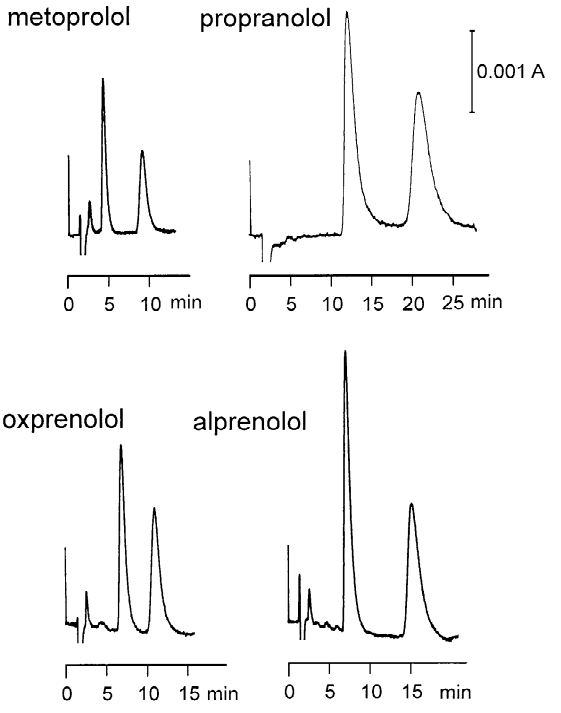

Fig. 2. Enantioseparation of the β-blockers on CBH 58-CSP. Mobile phase: sodium acetate buffer, pH 5.0, $I=0.01$ ($I=0.1$ in the case of propranolol); flow-rate, 0.2 ml/min. Solutes: *rac*-metoprolol, 10^{-5} *M*; *rac*-propranolol, 10^{-5} *M*; *rac*-oxprenolol, 2×10^{-5} *M*; *rac*-alprenolol, 5×10^{-5} M.

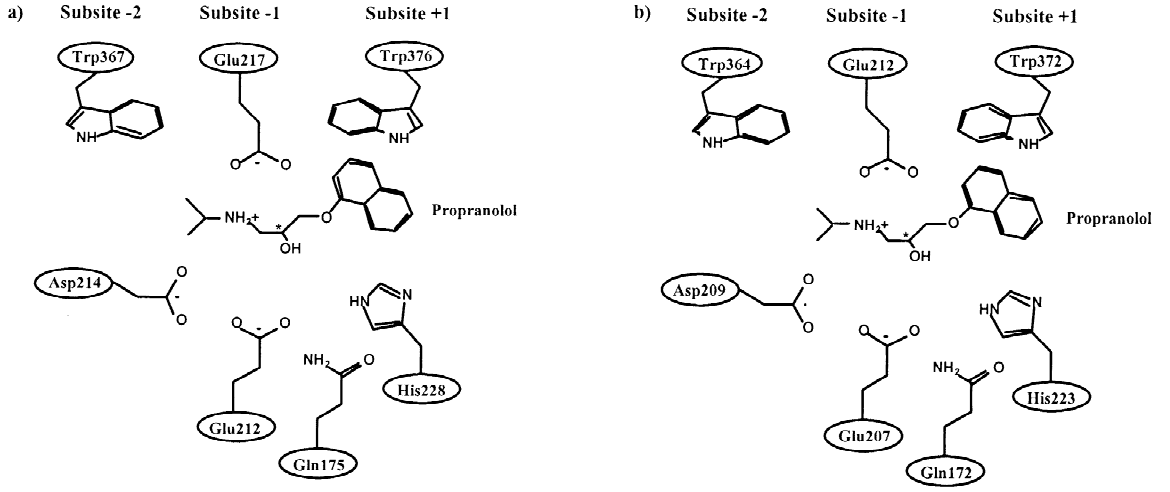

Fig. 3. Schematic representation for the binding of (*R*)/(*S*)-propranolol in the active site of CBH I (a) and CBH 58 (b). The residues around the active site are shown including the proposed acid–base catalyst, Glu217 in CBH I and Glu212 in CBH 58, and the nucleophile Glu212 in CBH I and Glu207 in CBH 58. The acidic residues are shown in their deprotonated state, and propranolol in its protonated state ($pK_a = 9.5$). In the molecule of propranolol the asymmetric center is marked with an asterisk. This model has been built manually, based on the information of the resolution of the 3-D structure of CBH I–(*S*)-propranolol (Ståhlberg et al., unpublished). The best fit of both forms of propranolol was obtained when the two catalytic glutamates interact with the nitrogen group, and the tryptophan at the +1 subsite (Trp376 in CBH I and Trp372 in CBH 58), stacks with the naphthyl group of the ligand.

cellobiohydrolases implies their similar functional properties as regards both catalysis and chiral recognition. Therefore, it is not surprising that most of the compounds resolved on the CBH I-phase were also well separated on the CBH 58-phase. However, all amino alcohols and amines analyzed here are more retained on CBH 58-silica, some of them even with higher selectivities, than on CBH I-silica (Table 1). The slight difference in coupling yield is not sufficient to account for this effect, meaning that there are intrinsic differences between the proteins. As found previously, both the enantioselective and non-selective binding capacity factors on the CBH 58-CSP were larger than those on the CBH I-CSP under the same conditions [18]. Furthermore, this can be confirmed by the determination of the inhibitory constants (K_i) for some of the solutes (Table 2). *Rac*-atenolol, (*R*)-propranolol and (*S*)-propranolol have lower K_i values, i.e., stronger binding to CBH 58 than to CBH I. We can also see that the enantioselectivity is related to the hydrophilicity of the compounds. The more hydrophilic β-blockers like metoprolol and atenolol, or β-adrenergic agonists, such as terbutalin, D2250 and D2253 are more retained with better selectivities on the CBH 58-phase than on the CBH I-phase. Practically all solutes are more retained on the CBH 58-phase and it is even true for the acidic compounds. On the other hand, amino alcohols with substituents in ortho position are better resolved on the CBH I-phase, whereas the CBH 58-phase prefers *para*-substituted solutes.

Table 2
Inhibitory effects on CBH I and CBH 58 of some chiral compounds[a]

Inhibitors	Parameters	CBH I	CBH 58
(a) at pH 5.0			
No inhibitor	K_M (m*M*)	0.93±0.04	5.13±0.73
	k_{cat} (s^{-1})	0.098±0.001	0.17±0.015
rac-Mexiletine	K_i (m*M*)	2.74±0.65	3.53±0.78
rac-Atenolol	K_i (m*M*)	3.25±1.07	0.57±0.05
(*R*)-Propranolol	K_i (m*M*)	0.50±0.03	0.27±0.01
(*S*)-Propranolol	K_i (m*M*)	0.072±0.004	0.068±0.005
(b) at pH 4.0			
No inhibitor	K_M (m*M*)	1.48±0.06	4.40±0.21
	k_{cat} (s^{-1})	0.078±0.001	0.091±0.002
rac-Warfarin	K_i (m*M*)	0.57±0.06	–[b]

[a] The experiment conditions: 10 m*M* sodium acetate buffer; enzyme concentration, 2 μ*M*; incubation, 15 min at 25°C.
[b] No inhibitory effect was observed.

In contrast to CBH I-silica, the CBH 58-phase could resolve mexiletine and atropine into their enantiomers at pH 7.0, indicating that CBH 58 has a broader selectivity spectrum for the basic compounds.

It was reported earlier that warfarin, an acidic compound, could be separated on CBH I-CSP [3]. Another investigation showed that the separation of warfarin on CBH I was impaired by adding cellobiose or lactose to the mobile phases (unpublished result). Warfarin was a weak competitive inhibitor ($K_i = 0.57$ mM) of CBH I but has no inhibitory effect on CBH 58 (Table 2). These results suggest that the mechanism for (chiral) recognition of warfarin is different for CBH I and CBH 58.

3.2. Use of selective competitors

Addition of the classical inhibitors cellobiose and lactose (Table 3 and Fig. 4) to the mobile phase decreased the retention of the β-blockers propranolol and atenolol, indicating that these drugs bind to the same site as cellobiose, most probably in the active site of CBH 58. This overlap of the enantioselective and enzymatic sites was in agreement with the earlier findings for CBH I [18]. The experimental capacity factor of each enantiomer showed a close fit to Eq. (1) above (Fig. 5) and thus Eq. (1) could be used to evaluate the enantioselective and non-selective capacity factors, which are listed in Table 4. The experimental dissociation constants (K_d) determined for the inhibitory effect of cellobiose on the retention of the model compounds showed little variation (103–124 μM) on the CBH 58-CSP, indicating that these compounds bind to the same site and have similar chiral recognition mechanisms. When lactose was used as competitor, the K_d values (in the range of 70–80 μM) were also virtually constant.

The relative retention (k') of *rac*-mexiletine, also followed Eq. (1) well, indicating that mexiletine bound to the same site as cellobiose, lactose and the β-blockers, although its enantiomers could not be separated at pH 5.0. At pH 7.0, however, mexiletine could be resolved into its enantiomers, and the

Table 3
Influence of disaccharides in the mobile phase on the chiral separation on CBH 58-CSP[a]

Competitor conc. (μM)	Capacity factors	Solutes							
		Propranolol	Atenolol			Mexiletine		Warfarin	
		CB	CB	CB[1]	LAC	CB	LAC	CB	LAC
0	k'_1	11.7	1.20	0.91	1.20	1.54	1.54	3.93	3.93
	k'_2	19.2	3.90	2.85	3.90				
10	k_1'	11.1	1.15	0.82	1.12	1.51	1.52	3.85	3.96
	k'_2	18.2	3.60	2.48	3.41				
50	k'_1	9.82	1.01	0.72	0.95	1.45	1.45	3.86	3.89
	k'_2	15.2	2.95	2.07	2.56				
100	k'_1	8.81	0.91	0.67	0.86	1.41	1.41	3.82	3.88
	k'_2	12.9	2.40	1.81	2.10				
250	k'_1	7.59	0.82	0.60	0.76	1.36	1.35	3.85	3.89
	k'_2	9.96	1.63	1.30	1.38				
500	k'_1	6.83	0.69	0.56	0.66	1.32	1.32	3.87	3.83
	k'_2	8.28	1.22	0.98	1.04				
1000	k'_1	6.51	0.64	0.54	0.63	1.29	1.31	4.00	3.81
	k'_2	7.03	0.91	0.79	0.81				
2500	k'_1	ND	ND	0.54[b]	0.66	ND	ND	ND	ND
	k'_2			0.58[b]	0.66				
5000	k'_1	5.97	0.63	0.53	ND	1.29	1.31	3.79	3.89
	k'_2	6.02	0.63	0.53					

[a] CB, cellobiose; LAC, lactose; ND, not determined. Mobile phase: sodium acetate buffer, pH 5.0, $I = 0.01$ (CB[1] with 0.5 M 2-propanol) with cellobiose in various concentration.

[b] Cellobiose concentration is 2000 μM.

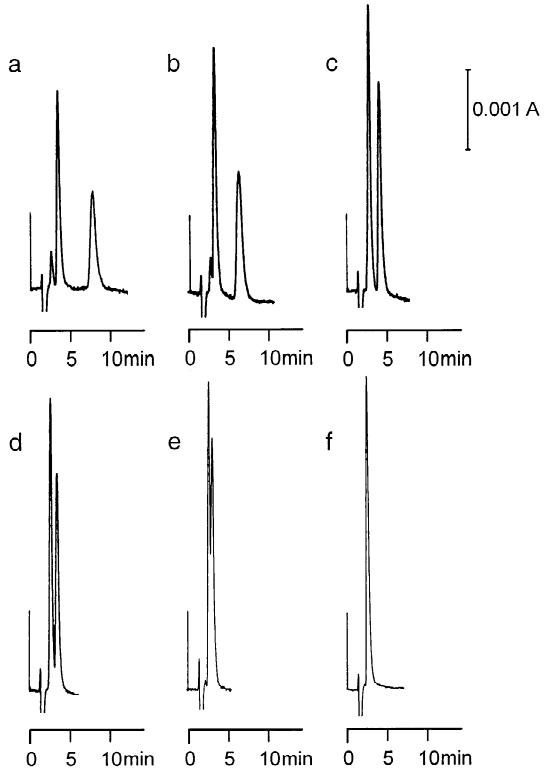

Fig. 4. Chiral separation of *rac*-atenolol on CBH 58-CSP with cellobiose as a selective competitor in the mobile phase. Mobile phase: sodium acetate buffer, pH 5.0, $I=0.01$ with cellobiose in concentrations from 0 to 5 m*M*: (a) cellobiose, 0; (b) cellobiose, 0.05 m*M*; (c) cellobiose, 0.25 m*M*; (d) cellobiose, 0.5 m*M*; (e) cellobiose, 1 m*M*; (f) cellobiose, 5 m*M*. Flow-rate, 0.2 ml/min. Solute, *rac*-atenolol, 10^{-5} M.

enzyme kinetics showed that mexiletine is a competitive inhibitor. The mechanism for separation of mexiletine and β-blockers may thus be similar.

Although no separation of warfarin can be obtained on CBH 58-silica, its retention on the column was comparable to that on CBH I-silica, a good chiral selector for warfarin. Interestingly, the retention was not affected at all by cellobiose or lactose (Table 3), meaning that the active site (more actually the specific binding site for cellobiose or lactose) of CBH 58 is not involved in the binding of this acidic compound. This is further confirmed by the fact that warfarin had no inhibitory effect on the activity of the enzyme (Table 2).

Enzyme kinetics showed that the basic compounds mentioned above are competitive inhibitors of CBH 58 as found for CBH I [22]. The inhibition constants (K_i) for these compounds are listed in Table 2. The data confirm that the enantiomers bind to the protein competitively at the same site, the active site, as the disaccharides.

3.3. Effect of pH on retention and separation

As found previously in the case of CBH I [3,13,21], the retentions of the solutes are strongly dependent on pH (Table 1). Roughly, basic solutes — like the β-blockers ($pK_a>9$) — show increased retention at increasing pH, whereas the opposite is true for acidic compounds. Obviously electrostatic interactions are crucial for the retention and the chiral separation. Although the total charge of the protein might have an influence on the retention, the pH dependence is expected to reflect mainly the pK_a values of the three carboxylic residues responsible for the catalysis [14]. When all of the carboxylic groups on CBH I were covalently modified, both the enzymatic activity and the enantioselectivity were severely impaired. However, when the carboxylic groups in the active site were protected by the presence of cellobiose, most of the enzyme activity as well as the enantiomer retention and selectivity were preserved [23].

Cellobiose can be used as a selective competitor upon chromatography on CBH I- and CBH 58-CSPs. Saturation of the cellobiose-binding sites eliminates the enantioselective binding of propranolol (Table 5), whereas the non-selective binding is unaffected. From Table 5 we can see that the non-selective binding has a strong influence on the total capacity factor (k') at pH 5, but the relative effect has decreased considerably at pH 6, meaning that the lower α at pH 5 is largely an effect of unspecific binding (see Eq. (1)). The previous results, together with the data presented herein, suggest that the strong increase in capacity factor between pH 5 and 7 is actually due to an increase in affinity for the enantioselective site caused by a deprotonation of one of the carboxylic groups discussed above, probably Glu217 (Glu212 in CBH 58), which is considered to be the proton donor in the proposed catalytic mechanism and thus is expected to titrate in that pH range.

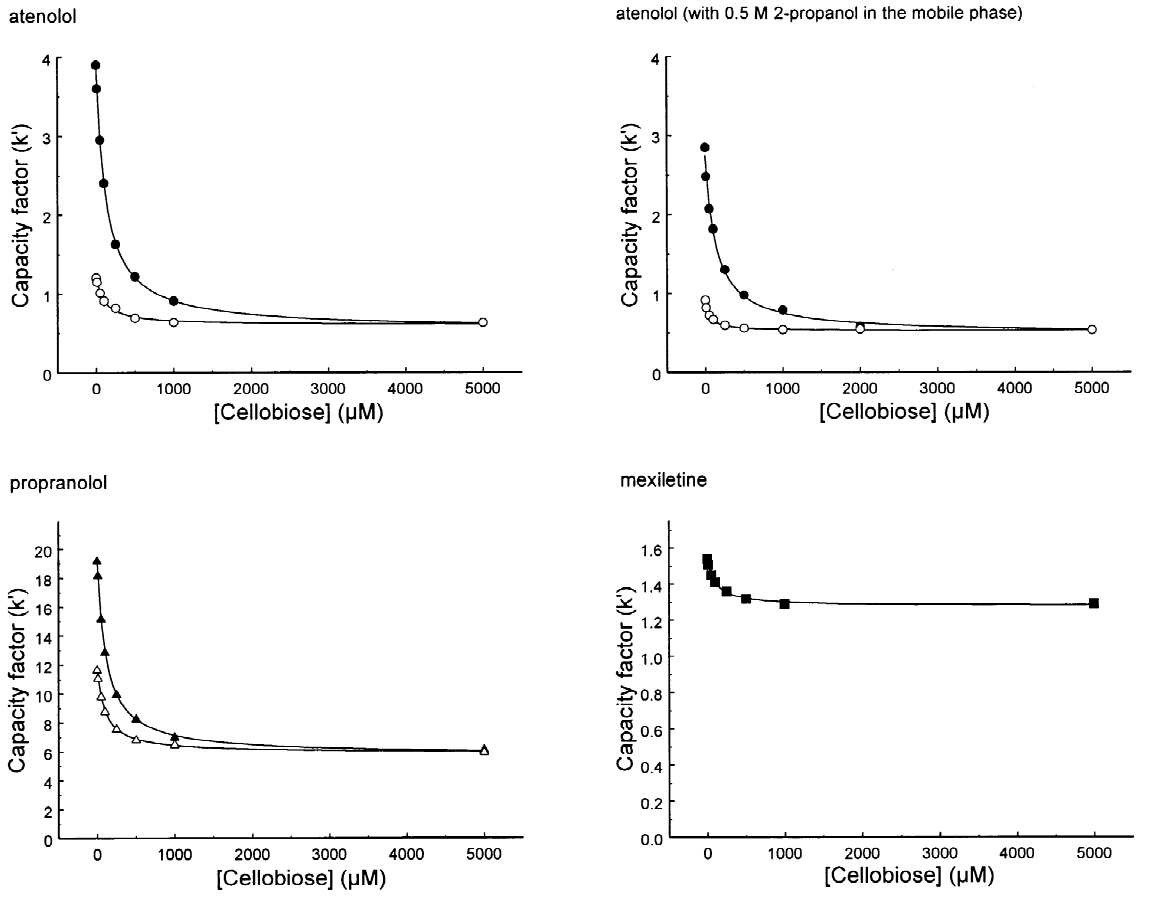

Fig. 5. Discrimination of the enantiomers binding on CBH 58-CSP using cellobiose as a selective competitor by an adapted function:

$$k'_x = k'_{ns,x} + \frac{k'_{es,x}}{1 + [\text{competitor}]/K_d}$$

The lines represent the regressions calculated from the equation and the spots (solid for *S*-form and empty for *R*-form) from the chromatographic experiments. Experimental conditions are the same as mentioned in Section 2 and Table 3.

3.4. Other factors regulating the retention and separation (type of ions and ionic strength, addition of organic modifier)

The influence of ionic strength and buffer ions on the retention and separation of propranolol and atenolol is shown in Table 6. At pH 5, an increase in the ionic strength of acetate buffer from 0.01 to 0.1 resulted in a decrease in retention and an increase in enantioselectivity of the solutes, as one might expect. The peak symmetry was improved for propranolol but unaffected for atenolol. The decrease in retention at higher ionic strengths is consistent with an electrostatic interaction [3,23–25]. Compared with acetate buffer, the change to phosphate buffer at pH 5.0 slightly shortened the retention of propranolol but had the opposite effect on atenolol. The peak symmetry of propranolol was improved whereas it was almost unchanged for atenolol. The enantioselectivities of the two compounds were nearly the same in both buffer systems.

Both the retention and selectivity of the chiral compounds were influenced by addition of 2-propanol to the mobile phase (Table 7). The retention of

Table 4
Enantioselective and non-selective binding of some basic compounds on CBH 58 phase (with comparison to the chromatography) and dissociation constants for the selective competitors[a]

Parameter	Propranolol cellobiose	Atenolol			Mexiletine	
		Cellobiose	Cellobiose[b]	Lactose	Cellobiose	Lactose
$k'_{ns,1}$	5.88	0.60	0.53	0.60	1.28	1.29
$k'_{ns,2}$	5.75	0.54	0.48	0.58		
$k'_{es,1}$	5.76	0.60	0.37	0.58	0.26	0.25
$k'_{es,2}$	13.5	3.34	2.26	3.27		
$K_{d,(1)}$ (μM)	104.9	112.7	54.2	69.7	102.5	82.0
$K_{d,(2)}$ (μM)	113.7	124.4	134.8	80.0		
k'_1	11.64	1.20	0.90	1.18	1.54	1.54
$k'_{1,c}$	11.66	1.20	0.91	1.20	1.54	1.54
k'_2	19.26	3.88	2.74	3.85		
$k'_{2,c}$	19.21	3.90	2.85	3.90		

[a] The experimental condition was the same as in Table 2. The parameters were calculated from the adapted function:

$$k'_x = k'_{ns,x} + \frac{k'_{es,x}}{1 + [\text{competitor}]/K_d}$$

except $k'_{1,C}$ and $k'_{2,C}$. Where $k'_{ns,1}$ is non-selective binding capacity factor of the first eluted enantiomer; $k''_{es,1}$ enantioselective binding capacity factor of the first eluted enantiomer; $k'_{ns,2}$ is unspecific binding capacity factor of the second eluted enantiomer; $k''_{es,2}$ is enantioselective binding capacity factor of the second eluted enantiomer; $K_{d,(1)}$ is dissociation constant of the competitor to the protein calculated from the first eluted enantiomer; $K_{d,(2)}$ is dissociation constant of the competitor to the protein calculated from the second eluted enantiomer; k'_1 is total binding capacity factor of the first eluted enantiomer; k'_2 is total binding capacity factor of the second eluted enantiomer; $k'_{1,C}$ is capacity factor of the first eluted enantiomer from chromatography; $k'_{2,C}$ is capacity factor of the second eluted enantiomer from chromatography.
[b] With 0.5 *M* 2-propanol in the mobile phase.

Table 5
Effect of pH on selective and non-selective binding of propranolol to the CBH-CSPs

	CSP					
	CBH I			CBH 58		
pH of mobile phase:	pH 5.0	pH 6.0*	k'_{pH6}/k'_{pH5}	pH 5.0	pH 6.0[a]	k'_{pH6}/k'_{pH5}
(a) Total binding capacity factor and selectivity (no cellobiose)						
k'_R	5.45	14.2	2.60	11.7	48.6	4.15
k'_S	11.4	50.7	4.45	19.3	99.0	5.13
α	2.09	3.57		1.65	2.04	
(b) Non-selective binding capacity factor and selectivity (saturated by cellobiose)						
k'_{nR}	4.54	10.1	2.22	5.97	12.3	2.06
k'_{nS}	4.81	10.4	2.16	6.02	12.8	2.09
α	1.06	1.03		1.01	1.04	
(c) Enantioselective binding capacity factor and selectivity						
k'_{eR}	0.91	4.1	4.51	5.73	36.3	6.34
k'_{eS}	6.59	40.3	6.11	13.3	86.2	6.48
α	7.24	9.83		2.32	2.37	

[a] The data are from Henriksson et al. [18].

Table 6
Influence of ionic strength and buffer ions on the separation of enantiomers on CBH 58-CSP[a]

Solute	Parameter	Buffer		
		Acetate ($I=0.1$)	Acetate ($I=0.01$)	Phosphate ($I=0.01$)
Propranolol	k'_2	11.5	23.8	20.5
	α	1.82	1.68	1.68
	R_s	2.78	2.67	2.54
	asf_2	1.73	1.99	1.53
Atenolol	k'_2	2.41	3.97	4.27
	α	4.29	3.24	3.20
	R_s	4.04	4.72	4.24
	asf_2	1.40	1.41	1.40

[a] Mobile phase: sodium acetate buffer, pH 5.0; flow-rate, 0.2 ml/min.

propranolol, atenolol and mexiletine decreased with increasing concentrations of 2-propanol and a better peak symmetry was observed. No trend could be observed concerning selectivity. The selectivity and resolution were enhanced in the case of propranolol but decreased for atenolol. Further investigations of using cellobiose as a competitor to isolate the enantioselective and non-selective capacity factors showed that this organic modifier affected both factors on the CBH 58-CSP (Tables 3 and 4) and the effect was stronger on the enantioselective binding. As for CBH I [3], this suggests that hydrophobic interaction is also involved in the chiral recognition on CBH 58. The data in Tables 4 and 5 indicate that both electrostatic and hydrophobic interactions contribute to the non-selective binding.

Table 7
Influence of 2-propanol on the enantioseparation on CBH 58-CSP[a]

Solute	Parameter	Concentration of 2-propanol (M)			
		0	0.1	0.5	1
Propranolol	k'_2	23.8	16.5	13.3	10.7
	α	1.68	1.76	1.83	1.85
	R_s	2.67	3.00	3.06	2.82
	asf_2	1.99	1.42	1.38	1.44
Atenolol	k'_2	3.97	3.47	2.84	2.10
	α	3.24	3.26	3.12	3.00
	R_s	4.72	4.31	3.19	2.95
	asf_2	1.41	1.33	1.68	1.33
Mexiletine	k'	1.54	1.33	1.17	0.97
	α	1	1	1	1
	asf	1.61	1.44	1.43	1.36

[a] Mobile phase: 2-propanol in sodium acetate buffer, pH 5.0 ($I=0.01$); flow-rate, 0.2 ml/min.

4. Conclusions

CBH 58, similarly to CBH I from *T. reesei*, is an excellent chiral selector for β-receptor blocking agents and even expresses a broader enantioselectivity for other basic compounds. It also, in general, displays higher retentions for the solutes, which can be an advantage to be applied in bioanalysis. The protein can easily be obtained in large amounts, giving it a commercial potential. The drugs that could be resolved into their enantiomers on the protein phases compete for the same binding site, the active site, with the substrate (*p*NPL) or the natural product from degradation of cellulose–cellobiose. The enantioselective and non-selective binding capacity factors can be determined by using cellobiose or lactose as a selective competitor, allowing a quantitative study of both the enantioselective and non-selective binding when the chiral separation is regulated by pH, organic modifier and other changes in the mobile phases. The mechanism of retention and chiral recognition of the enantiomers on CBH 58-silica is mainly the same as that of CBH I-silica. It should indeed be possible to design a compound based on the general β-blocker structure that could serve as a selective and very strong non-carbohydrate inhibitor useful for studies of polysaccharide degrading enzymes. The structural information is fundamental to an understanding of chiral recognition by cellulases.

5. Nomenclature

k'_x	Total capacity factor of an enantiomer calculated from the adapted function; $x=1$, 2 for the less and more retained enantiomer, respectively.
$k'_{ns,x}$	Calculated non-selective binding capacity factor of an enantiomer
$k'_{es,x}$	Calculated enantioselective binding capacity factor of an enantiomer
K_d	Dissociation (inhibition) constant for the competitor-protein binding

$k'_{x,C}$	Experimental capacity factor of an enantiomer from chromatography
K_M	Michaelis constant
k_{cat}	Catalytic constant (turnover number)
K_i	Inhibition constant
ns	Non-selective site
es	Enantioselective site
CBH	cellobiohydrolase
CSP	chiral stationary phases
HPLC	high-performance liquid chromatography

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References

[1] B. Henrissat, T.T. Teeri, R.A.J. Warren, FEBS Lett. 425 (1998) 352.
[2] P. Erlandsson, I. Marle, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Am. Chem. Soc. 112 (1990) 4573.
[3] I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr. 586 (1991) 233.
[4] S. Hjertén, Y.-M. Li, J.-L. Liao, J. Mohammad, K. Nakazato, G. Pettersson, Nature 356 (1992) 810.
[5] J. Mohammad, Y.-M. Li, M. El-Ahmad, K. Nakazato, G. Pettersson, S. Hjertén, Chirality 5 (1993) 464.
[6] L. Valtcheva, J. Mohammad, G. Pettersson, S. Hjertén, J. Chromatogr. 638 (1993) 263.
[7] M. Hedeland, R. Isaksson, C. Pettersson, J. Chromatogr. A 807 (1998) 297.
[8] S. Jönsson, A. Schön, R. Isaksson, C. Pettersson, G. Pettersson, Chirality 4 (1992) 505.
[9] M. Hedeland, H. Henriksson, P. Bäckman, R. Isaksson, G. Pettersson, Thermochim. Acta 356 (2000) 153.
[10] T. Fornstedt, P. Sajonz, G. Guiochon, J. Am. Chem. Soc. 119 (1997) 1254.
[11] C. Divne, J. Ståhlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J.K.C. Knowles, T.T. Teeri, T.A. Jones, Science 265 (1994) 524.
[12] J. Ståhlberg, C. Divne, A. Doivula, K. Piens, M. Claeyssens, T.T. Teeri, T.A. Jones, J. Mol. Biol. 264 (1996) 337.
[13] H. Henriksson, J. Ståhlberg, A. Koivula, G. Pettersson, C. Divne, L. Valtcheva, R. Isaksson, J. Biotechnol. 57 (1997) 115.
[14] J. Ståhlberg, H. Henriksson, C. Divne, R. Isaksson, G. Pettersson, G. Johansson, T.A. Jones, Paper V in thesis Chiral recognition on cellulases: the active sites of the enzymes are involved by Hongbin Henriksson, Acta Universitatis Upsaliensis: Comprehensive summaries of Uppsala Dissertation from the Faculty of Science and Technology 506, Uppsala, ISBN91-554-4643-4, 2000.
[15] E. Uzcategui, M. Raices, R. Montesino, G. Johansson, G. Pettersson, K.-E. Eriksson, Biotechnol. Appl. Biochem. 13 (1991) 323.
[16] E. Uzcategui, A. Ruiz, R. Montesino, G. Johansson, G. Pettersson, J. Biotechnol. 19 (1991) 271.
[17] I.G. Muñoz, J. Ståhlberg, C. Divne, G. Kleywegt, H. Henriksson, G. Johansson, S.L. Mowbray, T.A. Jones, presentation in the conference 'The role of cell wall degrading enzymes of *Trichoderma harzianum* in biocontrol' in Savalen Fjellhotell, Tynset, Norway, 31 July to 3 August 1998.
[18] H. Henriksson, G. Pettersson, G. Johansson, J. Chromatogr. A 857 (1999) 107.
[19] R. Bhikhabhai, G. Johansson, G. Pettersson, J. Appl. Biochem. 6 (1984) 336.
[20] I.J. Szabo, G. Johansson, G. Pettersson, J. Biotechnol. 48 (1996) 221.
[21] H. Henriksson, S. Jönsson, R. Isaksson, G. Pettersson, Chirality 7 (1995) 415.
[22] H. Henriksson, J. Ståhlberg, R. Isaksson, G. Pettersson, FEBS Lett. 390 (1996) 339.
[23] M. Hedeland, H. Henriksson, R. Isaksson, G. Pettersson, Chirality 10 (1998) 760.
[24] S. Allenmark, B. Bomgren, H. Borén, J. Chromatogr. 316 (1984) 617.
[25] M. Hedeland, S. Jönsson, R. Isaksson, C. Pettersson, Chirality 10 (1998) 513.