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Journal of Chromatography B, 745 (2000) 149–157

JOURNAL OF CHROMATOGRAPHY B

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Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein
VIII. Chiral recognition ability of partially and completely deglycosylated ovoglycoprotein

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Abstract

The influence of sugar moieties of ovoglycoprotein from chicken egg white (OGCHI) on chiral discrimination of various solutes has been investigated. Partially deglycosylated OGCHI (pd-OGCHI) and completely deglycosylated OGCHI (cd-OGCHI) were obtained by treatments of OGCHI with *N*-glycosidase, and a mixture of endoglycosidase and *N*-glycosidase, respectively. The average molecular masses of OGCHI, pd-OGCHI and cd-OGCHI were estimated to be about 30 000, 28 400 and 21 400, respectively, by matrix-assisted laser desorption time-of-flight mass spectrometry. The isoelectric points of OGCHI, pd-OGCHI and cd-OGCHI were in the ranges 4.37–4.51, 4.34–4.44 and 4.17–4.43, respectively, by isoelectric focusing. The OGCHI, pd-OGCHI and cd-OGCHI were bound to aminopropyl-silica gels activated with *N,N′*-disuccinimidylcarbonate to compare retentive and enantioselective properties of the three columns. It was found that pd-OGCHI showed excellent chiral recognition abilities comparable to OGCHI, and that the retentivity and enantioselectivity of basic solutes tested on the pd-OGCHI column were higher than those on the OGCHI column, while those of acidic solutes tested on the pd-OGCHI column were lower. Further, cd-OGCHI still showed chiral recognition abilities for various solutes tested. These results reveal that the chiral recognition site(s) for OGCHI exists on the protein domain of OGCHI.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Ovoglycoprotein

1. Introduction

Chiral stationary phases based on a glycoprotein are of special interest because of their unique properties of stereoselectivity and because they are suited for separating a wide range of enantiomeric mixtures. Those have included α_1-acid glycoprotein (AGP) [1,2], ovomucoid [3], avidin [4], ovoglycoprotein [5,6], cellobiohydrolase I [7], ovotransferrin [8] and flavoprotein [9,10]. Recently, we found that ovomucoid from chicken egg whites (OMCHI) used in previous studies was crude, and that crude OMCHI included about 10% of other glycoprotein [5]. In addition, we isolated and characterized the glycoprotein from the crude OMCHI

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00070-0

preparations and chicken egg whites [5]. We named the isolated protein OGCHI (which means ovoglycoprotein from chicken egg whites). The glycoprotein, OGCHI, had an average molecular mass of 29 700 and sugar content of 25%. Further, isolated OMCHI and OGCHI, respectively, were bound to aminopropyl-silica gels and the chiral recognition abilities of the two columns were compared. It was found that the chiral recognition ability of impure OMCHI reported by Miwa et al. [3] came from OGCHI, and that OGCHI gave significantly higher chiral recognition abilities than the impure OMCHI.

A glycoprotein consists of a protein domain and sugar moieties, both of which are chiral components. With regard to the chiral recognition mechanism on a glycoprotein-based stationary phase, the role of sugar moieties in enantioselective bindings by a glycoprotein has not been precisely investigated. It was thought that drug binding to AGP occurred at a single hydrophobic pocket or cleft within the protein domain of the molecule [11,12]. Recently, Shiono et al. [13] reported that sialic acid residues influence the enantioselective binding of basic drugs in different ways. They are not involved in the enantioselective verapamil-AGP binding, but participate in the binding of (*S*)-propranolol but not (*R*)-propranolol.

The domains of ovomucoid from turkey egg whites (OMTKY), which exist as three tandem, independent domains [14], were isolated, purified and characterized [15]. Furthermore, columns were made with purified OMTKY domains to test chiral recognition properties. The third domains of OMTKY consisted of glycosylated and unglycosylated (OMTKY3) domains. The third domains of OMTKY were found to be enantioselective to at least two classes of compounds, benzodiazepins and 2-arylpropionic acid derivatives. The chiral recognition mechanism of OMTKY3 was elucidated by using ^{1}H-nuclear magnetic resonance (NMR) measurements, molecular modeling and computational chemistry, where electrostatic, hydrogen bonding and hydrophobic interactions played an important role in chiral recognition of drug enantiomers [14]. Further, glycosylation of the third domain did not affect chiral recognition.

In this paper, we prepared pd-OGCHI and cd-OGCHI with enzymatic cleavage of the sugar moieties of OGCHI, and characterized them by isoelectric focusing (IEF), high-performance capillary electrophoresis (HPCE), reversed-phase high-performance liquid chromatography (HPLC) and matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Next, OGCHI, pd-OGCHI and cd-OGCHI were bound to aminopropyl-silica gels activated with *N*,*N*′-disuccinimidylcarbonate (DSC). The chiral recognition abilities of the three columns were compared, and the enantioselective binding properties of three columns were discussed. In addition, we clarify which parts of OGCHI, a protein domain or sugar moieties, play an important role in the chiral recognition ability of OGCHI.

2. Experimental

2.1. Reagents and materials

Benzoin, ketoprofen, DSC and Tween 20 were purchased from Wako (Osaka, Japan). Ibuprofen was donated by Chugai (Tokyo, Japan). Hexobarbital was donated by Teikoku (Tokyo, Japan). Chlorpheniramine maleate, alprenolol hydrochloride, propranolol hydrochloride, oxprenolol hydrochloride and endoglycosidase F/*N*-glycosidase F were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Recombinant *N*-glycosidase F was purchased from Boehringer Mannheim (Mannheim, Germany). D-Glucosamine hydrochloride was from Nacalai Tesque (Kyoto, Japan). A capillary coated with dimethyl polysiloxane (DB-1 capillary) (50 μm I.D., 0.1 μm film thickness) was obtained from GL Sciences (Tokyo, Japan). The silica gels (Ultron-12, 5 μm diameter, 12 nm pore size) were from Shinwa (Kyoto, Japan). Sephadex G-25 (fine) and SP Sepharose FF were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Other reagents and solvents of analytical- or HPLC-grade were obtained from Wako (Osaka, Japan) and used without further purification.

Water, purified using a Nanopure II unit (Barnstead, Boston, MA, USA), was used for the preparation of the eluent and the sample solution.

2.2. Isolation of OGCHI from egg whites

OGCHI was isolated as reported previously [16]. Briefly, crude OMCHI was precipitated from the

corresponding egg whites with ethanol according to the procedures modified slightly from those of Fredericq and Deutsch [17]. The obtained crude OMCHI was further purified by cation-exchange chromatography. A 2-g amount of crude OMCHI was applied to SP Sepharose FF column (12 cm×5 cm I.D.) that was equilibrated with 10 m*M* CH_3COONH_4 (pH 4.6) applying a linear gradient to 700 m*M* CH_3COONH_4 (pH 4.6) for 6 h at flow-rate of 100 ml/h, and then the eluent was changed to 1000 m*M* CH_3COONH_4 (pH 4.6). The eluent was monitored at 280 nm with a Model AC-500 spectrophotometric monitor (Atto, Tokyo, Japan). The separation was performed at 4°C. The OGCHI was desalted with a Sephadex G-25 (fine) column (20 cm×5 cm I.D.) using 15 m*M* NH_4HCO_3 (pH 9.0) as the eluent with an average flow-rate of 120 ml/h. The eluate was collected and lyophilized.

2.3. Preparation of pd-OGCHI and cd-OGCHI

A 30-mg amount of OGCHI was mixed with one unit of recombinant *N*-glycosidase F in 3 ml of 100 m*M* phosphate buffer (pH 7.2) and incubated at 37°C for 24 h. The pd-OGCHI obtained was dialyzed against 15 m*M* NH_4HCO_3 solution and lyophilized.

A 30-mg amount of OGCHI was reacted with two units of endoglycosidase F/*N*-glycosidase F in 3 ml of 100 m*M* acetate buffer (pH 5.0) at 37°C for 48 h. The obtained precipitate, cd-OGCHI, was dissolved in 15 m*M* NH_4HCO_3 solution, dialyzed against the same solution and lyophilized.

2.4. Preparation of OGCHI, pd-OGCHI and cd-OGCHI materials

Aminopropyl-silica gels were prepared from silica gels, and activated by DSC as reported previously [5]. OGCHI, pd-OGCHI and cd-OGCHI were bound to aminopropyl-silica gels activated by DSC as described previously [5]. Briefly, 1 g of the DSC-activated silica gels was slurried in 20 m*M* sodium phosphate buffer (pH 6.8). 0.666 μmol of OGCHI, pd-OGCHI and cd-OGCHI, which corresponds to 20.0, 18.9 or 14.2 mg, respectively, was dissolved in 20 ml of the same buffer. Then the solution was slowly added to the silica gel slurry at room temperature for 1 h by adjusting the pH to 6.6, and the resulting mixture was further stirred for 15 h at 30°C. The reaction mixture was filtered using a glass filter and washed with water. The obtained materials were dissolved in 20 ml of a blocking solution (pH 6.6) including 300 m*M* D-glucosamine and was stirred slowly for 1 h at room temperature. Then the reaction mixture was filtered using a glass filter, and washed with water and methanol. The obtained materials were packed into a stainless steel column (100 mm×2.0 mm I.D.) using a slurry packing method. The slurry and packing solvent was water–ethanol (95:5, v/v).

2.5. Determination of the amount of OGCHI, pd-OGCHI and cd-OGCHI bound to silica gels

The bound amounts of OGCHI, pd-OGCHI and cd-OGCHI to DSC-activated aminopropyl-silica gels were determined as follows. After reaction with a respective protein, the materials were filtered using a glass filter, and washed with water. All filtrated solutions were collected, and their volumes were measured. The concentration of each protein was determined using a reversed-phase chromatographic method as described below, and the unbound amounts of the protein were estimated. The bound amounts of a protein were calculated by subtraction of the unbound amounts of the protein from the reacted amounts.

2.6. Evaluation of chiral recognition abilities of OGCHI, pd-OGCHI and cd-OGCHI

For chiral resolution of racemic solutes on the OGCHI, pd-OGCHI and cd-OGCHI columns, a HPLC system consisting of an LC-10Avp pump, an SPD-10Avp spectrophotometer, a Rheodyne 7125 injector with a 5-μl loop and C-R6A integrator (all from Shimadzu, Kyoto, Japan) was used. The flow-rate was maintained at 0.2 ml/min. Detection was performed at 210 nm. All separations were carried out at 25°C using a water bath (Thermo Minder JR-100, Taitec, Saitama, Japan). The eluents used were specified in the legends of the figures and tables.

2.7. Isoelectric focusing

IEF of proteins was carried out on a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech),

thermostated at 10°C using a Multitemp II thermostatic circulator. A Multidrive XL electrophoresis constant power supply was used in conjunction with a volt–hour integrator. Ampholine PAG plate pH 3.5–9.5 (Amersham Pharmacia Biotech) was used at 1500 V, 50 mA for 30 min with 1 *M* phosphoric acid as anolyte and 1 *M* sodium hydroxide as catholyte. After IEF was completed, the gel was removed and stained with Coomassie Brilliant Blue R-250.

2.8. High-performance capillary electrophoresis

Capillary electrophoresis was performed with a P/ACE 5010 capillary electrophoresis system (Beckman, Fullerton, CA, USA). A 20 m*M* sodium acetate (pH 5.4) buffer containing 0.5% Tween 20 was used as a running buffer. The detection window was made at 7 cm from the outlet of a DB-1 capillary [27 cm (20 cm effective length)×50 μm I.D.] by carefully removing the polyimide coating with a razor under a microscope, and the transparent portion was fixed on the detector block. The capillary was washed with methanol for 10 min and then with water for 10 min. Each analysis was performed after washing the capillary with the running buffer for 10 min. Injections were automatically performed at ca. 3500 Pa for 2 s from the cathodic end. The separation was carried out at 30°C. The applied potential was −25 kV. Detection was performed with the on-line mode while the UV absorption was monitored at 280 nm. Data were collected and analyzed with a standard System Gold software (Version 8.10) on an IBM personal computer.

2.9. Reversed-phase high-performance liquid chromatography

For reversed-phase chromatographic separations of OGCHI, pd-OGCHI and cd-OGCHI, the same HPLC systems as described above was used except that two pumps were used for gradient elution. The eluents used were as follows: eluent A, water–acetonitrile (80:20, v/v) including 0.1% trifluoroacetic acid (TFA); eluent B, water–acetonitrile (20:80, v/v) including 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. The column used was Cosmosil 5C18-AR (250 mm×4.6 mm I.D., Nacalai Tesque). Detection was carried out at 280 nm. The flow-rate was 1.0 ml/min. Loaded amounts of a protein were 50 μg. All separations were performed at 30°C using a water bath.

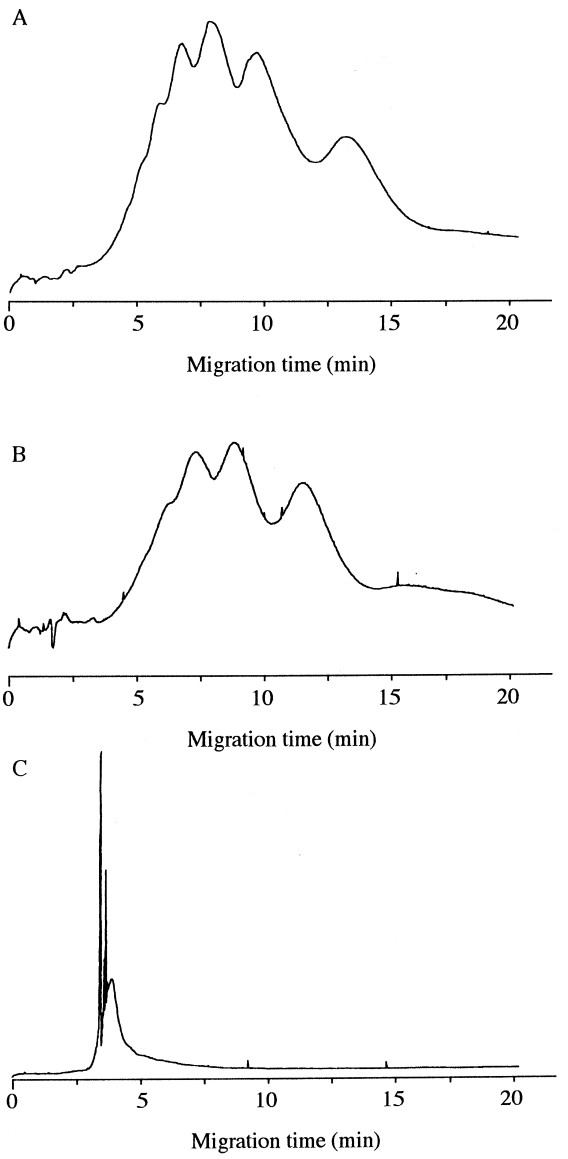

Fig. 1. Electropherograms of OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C). HPCE conditions: capillary, DB-1 fused-silica [27 cm (20 cm effective length)×50 μm I.D.]; running buffer, 20 m*M* sodium acetate (pH 5.4) containing 0.5% Tween 20; applied voltage, −25 kV; detection, UV absorption at 280 nm; sample concentration, 1 mg/ml; injection, ca. 3500 Pa for 2 s.

2.10. Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry

MALDI-TOF mass spectra were measured using a Vision 2000 reflector-type TOF instrument (Thermoquest, Tokyo, Japan) equipped with an N_2 laser operating at a wavelength of 337 nm with a pulse duration of 3 ns. The ions generated were accelerated to a potential of 5 kV in the ion source and postaccelerated to a potential of 20 kV for detection with a secondary ion multiplier. The MALDI-TOF mass spectra represent the accumulation of 15–20 single laser shots. They were calibrated externally by a standard sample (bovine serum albumin, molecular mass of 66 430) that was placed on the same target. The matrix used was 2,5-dihydroxybenzoic acid, dissolved in a 2:1 mixture of 0.1% aqueous TFA and acetonitrile at a concentration of 50 m*M*. Samples were dissolved in a water at a concentration of 10^{-6} *M*. A 0.5-μl portion of the sample solution was mixed with an equal volume of the matrix solution on the target, resulting in a used sample amount of 500 fmol. After deposition on the stainless steel target, the sample was air-dried and introduced into the mass spectrometer.

3. Results and discussion

3.1. Characterization of OGCHI, pd-OGCHI and cd-OGCHI

OGCHI, pd-OGCHI and cd-OGCHI were characterized by IEF, HPCE, reversed-phase HPLC and MALDI-TOF-MS. The isoelectric point (p*I*) of OGCHI was in the range 4.37–4.51 by IEF, while it was shown that the p*I* values of pd-OGCHI and cd-OGCHI were in the ranges 4.34–4.44 and 4.17–4.43, respectively. OGCHI had a slightly higher p*I* value than pd-OGCHI or cd-OGCHI. Fig. 1A–C shows electropherograms of OGCHI, pd-OGCHI and cd-OGCHI, respectively, where 20 m*M* sodium acetate buffer (pH 5.4) containing 0.5% Tween 20 was used as the running buffer solution. Since the capillary was coated with dimethyl polysiloxane, the value of electroosmotic flow, $3.00 \cdot 10^{-4}$ cm^2 V^{-1} S^{-1} at pH 5.0 using benzoin as the neutral marker, was negligible. The p*I* values of OGCHI, pd-OGCHI and cd-OGCHI being below 5.4, OGCHI, pd-OGCHI and cd-OGCHI electrophoretically migrated toward the anodic end. Broad peaks of OGCHI and pd-OGCHI should be due to the microheterogeneity of sugar moieties.

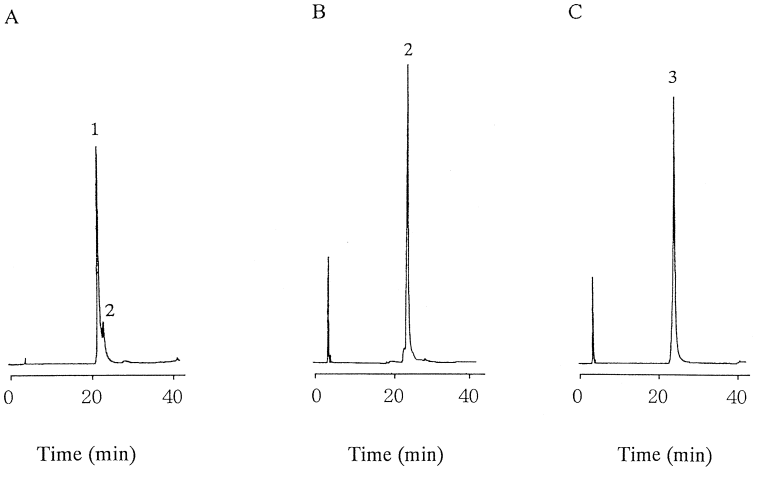

Fig. 2. Reversed-phase chromatograms of OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C). Column: Cosmosil 5C18-AR (250 mm×4.6 mm I.D.). Eluents: eluent A, water–acetonitrile (80:20, v/v) including 0.1% TFA; eluent B, water–acetonitrile (20:80, v/v) including 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. Flow-rate: 1.0 ml/min. Detection at 280 nm. Loaded amount: 50 μg.

Fig. 2A–C shows chromatograms of OGCHI, pd-OGCHI and cd-OGCHI, respectively, by reversed-phase chromatography. In a previous communication [18], we reported that OGCHI was present as fully and partially glycosylated OGCHIs with a ratio of ca. 4:1, which correspond to peaks 1 and 2, respectively, in Fig. 2A. With an increase in the incubation time of OGCHI with *N*-glycosidase, intensity of peak 1 in Fig. 2 is decreased with a concomitant increase in that of peak 2. After completion of the enzymatic reaction, peak 2 was mainly observed as shown in Fig. 2B. This result indicates that an *N*-linked sugar chain(s) of OGCHI is cleaved to yield pd-OGCHI, and that OGCHI is either fully or partially glycosylated with a ratio of ca. 4:1. With regard to complete deglycosylation of OGCHI, we used a 1:1 mixture of endoglycosidase and *N*-glycosidase. As shown in Fig. 2C, peak 3 having a longer retention time than OGCHI and pd-OGCHI appeared with an increase in the incubation time of OGCHI with the enzymes. However, when only endoglycosidase was used, the obtained protein gave the same retention times with OGCHI.

Fig. 3A–C shows MALDI-TOF mass spectra of OGCHI, pd-OGCHI and cd-OGCHI, respectively. The average molecular masses, averaged from $[M+H]^+$, $[2M+H]^+$ and $[3M+H]^+$ ions, are as follows; 30 004±90 for OGCHI, 28 362±54 for pd-OGCHI and 21 359±35 for cd-OGCHI. The MALDI-TOF mass spectra of the pd-OGCHI yielded an average molecular mass of 28 362, which is 1642 lower than the molecular mass of OGCHI. Taking into account the amount expected for a sugar chain [15], one sugar chain of OGCHI might be cleaved by *N*-glycosidase. The average molecular mass of the cd-OGCHI was estimated to be 21 359, which is 8645 lower than the molecular mass of OGCHI. Previously [5], we reported that the carbohydrate content of OGCHI was about 25%. Furthermore, the sugar moieties of OGCHI were completely cleaved by treatment with *N*-glycosidase following denaturation of OGCHI by sodium dodecyl sulfate. The average molecular mass of the obtained protein, estimated by MALDI-TOF-MS, was consistent with that of the cd-OGCHI. These results suggested that without denaturation of OGCHI, complete removal of the sugar moieties of OGCHI could be attained by treatment with a mixture of endoglycosidase and *N*-glycosidase.

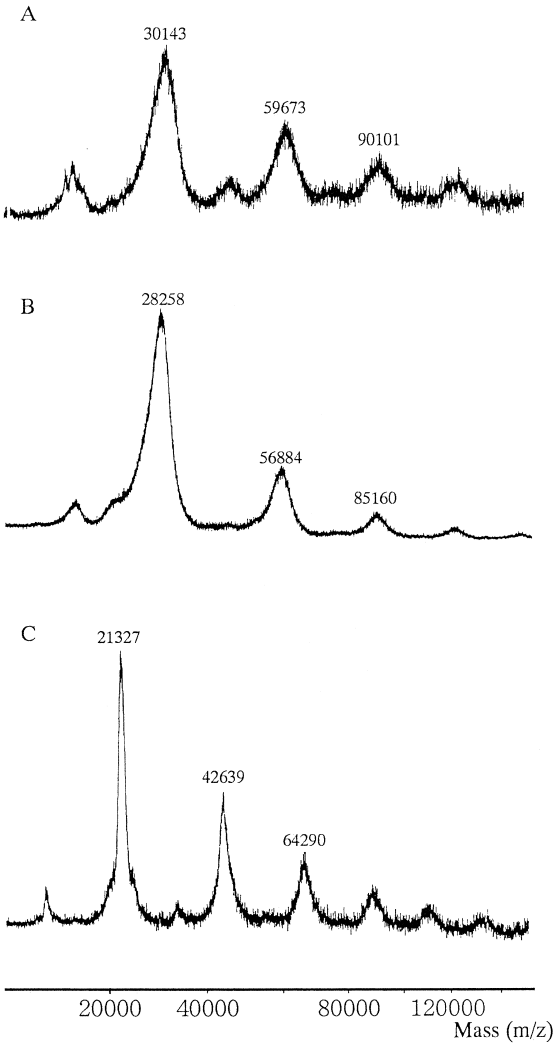

Fig. 3. MALDI-TOF mass spectra of OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C). Flight mode: reflector-type. Matrix: 2,5-Dihydroxybenzoic acid. Other conditions as in Experimental.

3.2. Comparison of retentivity, enantioselectivity and resolution of racemic solutes on OGCHI, pd-OGCHI and cd-OGCHI columns

OGCHI, pd-OGCHI and cd-OGCHI, respectively, were bound to DSC-activated aminopropyl-silica gels in order to compare chiral recognition properties of OGCHI, pd-OGCHI and cd-OGCHI. Since linear correlation is obtained between the retention factor of each enantiomer and the bound amount of OGCHI [16], it is essential to immobilize the same molar amount of OGCHI, pd-OGCHI and cd-OGCHI in

Table 1
Chiral resolution of various solutes on OGCHI, pd-OGCHI and cd-OGCHI columns[a]

Solute	Column								
	OGCHI			pd-OGCHI			cd-OGCHI		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
Neutral solute									
Benzoin	7.58	3.15	6.92	7.88	3.16	7.54	4.37	3.26	5.30
Hexobarbital	1.22	1.39	1.34	1.26	1.41	1.44	0.89	1.22	0.57
Basic solute									
Chlorpheniramine	4.14	2.69	5.32	5.47	2.85	6.57	3.07	2.57	4.75
Alprenolol	12.13	1.10	0.38	17.54	1.13	0.63	6.87	1.00	–[b]
Propranolol	32.41	1.00	–	45.62	1.10	0.38	15.96	1.16	0.45
Oxprenolol	6.47	1.20	1.86	9.50	1.26	1.57	3.47	1.22	0.72
Acidic solute									
Ibuprofen	6.44	1.28	1.86	5.05	1.25	1.53	4.75	1.15	0.81
Ketoprofen	20.50	1.38	2.78	14.70	1.35	2.60	10.71	1.21	1.29

[a] HPLC conditions: column, 100 mm×2.0 mm I.D.; eluent, 20 m*M* sodium dihydrogenphosphate–disodium hydrogenphosphate (pH 5.1)–ethanol (95:5, v/v); flow-rate, 0.2 ml/min; column temperature, 25°C; detection at 210 nm. The retention factor of the first-eluted enantiomer is calculated from the equation $k_1=(t_{R1}-t_0)/t_0$, where t_{R1} and t_0 are the retention times of the first-eluted enantiomer and unretained solute, respectively. The enantioseparation factor is calculated from the equation $\alpha=k_2/k_1$, where k_2 is the retention factor of the second-eluted enantiomer. Resolution is calculated from the equation $R_s=2(t_{R2}-t_{R1})/(w_1+w_2)$, where t_{R2} is the retention time of the second-eluted enantiomer, and w_1 and w_2 are the baseline peak widths.
[b] Not enantioseparated.

order to compare the retentivity and enantioselectivity of these columns. The reaction amounts of OGCHI, pd-OGCHI and cd-OGCHI per 1 g of the activated silica gel were 0.666 μmol, which corresponds to 20.0 mg, 18.9 mg and 14.2 mg for OGCHI, pd-OGCHI and cd-OGCHI, respectively. At these reactions, OGCHI, pd-OGCHI and cd-OGCHI were completely bound to aminopropyl-silica gels.

Table 1 shows the comparison of the retention factors, enantioseparation factors and resolution of neutral, acidic and basic solutes on OGCHI, pd-OGCHI and cd-OGCHI columns, where 20 m*M* sodium dihydrogenphosphate–disodium hydrogenphosphate (pH 5.1)–ethanol (95:5, v/v) was used as an eluent. Figs. 4–6 show typical chromatograms of benzoin, chlorpheniramine and ibuprofen, respec-

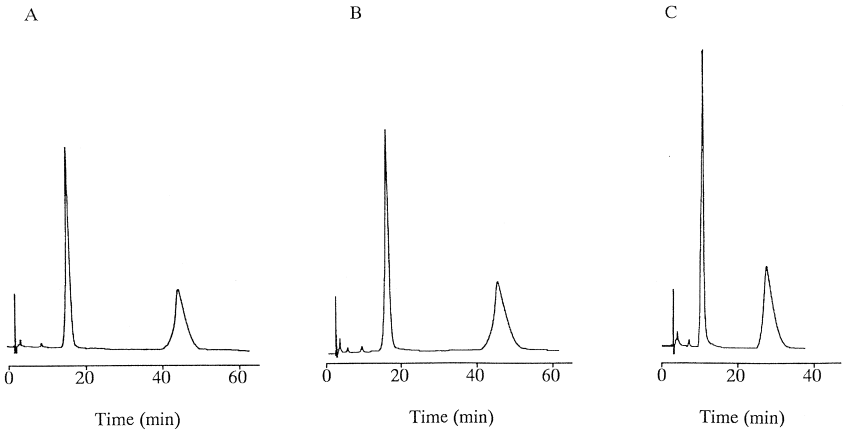

Fig. 4. Chromatograms of benzoin on OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C) columns. HPLC conditions as in Table 1.

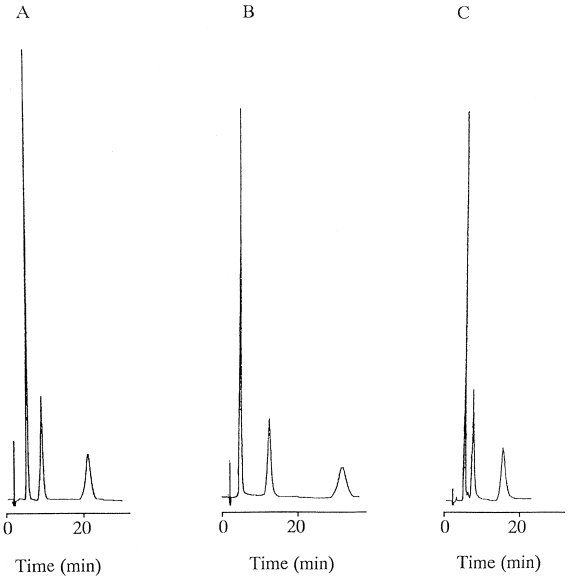

Fig. 5. Chromatograms of chlorpheniramine on OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C) columns. HPLC conditions as in Table 1.

tively, on these three columns. With regard to the retentivity of the solutes tested, the retention factors of neutral and basic solutes were in the order pd-OGCHI>OGCHI>cd-OGCHI. The enantioseparation factors and resolution of neutral and basic solutes were in the order pd-OGCHI>OGCHI>cd-OGCHI, except for benzoin, propranolol and oxprenolol. On the other hand, retention factors, enantioseparation factors and resolution of acidic solutes were in the order OGCHI>pd-OGCHI>cd-OGCHI. The results are summarized as follows. The OGCHI and pd-OGCHI columns give longer retentions, and higher enantioselectivity and resolution for solutes tested than the cd-OGCHI column, except for enantioseparation factors and/or resolution of benzoin, propranolol and oxprenolol. The OGCHI column shows better chiral recognition ability for acidic solutes tested than the pd-OGCHI column, and less chiral recognition ability for basic solutes tested. Further, cd-OGCHI still shows chiral recognition ability for various solutes tested.

Table 2 shows the effect of eluent pH on the retention factors and enantioseparation factors of various solutes. As reported previously [19], electrostatic and hydrophobic interactions mainly worked in the retentivity and enantioselectivity of various solutes on protein-based chiral stationary phases. Acidic solutes, whose pK_a values are ca. 4.5, were not retained at eluent pH>5 because of ionic repulsion and/or ionic exclusion, while basic solutes were well retained and resolved with an increase in eluent pH because of ionic and hydrophobic interactions. On the other hand, the retentivity and enantioselectivity of neutral solutes were not so affected by eluent pH changes. As shown in Table 2, there are almost similar tendencies of the retentivity and enantioselectivity of OGCHI, pd-OGCHI and cd-OGCHI columns toward pH changes.

In conclusion, OGCHI is either fully or partially glycosylated with a ratio of ca. 4:1. Fully and partially glycosylated OGCHI are different in chiral recognition properties; the former shows excellent chiral recognition ability for acidic solutes tested and the latter for basic solutes tested. The pd-OGCHI column showed similar stability against eluent pH change and repetitive injections of a solute with the OGCHI column. These two columns could be complementarily used for enantioseparations of various

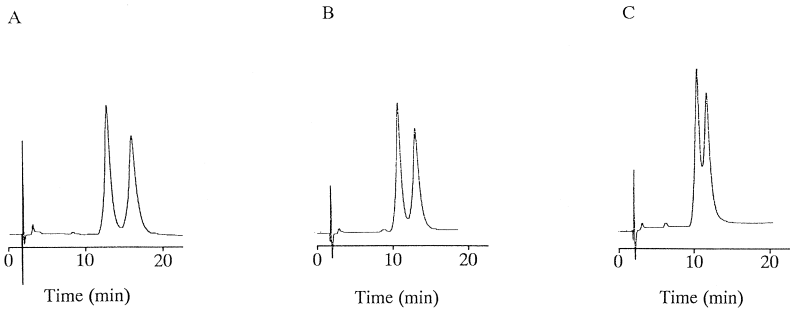

Fig. 6. Chromatograms of ibuprofen on OGCHI (A), pd-OGCHI (B) and cd-OGCHI (C) columns. HPLC conditions as in Table 1.

Table 2
Influence of eluent pH on the retention factors and enantioseparation factors of various solutes on OGCHI, pd-OGCHI and cd-OGCHI columns[a]

Solute	Eluent pH	Column					
		OGCHI		pd-OGCHI		cd-OGCHI	
		k_1	α	k_1	α	k_1	α
Benzoin	4.0	5.92	2.87	5.94	2.90	2.99	2.80
	5.1	7.58	3.15	7.88	3.16	4.37	3.26
	6.0	7.65	2.93	5.78	2.92	3.98	3.08
	7.0	8.37	2.41	5.11	2.44	4.57	2.58
Chlorpheniramine	4.0	0.33	2.67	0.26	2.89	0.07	2.00
	5.1	4.14	2.69	5.47	2.85	3.07	2.57
	6.0	18.79	2.99	18.85	2.89	9.12	2.67
Ibuprofen	4.0	12.46	1.48	12.43	1.51	8.97	1.30
	5.1	6.44	1.28	5.05	1.25	4.75	1.15
	6.0	1.90	1.14	1.51	1.00	1.35	1.00
	7.0	0.51	1.00	0.51	1.00	0.41	1.00

[a] HPLC conditions: column, 100 mm×2.0c mm I.D.; eluent, 20 m*M* sodium dihydrogenphosphate–disodium hydrogenphosphate–ethanol (95:5, v/v); flow-rate, 0.2 ml/min; column temperature, 25°C; detection at 210 nm.

solutes. Without denaturation of OGCHI, complete removal of the sugar moieties of OGCHI are attained by treatment with a mixture of endoglycosidase and *N*-glycosidase. Completely deglycosylated OGCHI shows chiral recognition ability for various solutes tested. These results reveal that the chiral recognition site(s) for OGCHI is present on the protein domain of OGCHI. Further study is in progress to clarify the chiral recognition site(s) and chiral recognition mechanism of OGCHI.

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