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Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein
V. Influence of immobilization method on chiral resolution

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Abstract

Ovoglycoprotein from chicken egg whites (OGCHI) was bound to aminopropyl-silica gels via an amino or carboxyl group(s) of OGCHI. In the former case, OGCHI was bound to N,N'-disuccinimidyl carbonate-activated aminopropyl-silica gels, while in the latter case OGCHI activated by a water-soluble carbodiimide and N-hydroxysulfosuccinimide was bound to aminopropyl-silica gels. The obtained OGCHI materials were compared with regard to the bound amounts, retentivity and enantioselectivity. The OGCHI materials prepared via a carboxyl group(s) of OGCHI are suitable for chiral resolution of acidic solutes, and those via an amino group(s) of OGCHI are suitable for chiral resolution of basic solutes. It is suggested that the electrostatic interaction between an amino or carboxyl group of OGCHI and a charged solute should play an important role in chiral recognition of the solute. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Miwa et al. [1] prepared chiral stationary phases based on ovomucoid from chicken egg whites (OMCHI) and utilized for the separation of enantiomeric compounds. Recently [2], we found that OMCHI used in previous studies was crude. In addition, we isolated a glycoprotein from chicken egg whites. The isolated protein was characterized by reversed-phase chromatography, N-terminal sequencing, matrix-assisted laser desorption time-of-

flight (MALDI-TOF) mass spectrometry, determination of sugar contents, and trypsin inhibitory activities [2]. Previously [3], Ketterer isolated a glycoprotein from chicken egg whites and termed it OGCHI (which means ovoglycoprotein from chicken egg whites). Much data support the belief that the protein isolated by us might be OGCHI, but the only discrepancy was the average molecular weight. We also termed the isolated protein OGCHI. It was found that about 10% OGCHI was included in crude OMCHI preparations [2]. In addition, the chiral recognition ability of OMCHI reported by Miwa et al. [1] came from OGCHI, and pure OMCHI had no chiral recognition ability. Further, we clarified that the good chiral recognition ability of a stationary

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phase based on crude OMCHI was due to preferential binding of OGCHI to *N*,*N*′-disuccinimidyl carbonate (DSC)-activated aminopropyl-silica gels compared with pure OMCHI [4].

The immobilization method employed so far has been based on DSC-activated aminopropyl-silica gels, where OGCHI was bound to silica gels via an amino group(s) of OGCHI. In this study, we tried to bind OGCHI via a carboxyl group(s) activated by a water-soluble carbodiimide and *N*-hydroxysulfosuccinimide to aminopropyl-silica gels. This paper reports the optimization of the immobilization method via a carboxyl group of OGCHI with regard to reaction pH, amount of activation reagents, and comparison of the bound OGCHI amounts and chiral recognition abilities of OGCHI materials prepared via amino and carboxyl groups of OGCHI.

2. Experimental

2.1. Reagent and materials

Benzoin and chlorpheniramine maleate were purchased from Nacalai Tesque (Kyoto, Japan). Ibuprofen and ketoprofen were donated by Chugai Pharmaceutical (Tokyo, Japan). Hexobarbital was donated by Teikoku Chemicals (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), DSC and D-glucosamine hydrochloride were purchased from Wako Pure Chemical Industry (Osaka, Japan). Sodium *N*-hydroxysulfosuccinimide (HSSI) was purchased from Pierce (Rockford, IL, USA). Sephadex G-25 (fine) and SP Sepharose FF were purchased from Pharmacia Biotech (Tokyo, Japan). Silica gels (Ultron-12, 5 μm diameter, 12 nm pore size, 300 m^2/g specific surface area) used were from Shinwa Chemical Industries (Kyoto, Japan). Other solvents and reagents were used without further purification.

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

2.2. Isolation of OGCHI from egg whites

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

2.3. Preparation of OGCHI materials

Aminopropyl-silica gels were prepared from silica gels as reported previously [5]. OGCHI was bound to aminopropyl-silica gels by two methods as shown in Fig. 1. In Fig. 1A the method includes activation of porous aminopropyl-silica gels by DSC, binding of OGCHI and blocking of the activated amino groups. The preparation method was optimized and reported previously [5]. Briefly, 1 g of the DSC-activated silica gels was slurried in 20 mM phosphate buffer (pH 6.8). To the mixture, OGCHI dissolved in 20 ml of the same buffer (4 mg/ml) was added slowly at room temperature for 1 h by adjusting the pH to 6.6, and further stirred for 15 h at 30°C. The reaction mixture was washed with water and dissolved in 20 ml of a blocking solution adjusted to pH 6.6, including 300 mM D-glucosamine, at room temperature for 1 h. The reaction mixtures were then filtered and washed with water and water–ethanol (95:5, v/v).

OGCHI was bound to aminopropyl-silica gels via a carboxyl group(s) of OGCHI activated by EDC and HSSI, as shown in Fig. 1B. A weight of 1 g of aminopropyl-silica gels was slurried in 20 mM phosphate buffer (pH 5.0). To the mixture, 20 ml of an OGCHI solution of the same buffer (4 mg/ml) was added slowly at room temperature for 1 h by adjusting the pH to 5.0, and further stirred for 24 h at
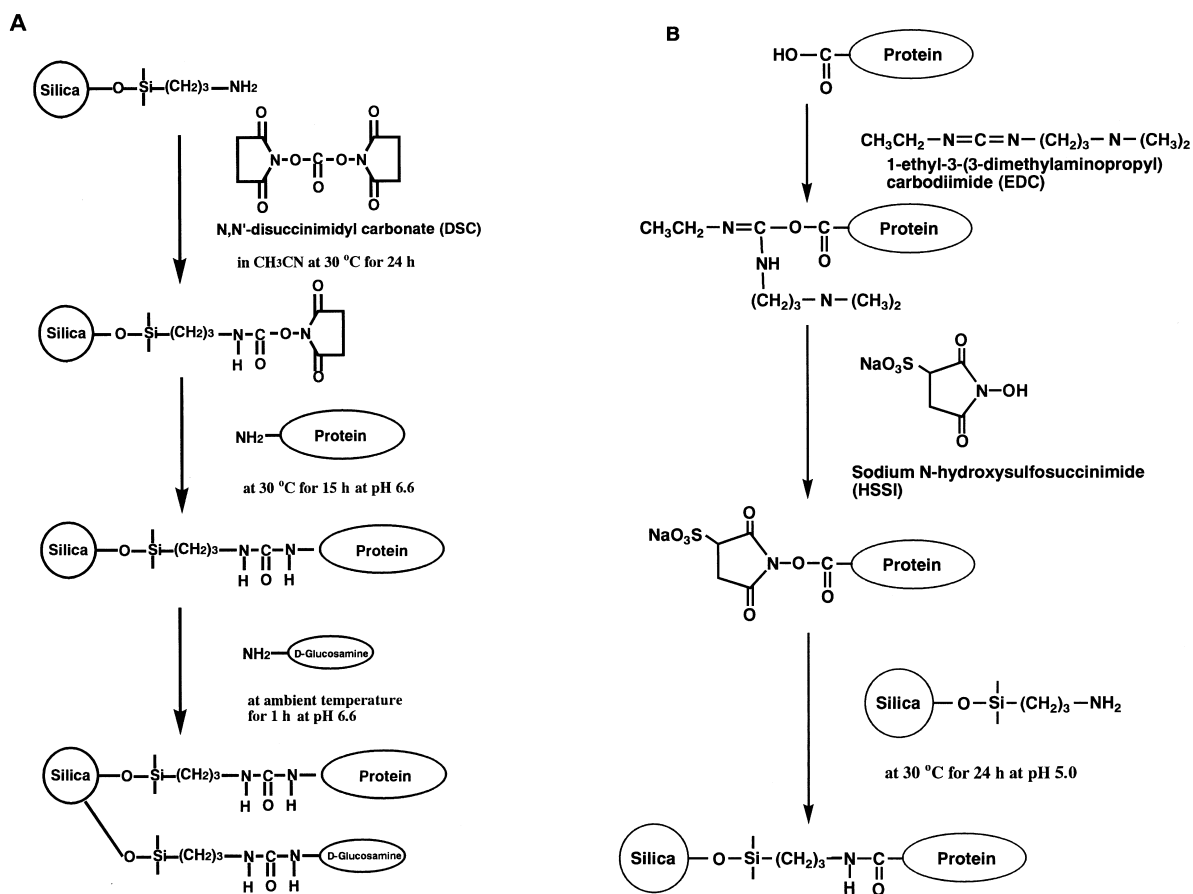
Fig. 1. Synthesis scheme for the preparation of OGCHI-based stationary phases. (A) Via an amino group of OGCHI; (B) via a carboxyl group of OGCHI.

30°C. Then 0.2 g of EDC and 0.1 g of HSSI dissolved in 0.5 ml of the same buffer was added and stirred for 24 h at 30°C. The reaction mixtures were filtered and washed with water and water–ethanol (95:5, v/v).

The obtained materials were packed into a 2.0 mm I.D.×100 mm stainless-steel column by the slurry packing method [7]. The slurry and packing solvents were water–ethanol (95:5, v/v).

2.4. Chromatography

2.4.1. Separation of enantiomer

For chiral resolution of racemic solutes on the OGCHI columns, the HPLC system used was composed of an LC-9A pump, an SPD-6A spec-

trophotometer, a Rheodyne 7125 injector with a 5-μl loop and a C-R6A integrator (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.2 ml/min. Detection was at 220 nm. The retention factor was calculated from the equation $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of retained and unretained solutes, respectively; k_1 and k_2 correspond to the retention factors of the first- and second-eluted peaks, respectively. The retention time of unretained solute, t_0, was measured by injecting a solution the organic modifier content of which was slightly different from that of the eluent used. The enantioseparation factor is calculated from the equation $\alpha = k_2/k_1$. Resolution is calculated from the equation $Rs = 2(t_2 - t_1)/(tw_1 + tw_2)$, where t_1 and t_2 are the retention times of the first- and second-eluted

peaks, respectively, and tw_1 and tw_2 are the baseline peak widths. All separations were carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluents were prepared using phosphoric acid, sodium dihydrogenphosphate or disodium hydrogenphosphate and ethanol. The eluents used are specified in the legends of figures and tables.

2.4.2. Separation of OGCHI

For reversed-phase chromatographic separations of OGCHI, the same HPLC system as described above was used except that two pumps were used for gradient elution. The eluents used were as follows: eluent A, water–CH_3CN (80:20, v/v) including 0.1% trifluoroacetic acid (TFA); eluent B, water–CH_3CN (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 (4.6 mm I.D.×250 mm, Nacalai Tesque, Kyoto, Japan). Detection was carried out at 280 nm. The flow-rate was 1.0 ml/min. All separations were performed at 30°C using a CO-1093C column oven (Uniflows, Tokyo, Japan).

2.5. Determination of OGCHI bound to silica gels

The amount of OGCHI bound to silica gels was determined as follows. After reaction with OGCHI, the obtained materials were washed with water. All wash solutions were collected and their volumes

were measured. A 50-μl portion of the solution was loaded onto the reversed-phase column under the conditions described in Section 2.4.2 and the OGCHI concentration was determined. The amount of OGCHI that reacted was determined by subtracting the amount of OGCHI measured in the wash solution after reaction from the amount initially added to the reaction.

2.6. Sample preparation

A known amount of a racemic solute was dissolved in methanol or water and the solution was diluted with the eluent at a concentration of 20 μg/ml. A 5-μl aliquot of the sample solution was loaded onto a column. The loaded amount was 0.5 nmol.

3. Results and discussion

3.1. Reaction conditions for immobilizing OGCHI via a carboxyl group(s) to aminopropyl-silica gels

In a previous paper [5], the influence of the pore size of the base silica gels and bound amounts of OGCHI on the chiral resolution of racemates was investigated, where aminopropyl-silica gels activated by DSC were used. The results obtained revealed that the OGCHI materials prepared with the 12-nm pore size silica gel gave the largest retention factor, and the highest enantioselectivity and resolution for

Table 1
Influence of EDC and HSSI amounts on bound OGCHI amounts and chiral resolution of benzoin, chlorpheniramine and ibuprofen[a]

Material	EDC/HSSI (g/g)	Bound amount (%)[b]	Solute[c]								
			Benzoin			Chlorpheniramine			Ibuprofen		
			k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
A	0.10/0.05	29.1	1.32	2.85	4.26	0.48	2.43	1.86	2.24	1.18	0.65
B	0.20/0.05	43.0	1.52	2.84	4.92	0.47	2.52	1.79	2.58	1.18	0.73
C	0.10/0.10	45.0	1.72	3.03	6.47	0.47	2.52	1.79	2.64	1.26	1.15
D	0.20/0.10	60.5	2.91	3.46	9.42	1.07	2.62	4.26	3.35	1.41	2.55
E	0.40/0.20	53.6	2.77	3.57	6.93	1.24	2.44		3.09	1.51	2.06

[a] Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HSSI, sodium *N*-hydroxysulfosuccinimide; N-OGCHI, OGCHI bound via an amino group(s); C-OGCHI, OGCHI bound via a carboxyl group(s).

[b] One gram of aminopropyl-silica gel and 80 mg of OGCHI are used for the reaction, and the reaction pH is 7.0.

[c] HPLC conditions: column, 100×2.0 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.1)–ethanol (90:10, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 220 nm.

Table 2
Influence of reaction pH on bound OGCHI amounts and chiral resolution of benzoin, chlorpheniramine and ibuprofen

Reaction pH	Bound amount (%)[a]	Solute[b]								
		Benzoin			Chlorpheniramine			Ibuprofen		
		k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
4.0	100	3.16	3.18	8.59	1.38	2.33	3.89	3.93	1.28	2.00
5.0	100	7.12	3.81	12.2	3.60	2.54	5.90	5.26	1.65	4.55
6.0	100	6.65	3.60	11.6	3.41	2.47	5.96	5.05	1.65	4.31
6.5	88.0	6.40	3.31	10.2	3.13	2.32	5.26	5.36	1.51	3.40
7.0	60.5	2.91	3.46	9.42	1.07	2.62	4.26	3.35	1.41	2.55
8.3	43.0	1.41	2.86	4.53	0.35	2.30	1.91	2.33	1.22	1.25

[a] One gram of aminopropyl-silica gel and 80 mg of OGCHI were used for the reaction.
[b] HPLC conditions as in Table 1.

the racemates tested. In this study, we compared OGCHI materials prepared via amino and carboxyl groups of OGCHI with regard to the bound amount of OGCHI, and retentivity and enantioselectivity of various racemates, where aminopropyl-silica gels with a 12-nm pore size were used.

With regard to the immobilization method via a carboxyl group(s) of a protein, Marle et al. [8] bound cellobiohydrolase I, via the carboxyl group(s) activated by EDC and HSSI, to aminopropyl-silica gels. For immobilizing OGCHI to aminopropyl-silica gels, we modified the method of Marle et al. [8] with regard to reaction pH. Table 1 shows the influence of EDC and HSSI amounts on bound OGCHI amounts and the chiral resolution of benzoin, chlorpheniramine and ibuprofen, where 1 g of aminopropyl-silica gel and 80 mg of OGCHI are used for the reaction, and the reaction pH is 7.0 as reported by Marle et al. [8]. Materials D and E gave higher OGCHI amounts bound than other materials, and

gave longer retentions for all solutes tested and higher enantioseparation factors and resolution. Thus, we used 0.2 g EDC and 0.1 g HSSI for the reaction of OGCHI. Table 2 shows the influence of reaction pH on bound OGCHI amounts and chiral resolution of benzoin, chlorpheniramine and ibuprofen, where 1 g of aminopropyl-silica gel and 80 mg of OGCHI were used for the reaction. At reaction pH <6.0, OGCHI was completely bound to aminopropyl-silica gels, while at reaction pH 7.0 half of the OGCHI used for the reaction was bound. At a reaction pH of 5.0, all solutes tested gave the longest retention factors and the highest enantioseparation factors and resolution except for the resolution of chlorpheniramine. In the following study, we used a reaction pH of 5.0. Marle et al. [8] used a reaction pH of 7.0 for immobilizing cellobiohydrolase I to aminopropyl-silica gels. We do not know whether the difference in the optimal reaction pH is due to a bound protein or other reasons.

Table 3
Reacted and bound OGCHI amounts, reaction ratios and surface coverages of various OGCHI materials

Material	Reacted amount (mg/g)	Bound amount (mg/g)	Reaction ratio (%)	Surface coverage (nmol/m^2)
N-OGCHI-2	20	20.0	100	2.2
N-OGCHI-4	40	40.0	100	4.4
N-OGCHI-8	80	79.6	99.6	8.8
N-OGCHI-16	160	102.6	64.1	11.4
C-OGCHI-2	20	20.0	100	2.2
C-OGCHI-4	40	40.0	100	4.4
C-OGCHI-8	80	80.0	100	8.9
C-OGCHI-16	160	132.8	83.0	14.8

3.2. Bound OGCHI amount

The optimization of the preparation method of OGCHI materials via an amino group(s) of OGCHI was reported previously [5]. OGCHI was bound to DSC-activated aminopropyl-silica gels via an amino group(s) of OGCHI. We compared the bound OGCHI amounts of various materials prepared via amino and carboxyl groups of OGCHI. Table 3 shows the

reacted and bound OGCHI amounts, reaction ratios and surface coverages of the various OGCHI materials. The reacted OGCHI amounts changed from 20 to 160 mg per 1 g of DSC-activated aminopropyl-silica gels or aminopropyl-silica gels. The materials obtained were abbreviated as, for example, N-OGCHI-2, which means that OGCHI is bound via an amino group(s), and that the reacted amount of OGCHI is 20 mg per 1 g of silica gels. With an
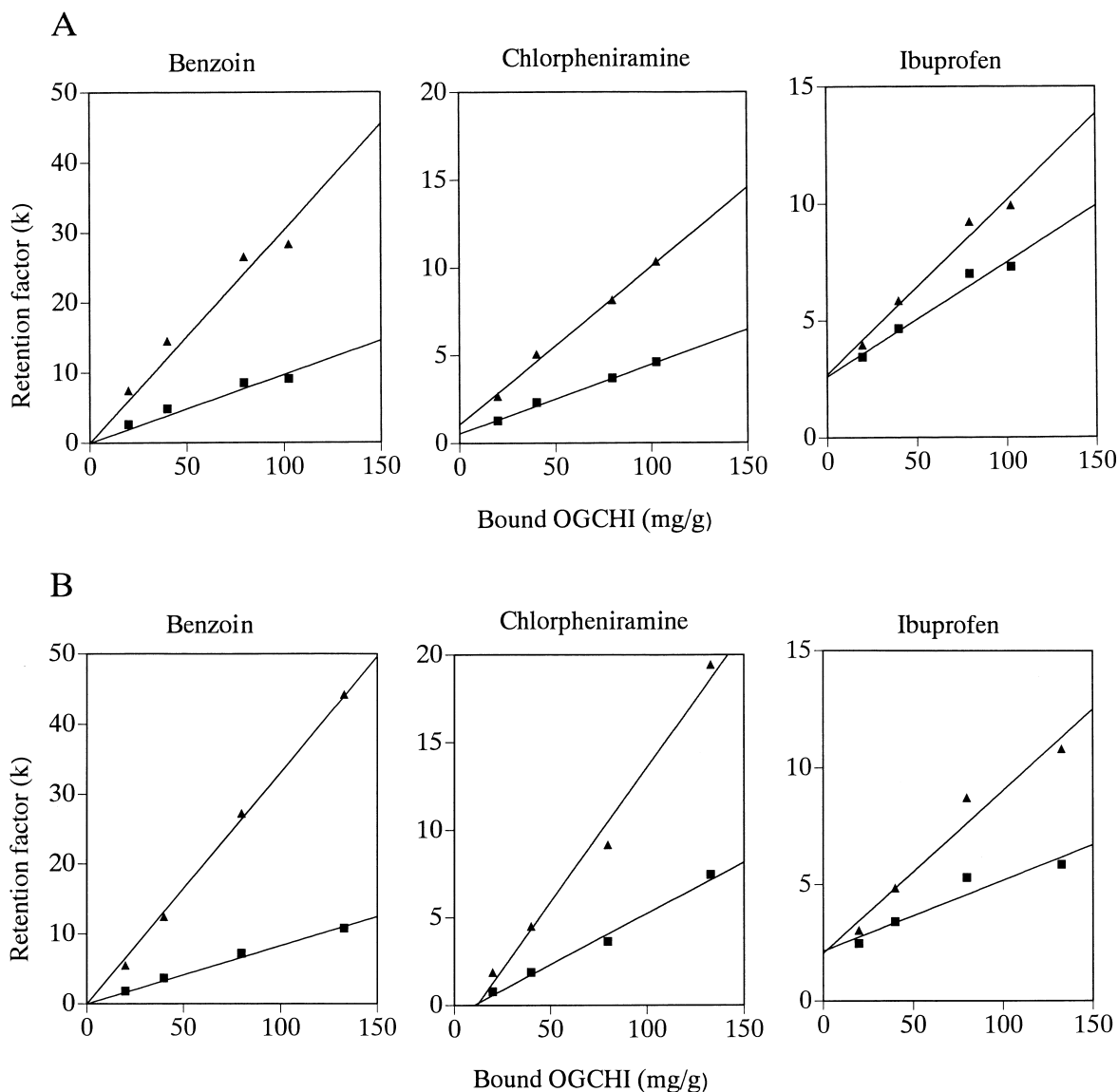

Fig. 2. Correlation of the bound amounts of OGCHI to the silica gels and the retention factors of benzoin, chlorpheniramine and ibuprofen enantiomers on N-OGCHI (A) and C-OGCHI (B) materials. (■) First-eluted enantiomer; (▲) second-eluted enantiomer.

Table 4
Chiral resolution of benzoin, chlorpheniramine and ibuprofen on various OGCHI materials[a]

Material	Solute								
	Benzoin			Chlorpheniramine			Ibuprofen		
	k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
N-OGCHI-2	2.54	2.90	6.14	1.24	2.12	2.96	3.42	1.15	0.68
N-OGCHI-4	4.75	3.04	7.47	2.27	2.21	3.06	4.62	1.26	1.60
N-OGCHI-8	8.48	3.12	11.0	3.67	2.21	5.34	6.97	1.32	2.92
N-OGCHI-16	9.00	3.14	8.23	4.58	2.25	4.48	7.27	1.36	2.89
C-OGCHI-2	1.71	3.17	7.46	0.74	2.50	2.98	2.43	1.23	1.40
C-OGCHI-4	3.59	3.44	10.9	1.85	2.42	4.94	3.37	1.43	2.57
C-OGCHI-8	7.12	3.81	12.2	3.60	2.54	5.90	5.26	1.65	4.55
C-OGCHI-16	10.7	4.12	12.9	7.43	2.61	6.80	5.82	1.85	5.49

[a] HPLC conditions as in Table 1.

increase in the reacted amount of OGCHI, the bound amounts of OGCHI increased. OGCHI was completely bound to silica gels with the OGCHI materials except for N- and C-OGCHI-16 materials. The N-OGCHI-16 material showed only 64% OGCHI binding, while the C-OGCHI-16 material showed 83% OGCHI binding. This indicates that the immobilization method via a carboxyl group of OGCHI is suitable for immobilizing a large amount of OGCHI to silica gels, compared with that via an amino group.

3.3. Comparison of retentivity, enantioselectivity and resolution of various racemates on OGCHI materials prepared via an amino or carboxyl group(s)

We selected benzoin, chlorpheniramine and ibu-

Table 5
Chiral resolution of various solutes on N- and C-OGCHI-8 materials[a]

Solute	Material					
	N-OGCHI-8			C-OGCHI-8		
	k_1	α	Rs	k_1	α	Rs
Neutral solute						
Benzoin[b]	6.40	2.87	10.4	5.03	3.57	11.1
Hexobarbital[b]	0.84	1.35	1.16	0.62	1.30	0.73
Acidic solute						
Ibuprofen[b]	15.5	1.46	4.35	11.8	1.89	6.43
Ketoprofen[b]	56.2	1.27	3.15	45.5	1.24	2.48
Pranoprofen[c]	8.13	1.17	1.17	4.54	1.24	1.34
Naproxen[b]	19.2	1.00		13.0	1.11	0.91
Fenoprofen[c]	25.1	1.23	1.50	17.7	1.30	1.91
2-Phenylpropionic acid[b]	3.05	1.00		2.77	1.58	1.96
Basic solute						
Alprenolol[d]	11.3	1.11	0.75	13.4	1.00	
Propranolol[d]	34.4	1.09	0.66	37.0	1.00	
Oxprenolol[d]	5.28	1.23	1.45	6.68	1.11	0.65
Chlorpheniramine[d]	3.67	2.21	5.34	3.60	2.54	5.90

[a] HPLC conditions as in Table 1 except for the eluent used.
[b] The eluent used is 20 mM phosphate buffer (pH 4.0)–ethanol (90:10, v/v).
[c] The eluent used is 20 mM phosphate buffer (pH 4.0)–ethanol (80:20, v/v).
[d] The eluent used is 20 mM phosphate buffer (pH 5.1)–ethanol (90:10, v/v).

profen as neutral, basic and acidic racemates, respectively. Fig. 2 illustrates the correlation of the bound amounts of OGCHI to the silica gels and the retention factors of benzoin, chlorpheniramine and ibuprofen enantiomers on N- and C-OGCHI materials, respectively. Table 4 shows the retention factor, enantioselectivity and resolution of benzoin, chlorpheniramine and ibuprofen on N- and C-OGCHI materials.

As shown in Fig. 2, a linear correlation was obtained between the retention factor of each enantiomer and bound amount of OGCHI with a correlation coefficient >0.95. The regression lines for benzoin and chlorpheniramine enantiomers passed near the origin, while the intercepts for ibuprofen enantiomers were about 3 and 2 with N- and C-OGCHI materials, respectively. These results

indicate that ibuprofen enantiomers interact more than benzoin and chlorpheniramine enantiomers with the base silica gels and/or spacers. Since aminopropyl-silica gels are used, it is plausible that ibuprofen enantiomers could interact with unreacted aminopropyl groups.

When the same amount of OGCHI was bound, the N-OGCHI materials gave slightly longer retentions than the C-OGCHI materials for all solutes tested, as shown in Table 4. With regard to enantioselectivity and resolution of the solutes, those of the N-OGCHI-2 material were lower than those with the N-OGCHI-4, -8 and -16 materials, which gave similar enantioselectivity and resolution. This is due to the superfluous achiral interaction of a racemate with the base silica gels and/or spacers because of lower protein coverage. On the other hand, in the case of C-OGCHI materials, the enantioselectivity and resolution of racemates were increased with an increase in the bound amount of OGCHI. Since a large number of amino groups remained unreacted because of no blocking of aminopropyl groups, superfluous achiral interaction of a racemate with the base silica gels and/or spacers might occur.
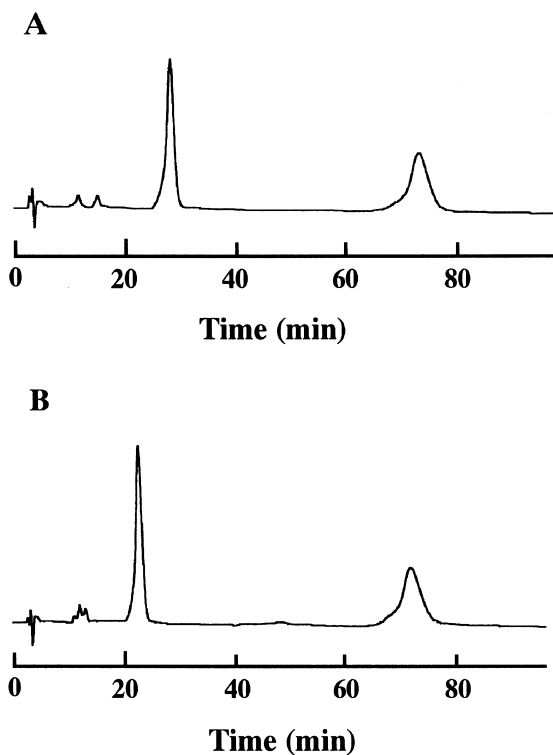

Fig. 3. Chromatograms of benzoin on the N-OGCHI-8 (A) and C-OGCHI-8 (B) materials. HPLC conditions: column, 2.0 mm I.D.×100 mm; eluent, 20 mM phosphate buffer (pH 4.0)–ethanol (90:10, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 220 nm.
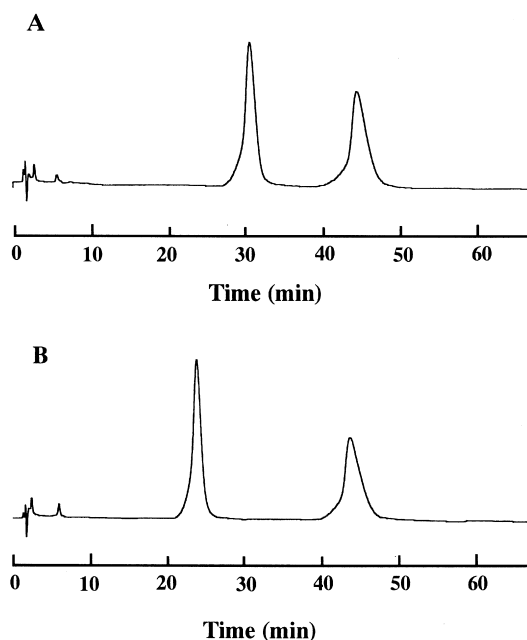

Fig. 4. Chromatograms of ibuprofen on the N-OGCHI-8 (A) and C-OGCHI-8 (B) materials. HPLC conditions as in Fig. 3.
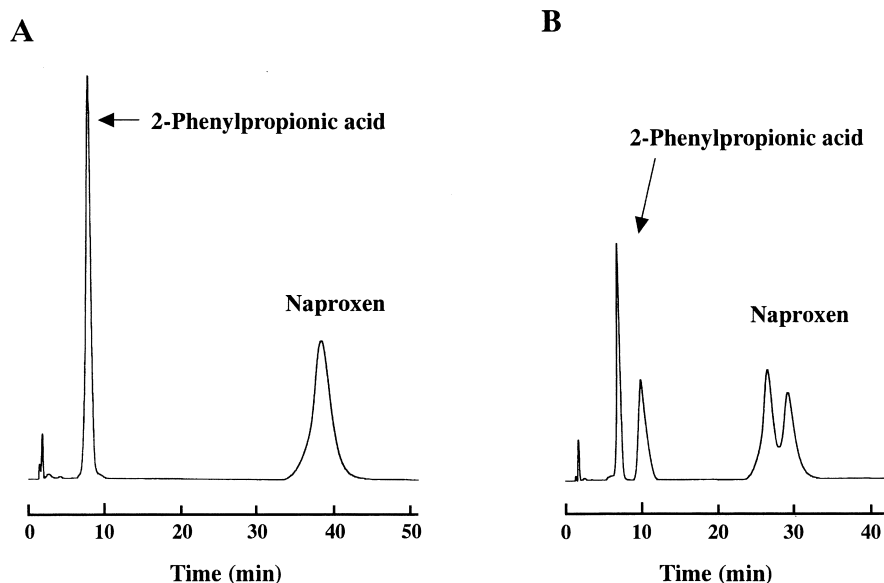
A

B

Fig. 5. Chromatograms of 2-phenylpropionic acid and naproxen on the N-OGCHI-8 (A) and C-OGCHI-8 (B) materials. HPLC conditions as in Fig. 3.

3.4. Comparison of OGCHI materials prepared via amino and carboxyl groups of OGCHI

Next, we compare the chiral recognition abilities, column efficiencies, column stability and batch reproducibility of N- and C-OGCHI-8 materials. Table 5 shows the chiral resolution of various solutes on N- and C-OGCHI-8 materials. Figs. 3 and 4 show chromatograms of benzoin and ibuprofen, respectively, on the N- and C-OGCHI-8 materials. Fig. 5 shows chromatograms of 2-phenylpropionic acid and naproxen on the N- and C-OGCHI-8 materials. With regard to the chiral resolution of neutral solutes, benzoin and hexobarbital, C-OGCHI-8 materials gave higher enantioselectivity for benzoin than N-OGCHI-8 materials, while the former gave lower enantioselectivity for hexobarbital than the latter. The C-OGCHI-8 materials gave higher enantioselectivity for all acidic solutes except for ketoprofen than the N-OGCHI-8 materials. It is interesting that 2-phenylpropionic acid and naproxen, which are not resolved on the N-OGCHI-8 materials, are resolved on the C-OGCHI materials. On the other hand, the C-OGCHI-8 materials gave higher enantioselectivity than N-OGCHI-8 materials for all basic solutes

except for chlorpheniramine. Alprenolol and propranolol were not resolved on the C-OGCHI-8 materials. These results reveal that the N-OGCHI materials are suitable for chiral resolution of basic solutes, and that C-OGCHI materials are suitable for chiral resolution of acidic solutes. It was reported that electrostatic interaction between an amino or carboxyl group of a protein and a charged solute should play an important role in chiral resolution of the solute on a protein-based stationary phase [9]. This was supported by the above findings: N-OGCHI

Table 6
The number of theoretical plates (N) of various solutes on N- and C-OGCHI-8 materials[a]

	N-OGCHI-8		C-OGCHI-8	
	N_1[b]	N_2[b]	N_1	N_2
Benzoin	3500	2650	3430	2500
Chlorpheniramine	2350	1370	1740	1660
Ibuprofen	2450	2150	2810	2600

[a] HPLC conditions as in Table 5.
[b] N_1 and N_2 are the number of theoretical plates of the first- and second-eluted enantiomers, respectively. The number of theoretical plates was calculated from the equation $N = 16 \times (t_R/t_W)^2$, where t_R is the retention time and t_W is the baseline peak width.

Table 7
Batch reproducibility of N- and C-OGCHI-8 materials[a]

	Benzoin		Chlorpheniramine		Ibuprofen	
	k_1	α	k_1	α	k_1	α
N-OGCHI-8						
Batch 1	8.45	3.09	4.17	2.23	6.51	1.34
2	7.96	3.10	3.94	2.21	5.93	1.34
3	8.48	3.12	3.67	2.21	7.27	1.32
Average±SD	8.30±0.30	3.10±0.02	3.92±0.25	2.22±0.01	6.57±0.67	1.33±0.01
C-OGCHI-8						
Batch 1	7.45	3.20	3.69	2.22	5.62	1.45
2	7.19	3.81	3.60	2.54	5.26	1.65
3	7.68	3.83	3.45	2.54	5.87	1.67
Average±SD	7.44±0.24	3.61±0.36	3.58±0.12	2.43±0.19	5.58±0.31	1.59±0.12

[a] HPLC conditions as in Table 1.

materials could not resolve some acidic solutes, and C-OGCHI materials could not resolve some basic solutes.

Table 6 shows column efficiencies of N- and C-OGCHI-8 materials. The numbers of theoretical plates of the first-eluted enantiomers of all solutes tested were larger than those of the second-eluted enantiomers. The N- and C-OGCHI-8 materials gave similar column efficiencies against the solutes tested. With regard to the column stability with respect to eluent pH changes, we first checked the retentivity and enantioselectivity of both materials using 20 m*M* phosphate buffer (pH 5.1)–acetonitrile (9:1, v/v) as the eluent. Next, five different eluents at different pH (from 3 to 7) were delivered for 300 column volumes, respectively, by injecting uncharged, basic and acidic solutes. The retentivity and enantioselectivity of both materials were then checked again. On the N-OGCHI-8 materials, the retention factors of benzoin, chlorpheniramine and ibuprofen were decreased by 0, 3 and 7%, respectively, before and after the eluent pH change, while the values were decreased by 10, 10 and 7%, respectively, on the C-OGCHI materials. On the other hand, there were almost no changes in the enantioselectivity of the solutes with both materials before and after the eluent pH change. However, if the same eluent was delivered for the same period described above, there were no changes in the retentivity and enantioselectivity of solutes with both materials. These results suggest that the N-OGCHI-8 materials are more stable against eluent pH changes than the C-OGCHI-8 materials.

Table 7 shows batch reproducibility of N- and C-OGCHI-8 materials. With regard to the retentivity, both materials gave similar reproducibility, while the N-OGCHI-8 materials showed a better reproducibility for enantioselectivity of solutes than the C-OGCHI-8 materials. These results reveal that N- and C-OGCHI materials could be reproducibly prepared.

In conclusion, the OGCHI materials prepared via a carboxyl group(s) of OGCHI gave higher enantioselectivity for acidic solutes than those prepared via an amino group(s), and gave less enantioselectivity for basic solutes. This reveals that the electrostatic interaction between an amino or carboxyl group of OGCHI and a charged solute should play an important role in chiral recognition of the solute. Further, the immobilization method is an important factor for preparing a protein-based stationary phase having an excellent chiral recognition ability.

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