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Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein
I. Influences of the pore size of base silica materials and bound protein amounts on chiral resolution

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Abstract

The influences of the pore size of base silica gels and the bound amounts of ovoglycoprotein (OGCHI) on the chiral resolution of racemates have been investigated. To the 12-, 20- and 30-nm pore size silica gels, aminopropyl groups were introduced, activated with N,N′-disuccinimidyl carbonate and then reacted with OGCHI followed by blocking with D-glucosamine. For the OGCHI material prepared with the same pore size silica gel, a linear correlation was obtained between the capacity factor of each enantiomer and the amount of bound OGCHI. Enantioselectivity and resolution obtained with the reaction of 40 mg OGCHI per 1 g silica gel were lower than those obtained with the reaction of 80, 160 and 320 mg OGCHI, when the same pore size silica gel was used. This is due to the superfluous achiral interaction with base silica gels and/or spacers because of lower protein coverage. With regard to comparison of the pore sizes of silica gels, the OGCHI materials prepared with the 12-nm pore size silica gel gave the largest capacity factor, and the highest eantioselectivity and/or resolution for the racemates tested, when the same amount of OGCHI was reacted. Thus, the OGCHI materials were prepared with the reaction of 80 mg OGCHI per 1 g silica gel having a 12-nm pore size followed by blocking with D-glucosamine. By diminishing the superfluous achiral interaction with base silica materials and/or spacers, much more efficient OGCHI materials should be obtainable.

Keywords: Chiral stationary phases, LC; Pore size; Ovoglycoprotein-based stationary phases; Chiral resolution; Enantiomer separation

1. Introduction

Stationary phases based on a protein have included albumins, such as bovine serum albumin [1] and human serum albumin [2], and mucoids, such as α_1-acid glycoprotein [3], ovomucoid [4] and ovoglycoprotein [5,6]. They have been developed for the separation of enantiomeric forms. Among these, an ovomucoid-bonded column was of special interest because of its long-term stability and because it was suited for separating a wide range of enantiomeric mixtures [7–9]. Recently, we isolated and characterized a new protein from chicken egg whites [6]. It was termed ovoglycoprotein (OGCHI, which means ovoglycoprotein from chicken egg whites). Also, it was included in crude ovomucoid (OMCHI, which

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means ovomucoid from chicken egg whites) preparations. We made OMCHI and OGCHI columns from isolated, pure proteins and compared the chiral recognition abilities of two columns. It was found [6] that the chiral recognition abilities of OMCHI reported previously [4] originated from OGCHI, and that OGCHI had stronger chiral recognition properties than OMCHI reported previously. It is well known that several factors, such as the physical properties of the base silica materials, the spacer length and the binding method, affect resolution of enantiomeric pairs on stationary phases based on a protein [10–15]. In this study, we precisely investigated the influences of the pore size of base silica materials and the amounts of bound OGCHI on chiral resolution of racemates on various OGCHI materials.

2. Experimental

2.1. Reagents and materials

Ibuprofen and chlorpheniramine maleate were kindly donated by Kaken Pharmaceutical (Tokyo, Japan) and Essex Nippon (Osaka, Japan), respectively. Benzoin and N,N′-disuccinimidyl carbonate (DSC) were purchased from Sigma (St. Louis, MO, USA). Sephadex G-25 (fine) and SP Sepharose FF were purchased from Pharmacia Biotech (Tokyo, Japan). D-Glucosamine hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethanol of HPLC grade was obtained from Wako Pure Chemical Industries (Osaka, Japan). Silica gels (Ultron-12, -20 and -30) used are from Shinwa Chemical Industries (Kyoto, Japan). Their physical properties are listed in Table 1. Other solvents and reagents were used without further purification.

Water purified with 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

Crude OMCHI was precipitated from egg whites with ethanol according to procedures modified slightly from those of Kato et al. [16]. The obtained crude OMCHI, which includes 10% OGCHI by weight, was further purified by cation-exchange chromatography. A 2-g amount of the OMCHI was applied to an SP Sepharose FF column (12×5 cm) that was equilibrated with 10 m*M* CH_3COONH_4 (pH 4.6) applying a liner gradient to 700 m*M* CH_3COONH_4 (pH 4.6) for 6 h at an average flow-rate of 100 ml/h; then the eluent was changed to 1000 m*M* CH_3COONH_4 (pH 4.6). The eluate was monitored at 280 nm with a Model AC-500 spectrophotometric monitor (Atto, Tokyo, Japan). The separation was performed at 4°C. Two fractions, OMCHI and OGCHI, were collected and lyophilized. The lyophilized OMCHI and OGCHI were desalted with a Sephadex G-25 (fine) column (20×5 cm) using 15 m*M* NH_4HCO_3 as the buffer with an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. OMCHI and OGCHI were characterized as reported previously [6].

2.3. Preparation of OGCHI materials

Ultron silica gel (5 g) was dried in vacuo over P_2O_5 at 150°C for 6 h and the dry silica gel was added to 120 ml of dry toluene. The mixture was heated to reflux until all the water had been removed as an azeotrope into a Dean–Stark-type trap. Next, 3-aminopropyltriethoxysilane, corresponding to 10 μmol/m^2 of the specific surface area, was added and reacted for 8 h. The reaction mixture was cooled to room temperature, filtered and washed with toluene

Table 1
Characteristics of silica particles used in this study

Silica	Particle diameter (μm)	Pore diameter (nm)	Specific surface area (m^2/g)
Ultron-12	5	12	300
Ultron-20	5	20	200
Ultron-30	5	30	100

and methanol. The isolated silica gels were dried in vacuo over P_2O_5 at 60°C for 2 h.

As shown in Fig. 1, OGCHI materials were prepared in three steps; activation by DSC, binding of OGCHI and blocking of the activated amino groups. First, the obtained aminopropyl-silica gels were activated by DSC. A 5-g amount of the gels was slurried in 70 ml of acetonitrile and reacted with 5 g of DSC for 24 h at 30°C. The reaction mixture was filtered and washed with acetonitrile, water, methanol and dichloromethane. The obtained silica gels were dried in vacuo over P_2O_5 at 60°C for 2 h.

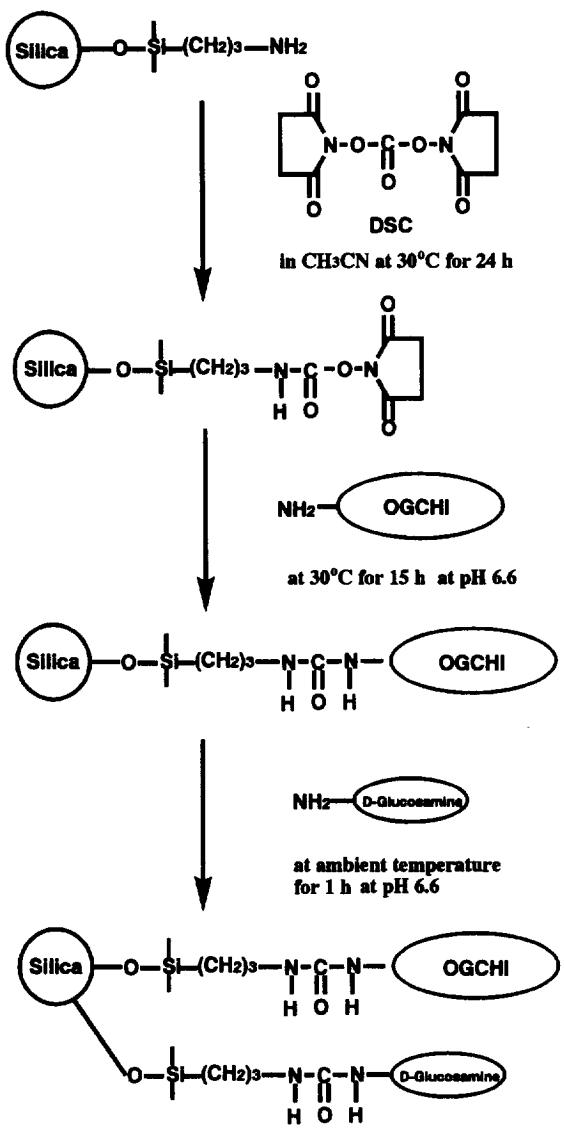

Fig. 1. Synthetic scheme for the preparation of OGCHI materials.

Second, OGCHI was bound to DSC-activated aminopropyl-silica gels as follows. A 1-g amount of the DSC-activated silica gels was slurried in 20 m*M* phosphate buffer (pH 6.8). To the mixture, 40, 80, 160 or 320 mg OGCHI dissolved in 20 ml of the same buffer was added slowly at room temperature for 1 h by adjusting pH to 6.6, and further stirred for 15 h at 30°C.

Third, the reaction mixture was washed with water and dissolved in 20 ml of a blocking solution adjusted to pH 6.6, including 300 m*M* D-glucosamine, at room temperature for 1 h. Then the reaction mixtures were filtered, washed with water and water–ethanol (95:5, v/v).

The obtained materials were packed into a 100× 2.0 mm I.D. stainless-steel column by the slurry packing method [17]. The slurry and packing solvents were water–ethanol (95:5, v/v).

2.4. Chromatography

For chiral resolution of racemic solutes on the OGCHI columns, the HPLC system used was composed of an LC-9A pump, an SPD-6A spectrophotometer, a Reodyne 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 performed at 220 or 254 nm. Capacity factors were calculated from the equation $k'=(t_R-t_0)/t_0$, where t_R and t_0 are retention times of retained and unretained solutes, respectively; k'_1 and k'_2 correspond to the capacity factors of the first- and second-eluted peaks, respectively. The retention time of unretained solute, t_0, was measured by injecting a solution whose organic modifier content 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 $R_s=2(t_2-t_1)/(t_{w1}+t_{w2})$, where t_1 and t_2 are retention times of the first- and second-eluted peaks, respectively, and t_{w1} and t_{w2} are the peak widths. All separations were carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluents are prepared by using sodium dihydrogenphosphate–disodium hydrogenphosphate

Table 2
Chiral resolution of benzoin, chlorpheniramine and ibuprofen on various OGCHI materials

Material	Solute								
	Benzoin			Chlorpheniramine			Ibuprofen		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
12-OGCHI-4	5.16	2.99	9.31	2.56	2.21	4.78	4.71	1.27	1.70
12-OGCHI-8	9.00	3.13	10.9	4.13	2.26	5.69	7.02	1.37	2.93
12-OGCHI-16	12.1	3.14	10.9	6.95	2.27	6.44	7.44	1.38	3.18
12-OGCHI-32	15.2	3.15	10.9	8.32	2.26	5.80	9.28	1.39	3.41
20-OGCHI-4	4.89	3.21	10.6	2.43	2.20	5.74	3.46	1.37	2.17
20-OGCHI-8	6.63	3.25	10.4	3.90	2.20	6.06	4.42	1.44	1.96
20-OGCHI-16	7.94	3.22	10.6	4.44	2.21	4.48	5.11	1.44	2.75
20-OGCHI-32	8.46	3.23	9.91	5.14	2.21	5.91	5.30	1.45	2.01
30-OGCHI-4	3.54	3.14	4.45	1.77	2.23	2.86	2.86	1.33	1.09
30-OGCHI-8	4.94	3.13	6.97	2.54	2.26	3.92	3.61	1.34	1.39
30-OGCHI-16	5.54	3.18	5.57	2.59	2.27	3.80	4.31	1.37	1.51
30-OGCHI-32	6.01	3.19	7.81	2.67	2.27	3.85	5.04	1.37	2.11

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 or 254 nm.

and ethanol. The eluent used is specified in the legend of Table 2.

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 are 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 (250×4.6 mm I.D.; 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 bound OGCHI to silica gels

The bound OGCHI amounts to silica gels were determined as follows. After reaction with OGCHI, OGCHI materials were washed with water. All washing 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. The concentration of OGCHI was determined based on a calibration graph constructed with concentration and peak area, and the unbound OGCHI amounts were estimated. The bound OGCHI amounts were calculated by subtraction of the unbound OGCHI amounts from the reacted OGCHI amounts.

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 to desired concentration. 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. Isolation of OGCHI from crude OMCHI

Previously [6], we isolated OGCHI by two-step ion-exchange chromatographic methods: anion- and cation-exchange chromatography. In this study, OGCHI was isolated by a one-step cation-exchange chromatographic method, as shown in Fig. 2. The method is simpler and faster than the previous one. The isolated OGCHI was confirmed by reversed-phase chromatography, N-terminal sequencing and

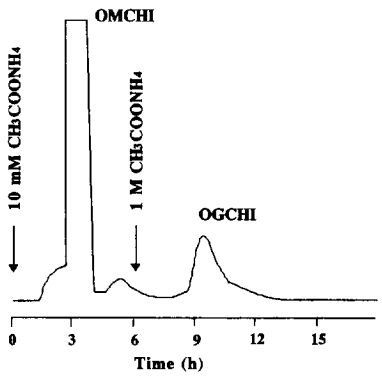

Fig. 2. Cation-exchange chromatographic separation of crude OMCHI. Separation conditions: column, SP Sepharose FF column (12×5 cm); eluent, 10 m*M* CH_3COONH_4 (pH 4.6) applying a linear gradient to 700 m*M* CH_3COONH_4 (pH 4.6) for 6 h and then changed to 1000 m*M* CH_3COONH_4 (pH 4.6); flow-rate, 100 ml/h.

matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, as reported previously [6]. The purity of OGCHI was estimated to be 99% based on the peak area by reversed-phase chromatography.

3.2. Bound OGCHI amounts and surface coverage of OGCHI materials

In a previous paper [15], the influences of the physical properties of base silica materials and spacer length on chiral resolution of various racemates on crude OMCHI materials have been investigated. The results obtained revealed that the 12-nm base silica gels were more suitable than 30-nm ones for obtaining higher enantioselectivity, and that an aminopropyl group was the most suitable among the spacers tested. In this study, we used aminopropyl-silica gels activated by DSC and precisely examined the influences of pore sizes of base silica materials and bound OGCHI amounts on chiral resolution of various racemates. The pore sizes of silica gels used were 12, 20 and 30 nm, and the reacted OGCHI amounts were 40, 80, 160 and 320 mg per 1 g silica gel. The obtained materials were abbreviated e.g., 12-OGCHI-4, which means that the pore size of a silica gel used is 12 nm, and that the reacted amount of OGCHI is 40 mg per 1 g silica gel. Table 3 shows the reacted and bound OGCHI amounts, reaction ratios and surface coverage of the various OGCHI materials.

As an increase in the reacted amount of OGCHI, the bound amount of OGCHI was increased. In the case of 12-OGCHI-4 and 12-OGCHI-8 materials, OGCHI was completely bound to the silica gels, while 12-OGCHI-16 and 12-OGCHI-32 materials gave only 60 and 40% OGCHI bindings, respectively. Similarly, the reaction ratio was further decreased as an increase in the reacted amount of OGCHI in the case of 20-OGCHI and 30-OGCHI materials.

With regard to comparison of the pore size of a silica gel, the bound amount of OGCHI was de-

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

Material	Pore size (nm)	Reacted amount (mg/g)	Bound amount (mg/g)	Reaction ratio (%)	Surface coverage (nmol/m^2)
12-OGCHI-4	12	40	40.0	100	4.3
12-OGCHI-8	12	80	76.5	95.6	8.5
12-OGCHI-16	12	160	102	63.8	11.3
12-OGCHI-32	12	320	130	40.6	14.4
20-OGCHI-4	20	40	39.9	99.8	6.7
20-OGCHI-8	20	80	71.1	88.9	11.9
20-OGCHI-16	20	160	93.9	58.7	15.7
20-OGCHI-32	20	320	98.2	30.7	16.4
30-OGCHI-4	30	40	35.7	89.3	11.9
30-OGCHI-8	30	80	47.4	59.3	15.8
30-OGCHI-16	30	160	57.0	35.6	19.0
30-OGCHI-32	30	320	69.3	21.7	23.1

creased with an increase in the pore size of a silica gel, when the same amount of OGCHI was used for the reaction. On the other hand, the surface coverage of OGCHI materials were increased with an increase in the pore size of a silica gel. The higher surface coverage of 30-OGCHI materials are ascribable to the fact that OGCHI should be more accessible to the inner surface of the silica gels than the other ones. Since the surface coverage of an aminopropyl group was 3.3–3.5 μmol/m^2 as reported previously [15], only 0.13–0.68% of the aminopropyl group was used for binding of OGCHI, assuming that OGCHI could be bound to aminopropyl-silica gels via only one amino group of OGCHI. This is due to the large steric interference because of the bulk of OGCHI.

3.3. Comparison of retention and enantioselectivity of various racemates on OGCHI materials different in pore sizes of base silica gels

We selected benzoin, chlorpheniramine and ibuprofen as neutral, basic and acidic racemates, respectively. Fig. 3 illustrates the correlation of the capacity factors of benzoin, chlorpheniramine and ibuprofen enantiomers, and the bound amounts of OGCHI to the 12-nm pore size silica gels. Table 2 shows the capacity factor, enantioselectivity and resolution of benzoin, chlorpheniramine and ibuprofen on various OGCHI materials.

As shown in Fig. 3, linear correlation was obtained between the capacity factor of each enantiomer and the bound amount of OGCHI. The regression lines for benzoin and chlorpheniramine enantiomers passed near the origin, while the intercepts for ibuprofen enantiomers were about 3. Similar results were obtained with 20- and 30-OGCHI materials. These results mean 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. The further reaction of OGCHI, 640 mg OGCHI per 1 g silica gel, resulted in a slight increase in the bound amount of OGCHI, and almost the same or slightly lower capacity factor and enantioselectivity of each racemate (data not shown). Further bound OGCHI did not improve chiral recognition. This result suggests that further bound OGCHI might not work effectively for the retention and chiral recognition of the racemate, and that there are bound OGCHI amounts suitable for chiral recognition.

With regard to comparison of the pore sizes of silica gels, the racemates were less retained with an increase in the pore size of a silica gel, when the same amount of OGCHI was reacted. This is reasonable because the capacity factor is dependent on the bound OGCHI amount as described above. Though almost the same amount of OGCHI was bound to the 12-OGCHI-4 and 20-OGCHI-4 materials, all racemates were more retained on the former than the latter. This is due to that the former is less bulky than the latter; that is, the packing density of the former to the same size column is higher than that of the latter.

As shown in Table 2, enantioselectivity and resolution obtained with the OGCHI-4 material were lower than those with the OGCHI-8, -16 and -32

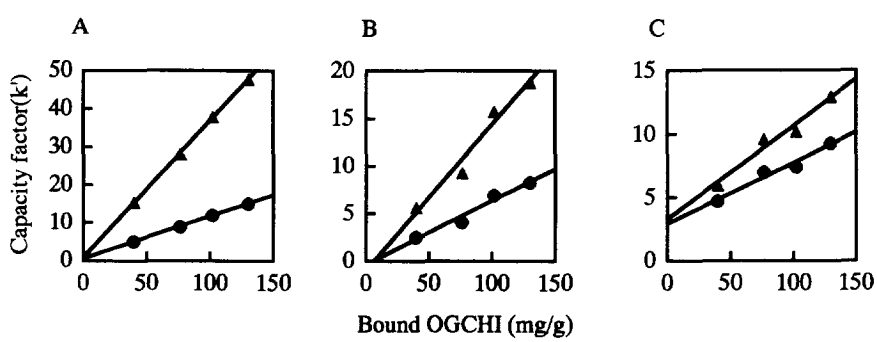

Fig. 3. Correlation of the capacity factors of benzoin (A), chlorpheniramine (B) and ibuprofen (C) enantiomers, and the bound amounts of OGCHI to the 12-nm pore size silica gels. Keys: (●) first-eluted enantiomer; (▲) second-eluted enantiomer.

materials. This is due to the superfluous achiral interaction of a racemate with the base silica gels and/or spacers because of lower protein coverage. Almost the same enantioselectivity and resolution were obtained with the OGCHI-8, -16 and -32 materials, when the same pore size silica gel was used. Since a higher density of chiral recognition sites should lead to a higher capacity, it is better to use the highly bound materials for preparative purposes. However, OGCHI-8 materials are more suitable for analytical purposes because of shorter retentions of racemates. Among 12-OGCHI-8, 20-OGCHI-8 and 30-OGCHI-8 materials, the 12-OGCHI-8 material gave the highest enantioselectivity and/or resolution. Thus, the OGCHI materials were prepared with the reaction of 80 mg OGCHI per 1 g silica gel having a 12-nm pore size followed by blocking with D-glucosamine. By diminishing the superfluous achiral interaction with base silica materials and/or spacers, much more efficient OGCHI materials should be obtainable.

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