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# Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein
## II. Comparison of chiral recognition properties with crude ovomucoid

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**Abstract**

Chiral stationary phases based on ovoglycoprotein from chicken egg whites (OGCHI) and crude ovomucoid from chicken egg whites (OMCHI) were compared with regard to the bound amounts of OGCHI and chiral recognition abilities. Crude OMCHI included 11% OGCHI, by weight. Since pure OMCHI had no appreciable chiral recognition ability, the chiral recognition ability of crude OMCHI originated from OGCHI, which was present in crude OMCHI preparations as an impurity. However, a chiral stationary phase based on crude OMCHI showed good chiral recognition ability, despite the 11% OGCHI content in crude OMCHI preparations. When crude OMCHI was reacted with N,N′-disuccinimidylcarbonate (DSC)-activated aminopropyl-silica gels, the ratio of bound OGCHI to that of totally bound protein was 0.23. These results reveal that the good chiral recognition ability of a stationary phase based on crude OMCHI is due to OGCHI being preferentially bound to DSC-activated aminopropyl-silica gels rather than the OMCHI. In addition, OMCHI did not contribute to the enantioselectivity of the solute at all and made little contribution to the retention characteristics. © 1997 Elsevier Science B.V.

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

## 1. Introduction

A stationary phase based on ovomucoid has been developed for the separation of enantiomeric forms [1–4] and is now commercially available as an Ultron ES-OVM column [5]. Recently, we isolated and characterized a new protein from chicken egg whites [6,7]. It was also included in crude ovomucoid (OMCHI, i.e., ovomucoid from chicken egg whites) preparations and was termed ovoglycoprotein (OGCHI, i.e., ovoglycoprotein from chicken egg whites). In addition, it was found that pure OMCHI had no appreciable chiral recognition ability, and that the chiral recognition ability of crude OMCHI originated from OGCHI, which was present in crude OMCHI preparations as an impurity [7]. A commercially available OMCHI column, Ultron ES-OVM, was made using crude OMCHI [5]. However, it is very strange that the crude OMCHI column shows good chiral recognition ability, despite the low

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content of OGCHI (11% by weight) in the crude OMCHI preparations. In this paper, OGCHI and crude OMCHI columns are compared regarding the amounts of bound OGCHI and chiral recognition abilities.

## 2. Experimental

### 2.1. Reagents and materials

Ibuprofen and chlorpheniramine maleate were kindly donated by Kaken Pharmaceuticals (Tokyo, Japan) and Essex Nippon (Osaka, Japan), respectively. Benzoin and N,N′-disuccinimidylcarbonate (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 Nacalai Tesque (Kyoto, Japan). Crude OMCHI was kindly donated by Eisai (Tokyo, Japan). Ethanol, of HPLC grade, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The silica gels (Ultron-12, 5 μm diameter, 12 nm pore size, 300 $m^2/g$) were from Shinwa Chemical Industries (Kyoto, Japan). Other solvents and reagents were 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 and OMCHI from egg whites

OGCHI and OMCHI were isolated from crude OMCHI, which was precipitated from egg whites with ethanol according to procedures that were modified slightly from those of Fredericq and Deutsch [8]. The crude OMCHI obtained was further purified as reported previously [9]. Briefly, 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) using a linear gradient to 700 m*M* $CH_3COONH_4$ (pH 4.6) for 6 h at an average flow-rate of 100 ml/h, and then the eluent was changed to 1000 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 using a Sephadex G-25 (fine) column (20×5 cm) using 15 m*M* $NH_4HCO_3$ as the buffer, and an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. The purity of the OMCHI and OGCHI obtained was estimated to be 100 and 99%, respectively, based on the peak area by reversed-phase chromatography under the conditions described below, as shown in Fig. 1.

### 2.3. Preparation of OGCHI, OMCHI and crude OMCHI 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 of 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 and methanol. The isolated silica gels were dried in vacuo over $P_2O_5$ at 60°C for 2 h.

As shown in Fig. 2, OGCHI, OMCHI or crude OMCHI materials were prepared by a three-step procedure, i.e., activation by DSC, binding of a

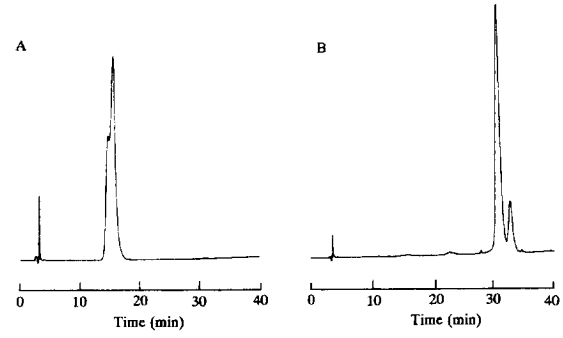


Fig. 1. Reversed-phase chromatograms of isolated OMCHI (A) and OGCHI (B). Column, Cosmosil 5C18-AR (250×4.6 mm I.D.). Eluent A, $H_2O$–$CH_3CN$ (80:20, v/v) containing 0.1% TFA; eluent B, $H_2O$–$CH_3CN$ (20:80, v/v) containing 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. Detection, 280 nm. Flow-rate, 1.0 ml/min. Column temperature, 30°C.

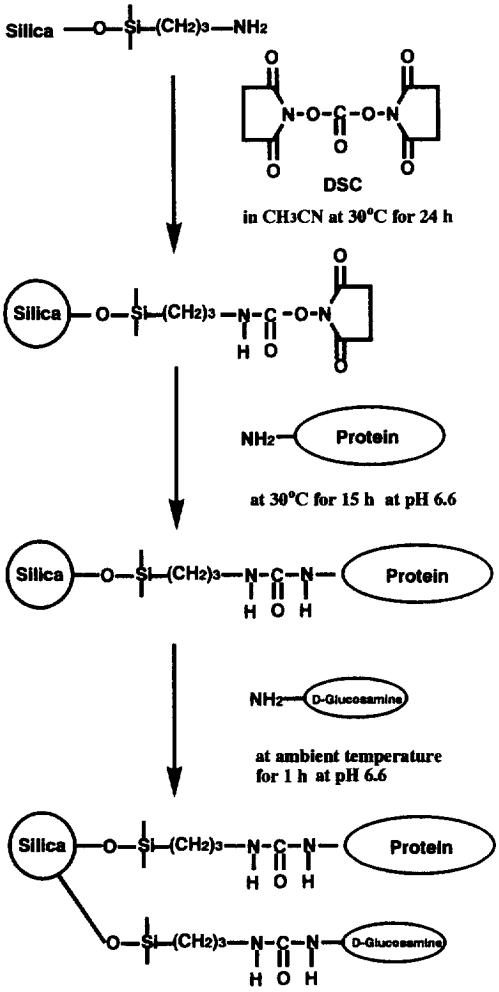


Fig. 2. Synthetic scheme for the preparation of OGCHI, OMCHI or crude OMCHI materials.

protein and blocking of the activated amino groups with D-glucosamine. First, the obtained aminopropyl-silica gels were activated by DSC. A 5-g amount of the gel 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.

Second, a protein was bound to DSC-activated aminopropyl-silica gels as follows. A 1-g amount of the DSC-activated silica gel was slurried in 20 m*M* phosphate buffer (pH 6.8). To the mixture, 20 ml of a protein solution in the same buffer was added slowly at room temperature for 1 h by adjusting the pH to 6.6, and this was 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 that had been adjusted to pH 6.6 and that 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 [10]. The slurry and packing solvent was water–ethanol (95:5, v/v).

### 2.4. Chromatography

For chiral resolution of racemic solutes on the OGCHI, OMCHI and crude OMCHI columns, the HPLC system used consisted of an LC-9A pump, an SPD-6A spectrophotometer, a Rheodyne 7125 injector with a 5-μl loop, 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. The capacity factor ($k'$), enantioseparation factor ($\alpha$) and resolution ($R_s$) of a racemate were calculated. All separations were carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluent was prepared using sodium dihydrogenphosphate–disodium hydrogenphosphate and ethanol. The eluent used is specified in the table legend.

For the reversed-phase chromatographic separation of OGCHI, OMCHI and crude OMCHI, 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, $H_2O$–$CH_3CN$ (80:20, v/v) containing 0.1% trifluoroacetic acid (TFA); eluent B, $H_2O$–$CH_3CN$ (20:80, v/v) containing 0.1% TFA; A linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min was used. The column used was a Cosmosil 5C18-AR column (250×4.6 mm I.D.) from Nacalai Tesque. 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 the amount of protein bound to silica gels

The amounts of protein bound to silica gels were determined as follows. After reaction with a protein, the obtained materials were washed with water. All wash solutions were collected and their volumes were measured. Protein concentration was determined using a reversed-phase chromatography system under the conditions described below. The amount of protein that reacted was determined by subtracting the amount of protein 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 eluent to the desired concentration. A 5-μl volume of the sample solution was loaded onto a column. The loaded amount was 0.5 nmol.

## 3. Results and discussion

### 3.1. Amounts of proteins bound to various materials

In a previous paper [9], we examined the influence of the pore size of base silica materials and the amount of OGCHI bound on the chiral resolution of various racemates. It was found that OGCHI materials prepared with a 12-nm pore size silica gel gave the largest capacity factor, and the highest enantioselectivity and/or resolution for the racemates tested. In this study, OGCHI, OMCHI and crude OMCHI materials were prepared using the 12-nm pore size aminopropyl-silica gel activated with DSC. Table 1 shows the amounts of protein of OGCHI and OMCHI bound, where 4, 8 and 16 indicate that the reacted amounts of OGCHI and OMCHI are 40, 80 and 160 mg, respectively, per 1 g of silica gel. On increasing the amounts of OGCHI and OMCHI reacted, the amounts of OGCHI and OMCHI that bound were increased. It is interesting that OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI, despite the fact that the average molecular masses of OGCHI and OMCHI are 30 000 and 27 000, respectively [7].

Table 2 shows the amounts of OMCHI and OGCHI that reacted and the amounts of OMCHI and OGCHI that bound, when crude OMCHI from Eisai was used for the reaction. Because a commercially available OMCHI column, Ultron ES-OVM, has been made with the crude OMCHI, the crude OMCHI included 10.9% OGCHI, by weight. On increasing the amount of crude OMCHI reacted, the amount of OGCHI that bound was increased, but that of OMCHI was slightly increased. Although the ratio of reacted protein, OGCHI/(OMCHI+OGCHI), was 0.11, the ratio of bound protein was 0.23 in the case of crude OMCHI-32 materials. This result supports the theory that OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI.

### 3.2. Comparison of retention and enantioselectivity of racemates on the various materials

We selected benzoin, chlorpheniramine and ibuprofen as neutral, basic and acidic solutes, respectively. Table 3 shows the capacity factor, enantioselectivity and resolution of these enantiomers on

Table 1
Amounts of OGCHI and OMCHI that were reacted and the amounts of OGCHI and OMCHI that were bound to silica gels

| Material | Reacted protein (mg/g) | Bound protein (mg/g) | Reaction ratio |
|---|---|---|---|
| OGCHI-4 | 40 | 40.0 | 1.00 |
| OGCHI-8 | 80 | 76.5 | 0.96 |
| OGCHI-16 | 160 | 102 | 0.64 |
| OGCHI-32 | 320 | 130 | 0.41 |
| OMCHI-4 | 40 | 33.2 | 0.83 |
| OMCHI-8 | 80 | 55.2 | 0.69 |
| OMCHI-16 | 160 | 88.8 | 0.56 |

Table 2
Amounts of OMCHI and OGCHI that were reacted and the amounts that were bound to silica gels in the reaction of crude OMCHI[a]

| Material | Reacted protein (mg/g) | | Bound protein (mg/g) | | Bound protein ratio |
|---|---|---|---|---|---|
| | OMCHI | OGCHI | OMCHI | OGCHI | OGCHI/(OMCHI+OGCHI) |
| Crude OMCHI-8 | 71.3 | 8.7 | 57.5 | 8.7 | 0.13 |
| Crude OMCHI-16 | 142.6 | 17.4 | 63.0 | 17.4 | 0.22 |
| Crude OMCHI-32 | 284.1 | 34.9 | 67.9 | 20.3 | 0.23 |

[a]Crude OMCHI included 10.9% OGCHI, by weight.

Table 3
Chiral resolution of benzoin, chlorpheniramine and ibuprofen on various OGCHI and OMCHI materials

| Material | Solute | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Benzoin | | | Chlorpheniramine | | | Ibuprofen | | |
| | $k_1'$ | $\alpha$ | $R_s$ | $k_1'$ | $\alpha$ | $R_s$ | $k_1'$ | $\alpha$ | $R_s$ |
| OGCHI-4 | 5.16 | 2.99 | 9.31 | 2.56 | 2.21 | 4.78 | 4.71 | 1.27 | 1.70 |
| OGCHI-8 | 9.00 | 3.13 | 10.9 | 4.13 | 2.26 | 5.69 | 7.02 | 1.37 | 2.93 |
| OGCHI-16 | 12.1 | 3.14 | 10.9 | 6.95 | 2.27 | 6.44 | 7.44 | 1.38 | 3.18 |
| OGCHI-32 | 15.2 | 3.15 | 10.9 | 8.32 | 2.26 | 5.80 | 9.28 | 1.39 | 3.41 |
| OMCHI-4 | 0.43 | 1.00 | – | 0.13 | 1.00 | – | 2.45 | 1.00 | – |
| OMCHI-8 | 0.45 | 1.00 | – | 0.13 | 1.00 | – | 2.51 | 1.00 | – |
| OMCHI-16 | 0.58 | 1.00 | – | 0.18 | 1.00 | – | 2.62 | 1.00 | – |

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.

various OGCHI and OMCHI materials. No chiral resolution of these racemates was obtained with the OMCHI materials. Table 4 shows the capacity factor, enantioselectivity and resolution of benzoin, chlorpheniramine and ibuprofen on various crude OMCHI materials. The results regarding the amount of protein that bound and the chiral recognition abilities reveal that good chiral recognition of the crude OMCHI materials occurred, despite the low content of OGCHI in crude OMCHI preparations, due to the preferential binding of OGCHI to the DSC-activated aminopropyl-silica gels.

Fig. 3A–C illustrates the correlation between the capacity factors of benzoin, chlorpheniramine and ibuprofen enantiomers, and the amounts of OGCHI that were bound. Fig. 4A–C illustrates the correlation between the capacity factors of benzoin, chlorpheniramine and ibuprofen, and the amounts of OMCHI that were bound. As shown in Figs. 3 and 4, linear correlations were obtained between the capacity factors of benzoin, chlorpheniramine and ibuprofen and the amounts of OGCHI and OMCHI that were bound. The correlation coefficients calculated from Figs. 3 and 4 were in the ranges 0.981–1.000 and 0.920–0.999, respectively. The results obtained reveal that OMCHI does not contribute to the

Table 4
Chiral resolution of benzoin, chlorpheniramine and ibuprofen on various crude OMCHI materials

| Material | Solute | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Benzoin | | | Chlorpheniramine | | | Ibuprofen | | |
| | $k_1'$ | $\alpha$ | $R_s$ | $k_1'$ | $\alpha$ | $R_s$ | $k_1'$ | $\alpha$ | $R_s$ |
| Crude OMCHI-8 | 1.65 | 2.48 | 4.69 | 0.67 | 1.93 | 1.78 | 3.37 | 1.13 | 0.66 |
| Crude OMCHI-16 | 2.81 | 2.75 | 5.50 | 1.24 | 2.01 | 2.46 | 3.93 | 1.20 | 1.04 |
| Crude OMCHI-32 | 3.10 | 2.75 | 5.72 | 1.32 | 1.99 | 2.76 | 4.41 | 1.21 | 1.19 |

HPLC conditions as in Table 3.

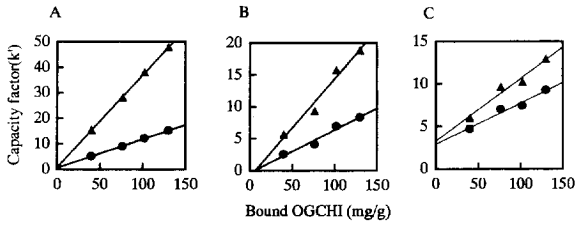


Fig. 3. Correlation of the capacity factors of benzoin (A), chlorpheniramine (B) and ibuprofen (C) enantiomers, and the amounts of OGCHI that were bound. (●) First-eluted enantiomer; (▲) second-eluted enantiomer.

enantioselectivity of these solutes and that OMCHI makes little contribution to retention. The intercepts of regression lines for ibuprofen on both OGCHI and OMCHI materials were larger than those for benzoin and chlorpheniramine. This means that ibuprofen interacts with the base silica gels and/or spacers more than benzoin and chlorpheniramine do.

### 3.3. Retentive and enantioselective properties of crude OMCHI materials

Assuming that there are no interactions between the immobilized proteins, the capacity factor of a solute on the crude OMCHI column can be expressed by

$$k'_{\text{crude OMCHI}} = k'_{\text{base}} + k'_{\text{OMCHI}} + k'_{\text{OGCHI}}, \quad (1)$$

where $k'_{\text{base}}$, $k'_{\text{OMCHI}}$ and $k'_{\text{OGCHI}}$ are the capacity factors due to base silica materials, and bound OMCHI and OGCHI, respectively, as reported previously [11]. Similarly, the enantioselectivity of a

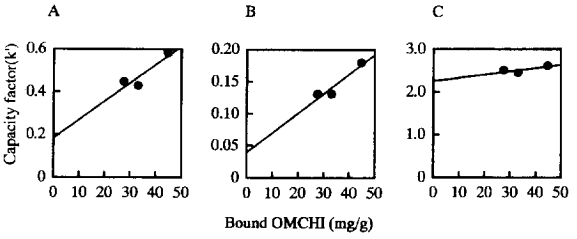


Fig. 4. Correlation of the capacity factors of benzoin (A), chlorpheniramine (B) and ibuprofen (C), and the amounts of OMCHI that were bound.

Table 5
Observed and calculated capacity factor ($k'$) and enantioselectivity ($\alpha$) on crude OMCHI-32 columns

| Solute | Crude OMCHI-32 column | | | |
|---|---|---|---|---|
| | Observed | | Calculated | |
| | $k'_1$ | $\alpha$ | $k'_1$ | $\alpha$ |
| Benzoin | 3.10 | 2.75 | 3.19 | 2.75 |
| Chlorpheniramine | 1.32 | 1.99 | 1.35 | 2.03 |
| Ibuprofen | 4.41 | 1.21 | 4.27 | 1.20 |

solute on the crude OMCHI column can be expressed by

$$\alpha_{\text{crude OMCHI}} = \frac{k'_{2,\text{ crude OMCHI}}}{k'_{1,\text{ crude OMCHI}}}, \quad (2)$$

where 1 and 2 are the first and second eluted enantiomers, respectively, on the crude OMCHI column. By substituting Eq. (1) into Eq. (2), the enantioselectivity on the crude OMCHI column can be expressed by

$$\alpha_{\text{crude OMCHI}} = \frac{k'_{2,\text{ base}} + k'_{2,\text{ OMCHI}} + k'_{2,\text{ OGCHI}}}{k'_{1,\text{ base}} + k'_{1,\text{ OMCHI}} + k'_{1,\text{ OGCHI}}} \quad (3)$$

Since each enantiomer of the solute was not resolved on the OMCHI column, $k'_{2,\text{ OMCHI}}$ is equal to $k'_{1,\text{ OMCHI}}$. Of course, $k'_{1,\text{ base}}$ is equal to $k'_{2,\text{ base}}$. Table 5 gives the observed $k'_1$ and $\alpha$ values of benzoin, chlorpheniramine and ibuprofen on the crude OMCHI-32 column and the calculated $k'_1$ and $\alpha$ values. The observed $k'_1$ and $\alpha$ values are consistent with the calculated values. These results support the theory that OMCHI should not contribute to the enantioselectivity of these solutes at all, and that OMCHI should make little contribution to retention. In addition, there could be no interaction between the immobilized proteins.

In conclusion, a chiral stationary phase based on crude OMCHI showed good chiral recognition, despite the low content of OGCHI in crude OMCHI. This is because OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI. In addition, OMCHI did not contribute to the enantioselectivity of the solutes tested and OMCHI made little contribution to the retention values.

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