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Retentive and enantioselective properties of ovomucoid-bonded silica columns. Influence of protein purity and isolation method

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Abstract

The influence of protein purity and isolation method on the chiral resolution of racemates on ovomucoid-bonded materials has been investigated. Chicken ovomucoid (OMCHI) and turkey ovomucoid (OMTKY) were obtained from commercial sources, and were isolated by precipitation with acetone from their corresponding egg whites. Further, crude OMCHI protein was fractionated by cation-exchange chromatography into two fractions, pure OMCHI and an unknown protein fraction. The OMCHI, fractionated OMCHI and OMTKY proteins were characterized by N-terminal sequencing and trypsin-inhibitory activity. Their purity was checked by reversed-phase chromatography. The results reveal that the commercial OMCHI, and the isolated OMCHI and OMTKY are crude preparations and that an unknown protein fraction is present in each crude ovomucoid protein. The OMCHI, fractionated OMCHI and OMTKY proteins were bound to aminopropyl-silica gels activated by N,N′-disuccinimidyl carbonate to test the retentive and enantioselective properties of those materials. The result suggests that the unknown protein fraction could be responsible for chiral recognition ability of the OMCHI and OMTKY columns.

1. Introduction

Protein-bonded stationary phases including albumins such as bovine serum albumin (BSA) [1] and human serum albumin [2], and glycoproteins such as α_1-acid glycoprotein [3] and chicken ovomucoid (OMCHI) [4] and avidin [5], and enzymes such as cellulase [6], trypsin [7], α-chymotrypsin [8] and lysozyme [9] have been developed for the separation of enantiomeric forms. Among these, an OMCHI-bonded silica column is of special interest because of its long-term stability [10] and because it is suited for separating a wide range of enantiomeric mixtures [10–12]. The column is now commercially available as an Ultron ES-OVM column.

It is well known that the protein purity and/or the isolation method affect the physiological activity of a protein. In this study, we investigated the influence of ovomucoid protein purity and isolation method on the retention and enantioselectivity of various solutes on OMCHI- and turkey ovomucoid (OMTKY)-bonded silica columns. Also, preliminary result on the origin of

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the chiral recognition ability of ovomucoid-bonded column will be discussed.

2. Experimental

2.1. Reagents and materials

Ketoprofen and chlorpheniramine maleate were kindly donated by Kaken Pharmaceutical Co. (Tokyo, Japan) and Essex Nippon (Osaka, Japan), respectively. Benzoin was purchased from Sigma (St. Louis, MO, USA). The Structures of the racemic compounds used in this study are shown in Fig. 1. Chicken ovomucoid (OMCHI) from Eisai Co. (Tokyo, Japan), Sigma, Taiyo Chemicals (Yokkaichi, Mie, Japan) were abbreviated OMCHI-C1, OMCHI-C2 and OMCHI-C3, respectively. OMTKY from Sigma was abbreviated OMTKY-C. Sephadex G-75, Sephadex G-25 (fine) and SP Sepharose FF were from Pharmacia Biotec (Tokyo, Japan). N,N'-Disuccinimidyl carbonate (DSC) was purchased from Sigma. Ethanol of HPLC grade was obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-Aminopropyltriethoxysilane was obtained from Chisso Co. (Tokyo, Japan). Silica gels (Ultron-120, 5-μm diameter, 120-Å pore size, 300 m^2/g) used were from Shinwa Chemical Industries (Kyoto, Japan). An Ultron ES-OVM column was kindly donated by Shinwa Chemical Industries. Other solvents and reagents of analytical-reagent grade 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 OMCHI and OMTKY from egg whites

OMCHI and OMTKY were isolated from their corresponding egg whites according to a modified form of the method reported previously [13]. A 900-ml volume of cold 0.5 M trichloroacetic acid–acetone (1:2, v/v) at pH 3.5 was added to 450 ml of the egg white, and the mixture was stirred for 4 h at 4°C. The solution was centrifuged at 5500 g rpm for 30 min at 4°C using polypropylene bottles. Two volumes of cold acetone were added to the supernatant and after stirring the solution for 2 h at 4°C, it was centrifuged at 5500 g rpm for 40 min at 4°C. The precipitate was dissolved and dialized against water and then lyophilized. The remaining product is further purified by the two methods described below.

The first purification method is a modification of the method reported by Kato et al. [4]. The remaining product was purified through a 90×5 cm Sephadex G-75 column equilibrated with 10 m*M* Tris buffer (pH 8.0) including 0.3 *M* NaCl at a flow-rate of 180 ml/h. The eluate was monitored at 280 nm. The separation was performed at 4°C. The eluted major component was fractionated and lyophilized. The lyophilized sample was desalted on a Sephadex G-25 (fine) column (20×5 cm) using 15 m*M* NH_4HCO_3 buffer with an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. The obtained product was termed OMCHI-I1 or OMTKY-I.

The second purification method is based on a modification of the method reported by Waheed and Salahuddin [15]. The lyophilized product was brought to 90% $(NH_4)_2SO_4$ saturation, pH

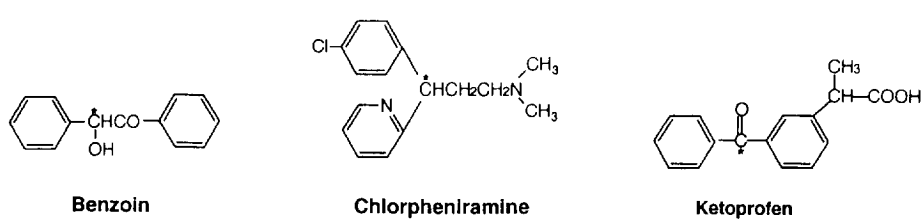

Fig. 1. Structures of the racemic compounds used in this study.

4.6. The solution was centrifuged at 5500 g for 40 min at 4°C. The precipitate was dissolved and dialized against water and then lyophilized. The remaining product was dissolved in water. The obtained solution was centrifuged again at 5500 g for 40 min at 4°C. The supernatant was lyophilized. The obtained product was termed OMCHI-I2.

2.3. Fractionation of crude OMCHI protein

The OMCHI, OMCHI-C1, from Eisai was further separated on a 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 500 m*M* CH_3COONH_4 (pH 4.6) for 4.5 h at an average flow-rate of 100 ml/h, and then the eluent was changed to 800 m*M* CH_3COONH_4 (pH 4.6). The separation was performed at 4°C. Detection was carried out at 280 nm. Two fractions were collected and lyophilized. The lyophilized samples were desalted with a Sephadex G-25 (fine) column as described above, and the eluate was collected and lyophilized. The isolated fractions were termed fractions A and B.

2.4. Characterization of OMCHI, fractionated OMCHI and OMTKY protein

N-Terminal sequencing

A 70-μg amount of each protein was reconstituted with 50 μl of water. A 5-μl portion of the dilution was spotted on a solid support for N-terminal sequencing using an ABI 473A Protein Sequencer (Applied Biosystems Division, Perkin Elmer Japan, Tokyo, Japan).

Trypsin-inhibitory activity

Trypsin-inhibitory activity measurement is based on a modification of the method reported by Waheed and Salahuddin [15].

2.5. Preparation of aminopropyl-silica gels

Ultron-120 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, 3.32 g of 3-aminopropyltriethoxylsilane, corresponding to 10 μmol/m^2 of the

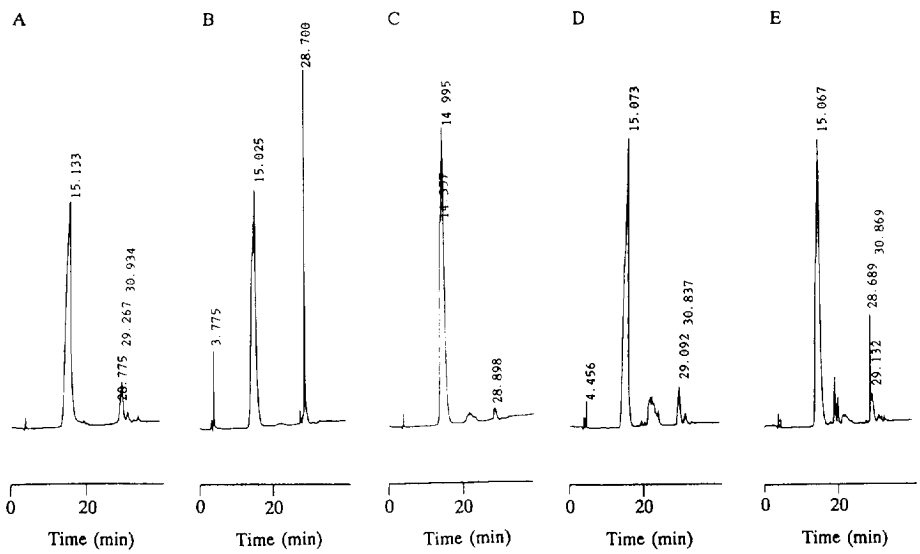

Fig. 2. Chromatograms of commercial and isolated OMCHI proteins on a C_{18} column. (A) OMCHI-C1; (B) OMCHI-C2; (C) OMCHI-C3; (D) OMCHI-I1; (E) OMCHI-I2. Column: Cosmosil 5C18-AR (250 × 4.6 mm I.D.). Eluents: eluent A, H_2O–CH_3CN (80:20, v/v) including 0.1% TFA; eluent B, H_2O–CH_3CN (20:80, v/v) including 0.1% TFA; linear gradient from 0% eluent B at 0 min to 100% eluent B at 90 min. Flow-rate, 1.0 ml/min; Detection, 280 nm; Loaded amount, 200 μg.

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.

2.6. Activation of aminopropyl-silica gels

Aminopropyl-silica gels were activated by DSC. Five grams of the gels were 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 and methanol. The obtained silica gels were dried in vacuo over P_2O_5 at 60°C for 2 h.

2.7. Preparation of ovomucoid-bonded materials

Each protein was bound to DSC-activated aminopropyl-silica gels as follows: 1.2 g of the DSC-activated silica gels were slurried in 20 ml of 20 m*M* phosphate buffer (pH 6.8). To the mixture, 400 mg of each ovomucoid protein, except for the fractionated protein portion (fraction B), dissolved in 5 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. For fraction B protein, 100 mg of protein were used and treated as described above. The reaction mixture was washed with water and further reacted with 2-aminoethanol–water (1:50) adjusted to pH 6.6 with hydrochloric acid at room temperature for 1 h. Then the reaction mixtures were filtered, and washed with water and methanol. The isolated materials were dried in vacuo over P_2O_5 at 40°C for 6 h.

The ovomucoid-bonded materials were packed into a 100 × 4.6 mm I.D. stainless-steel column by the slurry packing method using 5% ethanol as the slurry and packing solvent.

2.8. Elemental analysis

Elemental analysis of the ovomucoid-bonded silica materials was performed using ion-chromatography combined with oxygen-flask method for sulfur.

2.9. Chromatography

For the chiral resolution of solutes, the HPLC system consisted of an LC-9A pump, an SPD-6A spectrophotometer, an SIL-6B autoinjector, a

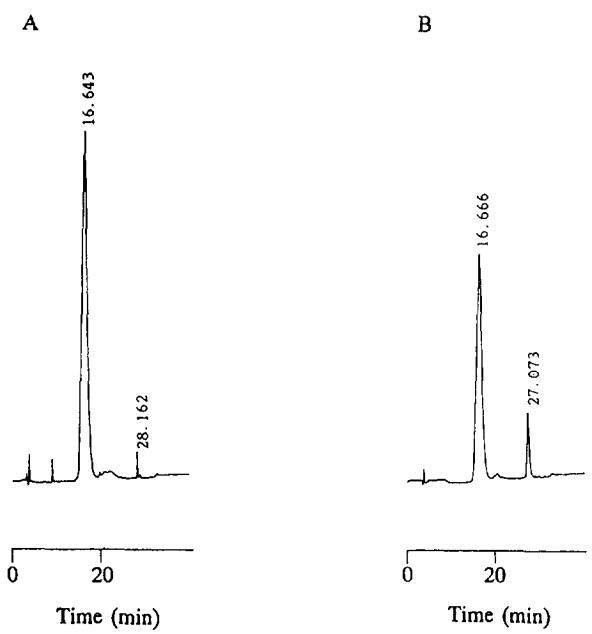

Fig. 3. Chromatograms of commercial and isolated OMTKY proteins on a C_{18} column. (A) OMTKY-C; (B) OMTKY-I. Other conditions as in Fig. 2.

Table 1
Amounts of bonded proteins on OMCHI and OMTKY Materials

Material	Sulfur content[a] (%)	Amount of bonded ovomucoid (μmol/g)
OMCHI-C1	0.15	2.3
OMCHI-C2	0.20	3.1
OMCHI-C3	0.23	3.6
OMCHI-I1	0.18	2.8
OMCHI-I2	0.16	2.5
OMTKY-C	0.23	3.6
OMTKY-I	0.21	3.3
OVM[b]	0.26	4.1

[a] Estimated from the elemental analysis data of sulfur.
[b] Commercial available Ultron-ES OVM column.

C-R4A integrator and an SCL-6B system controller (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.8 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 the elution times of retained and unretained solutes, respectively; k'_1 and k'_2 are the capacity factors of the first- and second-eluted peaks, respectively. The retention time of unretained solute, t_0, was measured by injection of 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 $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are the retention times of the first- and second-eluted peaks, respectively, and t_{w1} and t_{w2} are the peak widths. All separation were carried out at 25°C using a CO-1093C column oven (Uniflows, Tokyo, Japan). The composition of eluents, which are prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphosphate and organic modifier, are specified in the table and figure legends.

For reversed-phase chromatography of the ovomucoid protein, 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) including 0.1% trifluoroacetic acid (TFA); eluent B, H_2O–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 a Cosmosil 5C18-AR column (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.

3. Results and discussions

3.1. Characterization of ovomucoid proteins

Fig. 2A–E shows chromatograms of the separation of OMCHI-C1, OMCHI-C2, OMCHI-C3,

Table 2
Capacity factor, enantioselectivity and resolution of benzoin, chlorpheniramine and ketoprofen on several OMCHI and OMTKY columns[a]

Column	Benzoin			Chlorpheniramine			Ketoprofen		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
OMCHI-C1	2.86	2.37	5.64	1.30	1.77	1.52	10.8	1.06	0.54
OMCHI-C2	1.85	2.29	3.87	1.33	1.49	1.16	6.01	1.07	0.56
OMCHI-C3	1.52	1.90	3.30	0.75	1.42	1.28	6.39	1.00	–
OMCHI-I1	3.68	2.70	9.57	1.83	1.91	n.c.[b]	11.1	1.11	1.38
OMCHI-I2	2.45	2.33	4.49	1.53	1.76	3.11	8.10	1.14	1.06
OMTKY-C	0.61	1.00	–	0.33	1.00	–	3.83	1.00	–
OMTKY-I	2.80	2.03	6.21	1.97	1.20	n.c.	8.87	1.16	1.60
OVM	4.08	2.62	8.72	1.63	1.80	3.07	14.3	1.10	1.18
Fraction A	0.46	1.00	–	3.57	1.00	–	4.15	1.00	–
Fraction B	9.19	3.08	11.0	4.63	2.28	6.24	20.7	1.24	2.08

[a] HPLC conditions: column, each material packed into 100 × 4.6 mm I.D. stainless steel column except for the OVM column, which is the commercially available Ultron ES-OVM column (150 × 4.6 mm I.D.); eluent, 20 m*M* phosphate buffer (pH 5.0)–ethanol (90:10, v/v); flow-rate: 1.0 ml/min for the Ultron-ES OVM column and 0.8 ml/min for other columns; detection, 220 nm; t_0 was 2.1 min for the Ultron ES-OVM column and 1.9 min for the other columns.

[b] n.c. means that resolution is not calculated because of overlapping of the peaks of one enantiomer and maleic acid.

OMCHI-I1 and OMCHI-I2 proteins, respectively, on a C_{18} column. The first three OMCHI proteins were from commercial sources, while the last two proteins were isolated from chicken egg whites. It was found that the commercial and isolated OMCHI proteins are crude preparations, and that the OMCHIs contain one major protein fraction at a retention time of 15 min and one or two minor protein fractions at retention times of 30 min or 20 and 30 min.

Next, we separated the OMCHI-C1 into two fractions, fractions A and B, by cation-exchange chromatography. The obtained fractions A and B showed broad peaks at retention times of about 15 min and 30 min, respectively, on the same C_{18} column as used in Fig. 2. Note that the protein fraction giving the sharp peak at a retention time of 29 min observed with OMCHI-C2 and OMCHI-I2 proteins is different from the fraction B protein. The N-terminal sequencing results of the first 15 amino acid in proteins of fraction A are A-E-V-D-X-S-R-F-P-X-A-T-D-K-E, where X deviates cystine or glycosylated amino acid residue. These amino acid sequences are in good agreement with those of the OMCHI reported by Kato et al. [14]. However, the N-terminal sequencing results of the first 5 amino acid in proteins of fraction B are T-E-S-P-X. The second sequences were not observed with the N-terminal sequencing results of fractions A and B. Also, fraction A protein as well as commercial and isolated OMCHI proteins showed trypsin-inhibitory activity as reported previously [15], while fraction B protein had no trypsin-inhibitory activity. These results suggest that the major protein fraction at a retention time of 15 min should be the OMCHI, and that the minor protein fraction with a retention time of 30 min should be other protein. At present we have not yet identified the minor protein fraction, and thus this protein fraction is termed "unknown protein".

Fig. 3A, B shows chromatograms of OMTKY-C and OMTKY-I proteins, respectively, on a C_{18} column. The former OMTKY protein was from commercial sources, while the latter protein was isolated from turkey egg whites. Taking into account the results of the amino acid sequencing and the trypsin-inhibitory activity of the OMTKY, the protein fraction at a retention time of 16 min should be the OMTKY, and the minor protein fraction at a retention time of 27 min could be the unknown protein as in the case of the commercial and isolated OMCHI proteins. It is interesting that the OMTKY-C has no protein fraction at a retention time of 27 min.

We do not know how the commercial OMCHI and OMTKY are isolated. However, the above results reveal that the commercial and isolated OMCHI and OMTKY are crude, except for the commercial OMTKY, and that they include minor unknown protein fractions. Also, the isolation method affects the OMCHI and OMTKY purity.

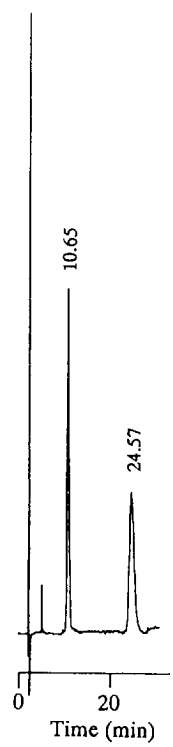

Fig. 4. Chiral resolution of benzoin on a commercial OMCHI-bonded column (Ultron ES-OVM column). HPLC conditions: column, 150 × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.0)–ethanol (90:10, v/v); flow-rate, 1.0 ml/min; detection, 220 nm.

3.2. Amounts of the bonded ovomucoid proteins

Next, we bound these proteins to DSC-activated aminopropyl-silica gels. Table 1 shows the amounts of bonded ovomucoid proteins on the various OMCHI and OMTKY materials. Assuming that the protein is pure, the amount of the bonded protein was estimated by elemental analysis of sulfur. The results show that the amounts of bonded OMCHI and OMTKY proteins range from 2.3 to 3.6 μmol/g. The amounts of bonded proteins on commercial OMCHI materials (Ultron ESOVM) was estimated to be 4.1 μmol/g.

3.3. Chiral recognition properties of the OMCHI, fractionated OMCHI and OMTKY columns

Next, we examined the chiral recognition properties of the OMCHI, fractionated OMCHI (fractions A and B) and OMTKY columns. Table 2 shows the capacity factor (k'_1), enantioselectivity (α) and resolution (R_s) of benzoin, chlorpheniramine and ketoprofen on the columns made with the various proteins. For comparison, those values on the commercial OMCHI-bonded column (Ultron ES-OVM) are also cited in Table 2. Figs. 4–6 show chromatograms of benzoin on Ultron ES-OVM, OMCHI and fractionated OMCHI (fractions A and B) columns, respectively. Fig. 7 shows chromatograms of benzoin on the OMTKY columns. It was found that commercial and isolated OMCHI columns give a chiral recognition ability comparable to that of a commercial Ultron ES-OVM column. Also, the pure OMCHI (fraction A) has no chiral recognition ability, but the unknown protein (fraction B) has. These results show that the chiral recognition ability of the crude OMCHI-bonded column originates from the unknown protein fraction in the crude OMCHI proteins. The differences in retention and enantioselective properties of the various OMCHI columns were easily elucidated. It depends on how much of the unknown protein is isolated from egg whites with the pure OMCHI. It is plausible that the isolation method and/or the protein purity much affect the chiral recognition

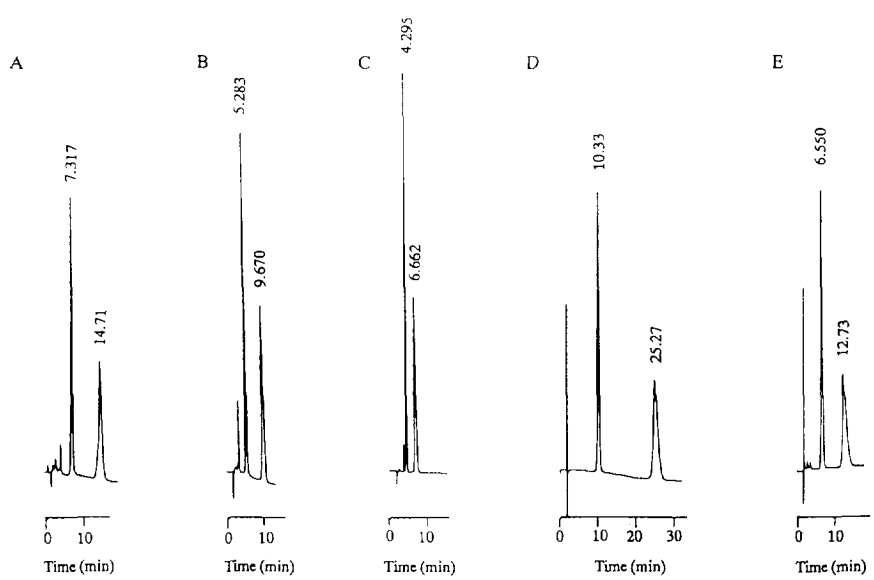

Fig. 5. Chiral resolution of benzoin on columns made with commercial and isolated OMCHI. (A) OMCHI-C1; (B) OMCHI-C2; (C) OMCHI-C3; (D) OMCHI-I1, (E) OMCHI-I2. HPLC conditions: column, 100 × 4.6 mm I.D.; flow-rate, 1.0 ml/min. Other conditions as in Fig. 4.

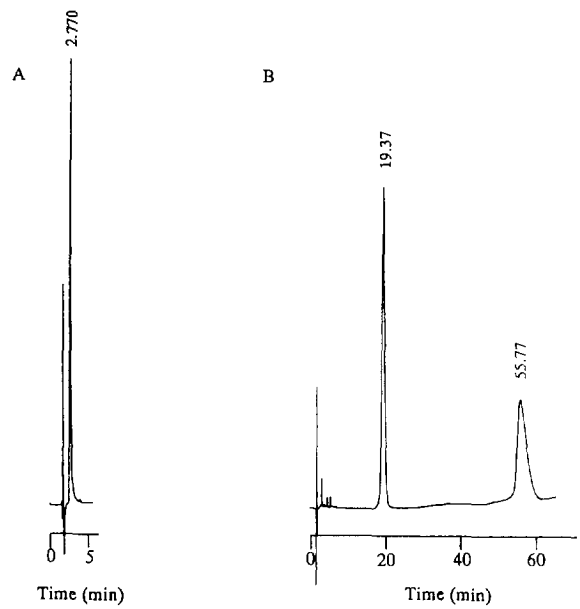

Fig. 6. Chiral resolution of benzoin on columns made with the fractionated OMCHI. (A) fraction A; (B) fraction B. Other conditions as in Fig. 5.

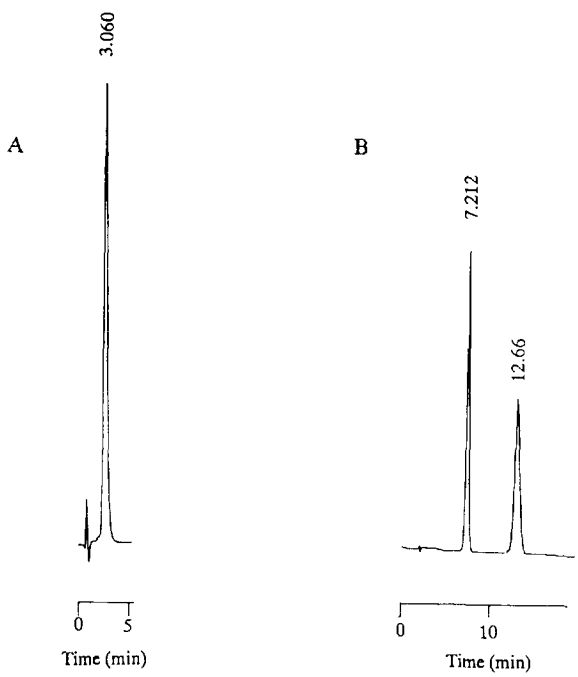

Fig. 7. Chiral resolution of benzoin on columns made with commercial and isolated OMTKY. (A) OMTKY-C; (B) OMTKY-I. Other conditions as in Fig. 5.

properties of the OMCHI columns made with crude proteins.

As shown in Table 2 and Fig. 7, the OMTKY-C column made with the commercial OMTKY had no chiral recognition ability, whereas the OMTKY-I column made with the isolated OMTKY showed good chiral recognition ability. The OMTKY-C column still gave no chiral recognition ability when the ethanol content in the eluent was decreased from 10% to 1%. If the chiral recognition ability of the OMTKY-I column should originate from the protein fraction with a retention time of 27 min, the presence and absence of the chiral recognition ability of the two OMTKY columns could be well explained in the case of the crude OMCHI columns. The lack of chiral recognition ability of the OMTKY-C column is due to the absence of the protein fraction with a retention time of 27 min.

In conclusion, an unknown protein fraction is present in crude ovomucoid protein from commercial sources and isolated from egg whites. This unknown protein fraction could be responsible for the chiral recognition of the solutes tested. Further work is on-going to characterize the unknown protein fraction in crude ovomucoid proteins.

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