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Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein
III. Effect of aggregation of ovoglycoprotein on chiral resolution

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Abstract

Ovoglycoprotein from chicken egg whites (OGCHI) subjected to 80°C has produced the associated OGCHI, which is a heat-induced aggregate of OGCHI. The molecular weight of the aggregate was estimated to be ca. 5.7 million daltons by a low-angle laser light-scattering detection. The heat-induced aggregate of OGCHI was found to dissociate reversibly to the OGCHI monomer. The OGCHI aggregate has little chiral recognition ability, or has much lower chiral recognition ability than the native OGCHI. Further, the OGCHI monomer from the reversibly dissociated OGCHI aggregate has chiral recognition ability comparable to that of the native OGCHI.

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

1. Introduction

The avian ovomucoid is a glycoprotein which is composed of three tandem, homologous, structural domains [1]. Since each domain had potent inhibitory activity toward serine proteinases [1], the physical property and biological activity of the ovomucoid were intensively investigated. It was reported that the average molecular weight of ovomucoid from chicken egg whites (OMCHI) was 27 000–32 000 daltons [2], and that OMCHI contained microheterogeneity in the carbohydrate moiety [2,3]. On the other hand, Miwa et al. [4] prepared chiral stationary phases based on OMCHI and utilized for the separation of enantiomeric compounds. Recently [5], we found that OMCHI used in previous studies was crude. The OMCHI preparations included about 10% of other glycoprotein. In addition, we isolated the glycoprotein from the crude OMCHI preparations and chicken egg whites [5]. The isolated glycoprotein 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. The glycoprotein had an average molecular weight of 29 700 daltons and sugar content of 25%. In addition, the N-terminal amino acid of the glycoprotein was Thr, and the glycoprotein had no trypsin inhibitory activity. Previ-

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ously [6], Ketterer isolated ovoglycoprotein from chicken egg whites (OGCHI). Many data supported that the protein isolated by us might be OGCHI, but the only discrepancy was the average molecular weight. We also termed the isolated glycoprotein OGCHI. About 10% OGCHI was included in crude OMCHI preparations. Pure OMCHI and OGCHI, respectively, were bound to aminopropyl–silica gels to evaluate chiral recognition ability. However, the pure OMCHI gave no chiral recognition, while the pure OGCHI gave significantly higher chiral recognition than the impure OMCHI reported by Miwa et al. [4]. This reveals that chiral recognition ability of the OMCHI reported previously comes from the OGCHI, which is present in crude OMCHI as an impurity. Further, a chiral stationary phase based on crude OMCHI (commercially available OVM column) showed good chiral recognition ability despite the low OGCHI content in crude OMCHI [7]. This is due to that OGCHI is preferentially bound to N,N′-disuccinimidylcarbonate (DSC)-activated aminopropyl-silica gels compared with OMCHI.

It was reported that OMCHI would not denature irreversibly when subjected to 100°C for one hour [8]. However, there is no report for heat stability of OGCHI. For the preparation of excellent chiral stationary phases based on OGCHI, it is important to clarify the physicochemical stability of the OGCHI. In this study, we examined the heat stability of OGCHI. This paper deals with heat-induced association of OGCHI, and chiral recognition ability of the associated OGCHI.

2. Experimental

2.1. Reagents and materials

Benzoin, chlorpheniramine maleate and tolperisone hydrochloride were purchased from Nacalai Tesque (Kyoto, Japan). Ibuprofen and ketoprofen were donated from Chugai Pharmaceutical (Tokyo, Japan). Hexobarbital was donated from Teikoku Chemicals (Tokyo, Japan). Sephadex G-25 (fine) and SP Sepharose FF were purchased from Pharmacia Biotech (Tokyo, Japan). DSC was purchased from Wako Pure Chemical Industry (Osaka, Japan). Silica gels (Ultron-30, 5-μm diameter, 30-nm pore size, 100 m^2/g specific surface area) used are 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 the preparations of the electrophoretic buffer, eluent and sample solution.

2.2. Isolation of OGCHI from egg whites

OGCHI was isolated as reported previously [9]. Briefly, crude OMCHI was precipitated from egg whites with ethanol according to procedures modified slightly from those of Fredericq and Deutsch [10]. 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 SP Sepharose FF column (5×12 cm) 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 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 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 (5×20 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. The purity of OGCHI obtained was estimated to be 99%, based on the peak area by reversed-phase chromatography as described below.

2.3. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) separations were performed with a Beckman P/ACE system 5500 equipped with a photodiode array detector (Fullerton, CA, USA). A fused silica capillary (75 μm I.D., effective length 50 cm) was used for separation. All capillaries were thermostated at 23°C by using a liquid coolant.

Electrophoretic buffer (running buffer) solutions used in this study were 25 m*M* borate buffer (pH 9.2) containing 5 m*M* 1,3-diaminopropane. The

running buffer solutions were filtered through a 0.45-μm membrane filter (Gelmann Sciences Japan, Tokyo, Japan) and degassed with a Branson model B-2200 ultrasonic bath (Yamato, Tokyo, Japan) prior to use. The capillary was rinsed with water for 1 mm, 0.1 *M* NaOH for 3 min, water for 1 min and the running buffer for 2 min prior to the run. The sample concentration was about 4 mg/ml. The sample solution was injected at ca. 3450 Pa for 1 s. Both ends of the capillary were dipped into the running buffer solution, and a constant voltage of 25.0 kV was applied for the separation. Detection was performed at 214 nm.

2.4. Liquid chromatography

The HPLC system used was composed of two LC-9A pumps, an SPD-6A spectrophotometer, an SIL-6B auto injector, a C-R4A integrator and an SCL-6B system controller (all from Shimadzu, Kyoto, Japan). Native OGCHI was dissolved in water at a concentration of 4 mg/ml and incubated at 80°C. With predetermined intervals, the sample was withdrawn and checked by anion-exchange, size-exclusion and reversed-phase chromatography, as described below.

2.4.1. Anion-exchange chromatography

The column used was DEAE-5PW (7.5 mm I.D.× 75 mm) (Tosoh, Tokyo, Japan). The flow-rate was 0.8 ml/min. A 20 μl aliquot of the sample was loaded onto the column. Detection was performed at 280 nm. All separations were carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The eluents used are as follows: eluent A, 20 m*M* phosphate buffer (pH 7.1); eluent B, 20 m*M* phosphate buffer (pH 7.1) including 400 m*M* Na_2SO_4; linear gradient from 0% eluent B at 0 min to 100% eluent B at 40 min.

2.4.2. Size-exclusion chromatography

The eluent used was 0.1 *M* NH_4HCO_3. The columns used were Shodex PROTEIN KW-802.5 (8.0 mm I.D.×300 mm) (Showa Denko, Tokyo, Japan). A 20 μl aliquot of the sample was loaded onto the column. Detection was performed at 280 nm. The separation was performed at ambient temperature. The protein standards used are bovine serum albumin [molecular weight (MW), 67 000 daltons], β-lactoglobulin (MW, 34 000 daltons), α-chymotrypsin (MW, 25 000 daltons), ribonuclease A (MW, 13 700 daltons), fetuin (MW, 48 400 daltons), OGCHI (MW, 29 700 daltons), OMCHI (MW, 27 000 daltons) [5], first and second combination domain of ovomucoid from turkey (OMTKY [2+3]) (MW, 15 150 daltons) [11] and glycosylated ovomucoid third domain (OMTKY3S) (MW, 8670 daltons) [11].

2.4.3. Reversed-phase chromatography

For the reversed-phase chromatographic separations, 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 Cosmosil 5C18-AR (4.6 mm I.D.× 250 mm) (Nacalai Tesque, Kyoto, Japan). A 5 μl aliquot of the sample was loaded onto the column. 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 molecular weights of OGCHI aggregate

Determination of molecular weights was performed with a low-angle laser light-scattering (LALLS) detector (LS-8000, Tosoh, Tokyo, Japan), where the size exclusion column, Shodex PROTEIN KW-803 (8.0 mm I.D.×300 mm) (Showa Denko, Tokyo, Japan), was used. The flow rate was 0.5 ml/min and other conditions were as described in Section 2.4.

2.6. Preparation of associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate

OGCHI was dissolved in water at a concentration of 4 mg/ml and incubated at 80°C for 60 min. Then, the solution was lyophilized. The obtained OGCHI was termed associated OGCHI, which included the aggregate and monomer of OGCHI at a weight ratio of 0.80:1.

The associated OGCHI was subjected to anion-

exchange chromatography under the conditions described above to isolate an OGCHI aggregate. The OGCHI aggregate fraction was isolated, lyophilized and dialyzed against water. The obtained dialyzate was lyophilized. The obtained protein was termed OGCHI aggregate, which includes the aggregate and monomer of OGCHI at a weight ratio of 1.76:1.

The associated OGCHI was incubated in 20 m*M* phosphate buffer solution (pH 6.6) at 30°C. To examine the time course of dissociation of the associated OGCHI, the sample was withdrawn with the predetermined intervals and checked by size-exclusion chromatography. The associated OGCHI was incubated in 20 m*M* phosphate buffer solution (pH 6.6) at 30°C for 30 h. The obtained protein was termed dissociated OGCHI aggregate, which includes the aggregate and monomer of OGCHI at a weight ratio of 0.30:1.

2.7. Preparation of native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate materials and evaluation of their chiral recognition ability

Aminopropyl-silica gels were prepared from silica gels, and activated by DSC as reported previously [5]. Native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate were bound to aminopropyl–silica gels activated by DSC as described previously [5]. Briefly, a weight of 1 g of the DSC-activated silica gels was slurried in 20 m*M* phosphate buffer (pH 6.8). To the mixture, 20 ml of a protein solution of the same buffer (1 mg/ml) was added slowly at room temperature for 1 h by adjusting 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 (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 2.0 mm I.D.×100 mm stainless-steel column by the slurry packing method. The slurry and packing solvents were water–ethanol (95:5; v/v).

For chiral resolution of various solutes on the native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate materials, the same HPLC system as described above was used except for a Rheodyne 7125 injector (Cotati, CA, USA) with a 5-μl loop and a C-R6A integrator (Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.2 ml/min. Detection was performed at 210 nm. Retention factors (k), enantioseparation factor (α) and resolution (Rs) of a racemate were calculated. The retention time of unretained solute, t_o, was measured by injecting a solution whose organic modifier content was slightly different from that of the eluent used. All separations were carried out at 25°C using a water bath. The eluent was prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphosphate and ethanol.

2.8. 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 above. 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.

3. Results and discussion

3.1. Heat stability of OGCHI

Though OMCHI was subjected to 80°C for 2 h, there was no difference in CZE behaviors before and after heating. It was reported that OMCHI would not denature irreversibly when subjected to 100°C for 1 h [8]. Our result is consistent with that reported previously [8]. Fig. 1, parts A and B, shows the electropherograms of native OGCHI and OGCHI subjected to 80°C for 60 min, respectively. The shoulder peak having longer migration time than native OGCHI was observed after heating. It was found that OGCHI was unstable against heating at 80°C, but that OMCHI was stable.

We tried to clarify what heat-induced change

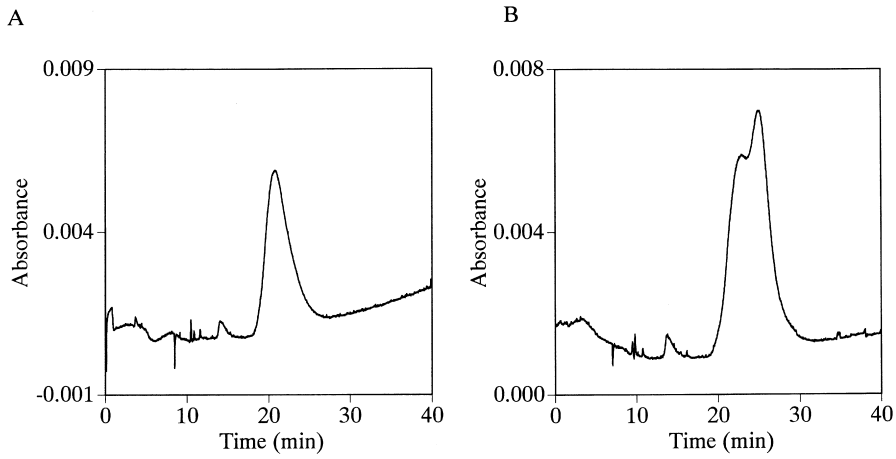

Fig. 1. Electropherograms of native OGCHI (A) and OGCHI subjected to 80°C for 60 mm (B). Conditions: capillary, 75 μm I.D., effective length 50 cm; running buffer solution, 25 m*M* borate buffer (pH 9.2) including 5 m*M* 1,3-diaminopropane; sample, 4 mg/ml each protein; applied voltage, 25 kV.

occurred to OGCHI. Fig. 2, parts A, B and C, shows size-exclusion, anion-exchange and reversed-phase chromatographic separations, respectively, of OGCHI subjected to 80°C for 0, 10, 20, 40 and 60 min. From these electrophoretic and chromatographic behaviors, it was found that a heat-induced change occurred to OGCHI with a size and charge; OGCHI subjected to 80°C was more negatively charged than native OGCHI, and the former had higher molecular weight than the latter. However, it is interesting that OGCHI subjected to 80°C showed the same retention times as native OGCHI on a reversed-phase column.

3.2. Determination of molecular weight of heat-induced product of OGCHI

Fig. 3 shows the relationship of retention times and logarithm of molecular weights for standard proteins on a size-exclusion column, where aqueous ammonium bicarbonate solutions are used as an eluent. As shown in Fig. 3, two calibration lines were obtained. The upper line is for hydrophobic, nonglycosylated proteins, and the lower line is for hydrophilic glycoproteins. Hefti [12] reported that when aqueous buffer solutions with no addition of ethanol were used, the plots of hydrophobic proteins such as bovine serum albumin and avidin were deviated from the calibration graph. When ethanol was added to the eluent, the plots of hydrophilic glycoproteins were deviated from the calibration graph prepared using hydrophobic, nonglycosylated proteins. Thus, we used the calibration line for hydrophilic glycoproteins to estimate the molecular weight of the associated OGCHI. The molecular weight of heat-induced associated OGCHI was estimated to be ca. 71 000 daltons. Fig. 4, parts A and B, shows size exclusion chromatograms of the native OGCHI and associated OGCHI, respectively, where solid and dotted lines indicate the refractive index (RI) and LALLS detections, respectively. The LALLS intensity of the aggregated OGCHI species in the associated OGCHI is much stronger than that in the native OGCHI. Though the native OGCHI had no RI response corresponding to the OGCHI aggregate, the LALLS detection showed the presence of the aggregated OGCHI. This indicates that the native OGCHI includes trace amounts of the aggregated OGCHI. Though the aggregated OGCHI gave a single peak by RI detection, the LALLS detection showed two peaks. The molecular weight of the major peak of the aggregated OGCHI was estimated to be ca. 5.7 million daltons. These results indicate that the native OGCHI produces the aggregated OGCHI by heating at 80°C, and that the molecular weight of the aggregated OGCHI can not be estimated precisely by size exclusion chromatography.

The aggregated OGCHI showed the same retention times as the native OGCHI on a reversed-phase

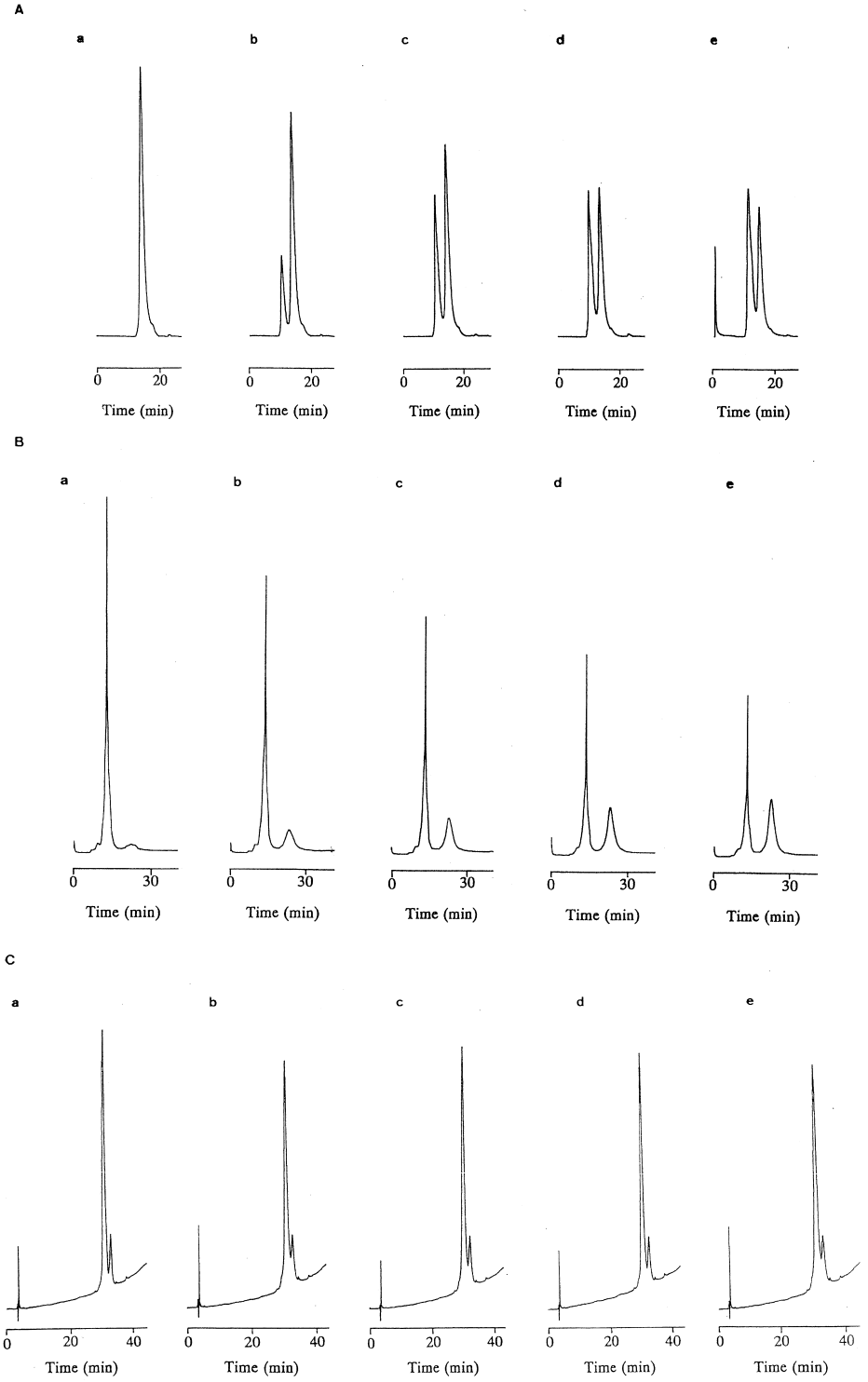

Fig. 2. Size-exclusion (A), anion-exchange (B) and reversed-phase (C) chromatographic separations of OGCHI subjected to 80°C. HPLC conditions, see Section 2. Reaction time: a, 0 min; b, 10 min; c, 20 min; d, 40 min; e, 60 min.

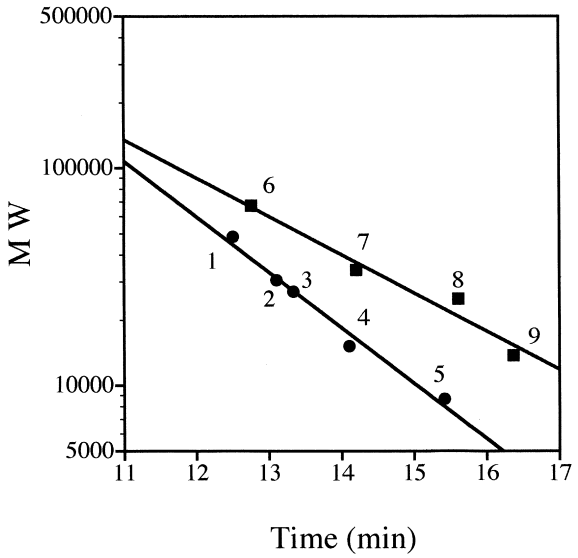

Fig. 3. Relationship of retention times and logarithm of molecular weights for standard proteins on a size-exclusion column. Keys: 1, fetuin; 2, OGCHI; 3, OMCHI; 4, OMTKY [2+3]; 5, OMTKY3S; 6, bovine serum albumin; 7, β-lactoglobulin; 8, α-chymotrypsin; 9, ribonuclease A. Column: PROTEIN KW-802.5 (8 mm I.D.×30 cm). Eluent: 0.1 *M* NH_4HCO_3. Flow-rate: 0.8 ml/min. Detection: 280 nm. Loaded amount: 25 μg.

column. This could be due to that the OGCHI aggregate dissociates to the monomer in a mixture of water and acetonitrile including TFA. This result suggests that association of OGCHI could be caused by hydrophobic, electrostatic and/or hydrogen bonding interactions.

3.3. Dissociation of OGCHI aggregate to OGCHI monomer

The fraction of OGCHI aggregate was isolated using an anion-exchange chromatographic method followed by desalting and lyophilization. The isolated OGCHI aggregate included OGCHI monomer as the weight ratio of 1.76:1. This result indicates that OGCHI aggregate dissociates to OGCHI monomer in the process of isolation. We examined the time course of dissociation of the OGCHI aggregate to the monomer in a phosphate buffer solution (pH 6.6) at 30°C, whose conditions are the same with those for preparing an OGCHI-bonded column. We assumed that the native OGCHI produced only the OGCHI aggregate by heating at 80°C, whose molecular weight is ca. 5.7 million daltons, and the OGCHI aggregate reversibly dissociated to the OGCHI monomer. Fig. 5 shows the time course of the dissociation of the OGCHI aggregate to the OGCHI monomer. After the incubation for 72 h, the aggregated OGCHI was almost completely dissociated to the OGCHI monomer.

3.4. Chiral recognition ability of native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate

Native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate were bound to activated aminopropyl–silica gels in order to examine chiral recognition abilities of the heat-induced associated OGCHI, according to the method reported previously [5]. The weight ratios of the aggregate and monomer of OGCHI used for the preparation of associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate materials were 0.80:1, 1.76:1 and 0.30:1, respectively. The reaction of a protein and activated silica gel was performed in phosphate buffer (pH 6.6) at 30°C for 15 h. Thus, the weight ratio of the OGCHI aggregate and monomer might change in the course of the binding reaction. When a weight of 20 mg of native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate per 1 g of the activated silica gels were reacted, the bound OGCHI amounts were ca. 17 mg (0.58 μmol) as a monomer for all materials prepared. In a previous paper, we reported that linear correlation was obtained between the capacity factor of each enantiomer and the bound amount of OGCHI [7]. It is essential to bind the same amount of a protein in order to compare the retentivity and enantioselectivity of these OGCHI materials. Table 1 shows the retentivity and enantioselectivity of native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate materials for neutral, acidic and basic solutes. With an increase in the weight ratio of the OGCHI monomer to the aggregate, the retentivity and enantioselectivity of various solutes were increased. These results reveal that the OGCHI aggregate has little chiral recognition ability, or has much lower chiral recognition ability than the native OGCHI. Further, the heat-induced OGCHI

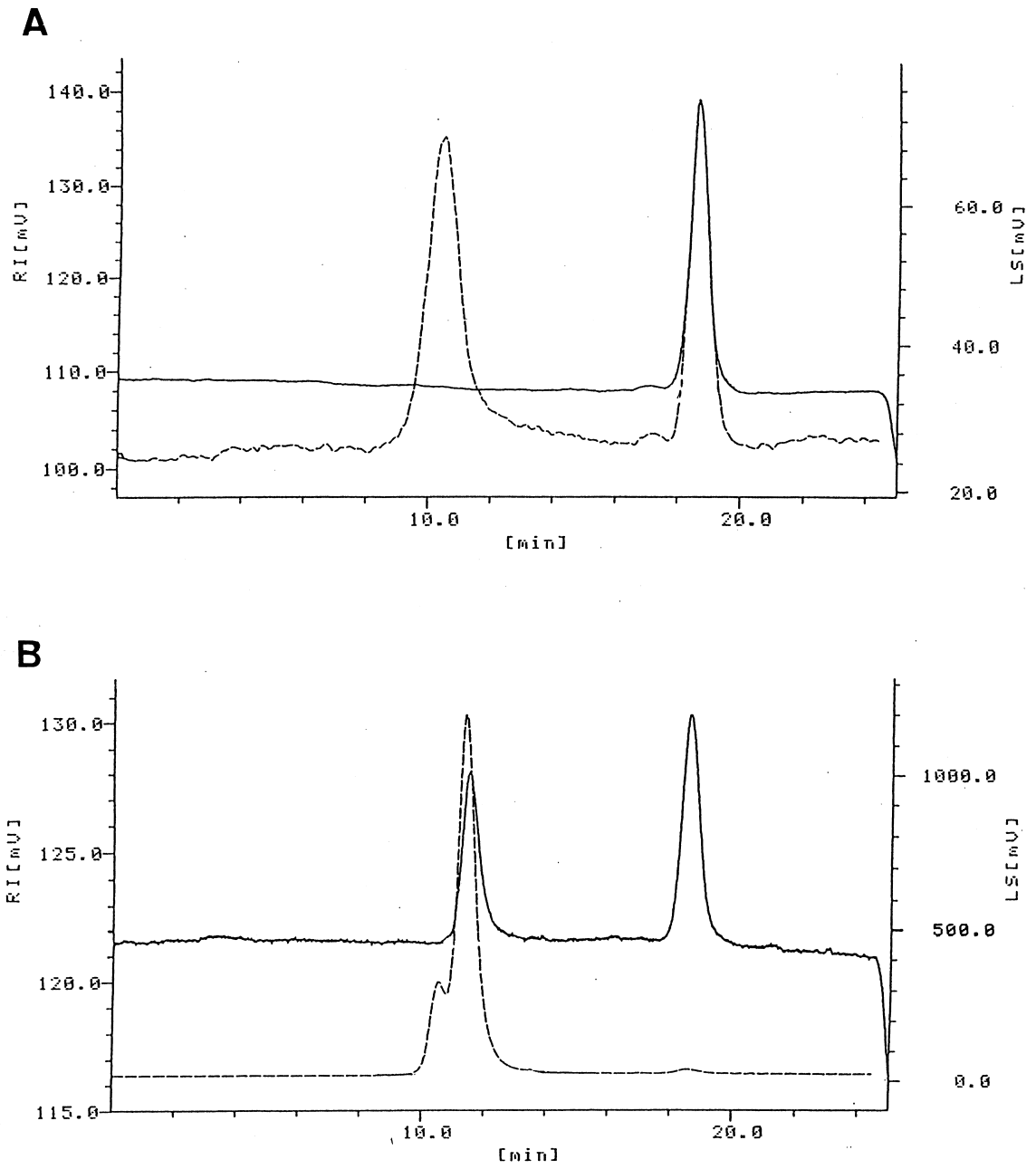

Fig. 4. Size exclusion chromatograms of native OGCHI (A) and associated OGCHI (B). Solid and dotted lines indicate the RI and LALLS detections, respectively. Column: PROTEIN KW-803 (8 mm I.D.×30 cm). Flow-rate: 0.5 ml/min. Other conditions as in Fig. 3.

aggregate reversibly dissociates to the monomer, which still has chiral recognition ability.

In conclusion, OGCHI suffered heat-induced aggregation. The aggregated OGCHI was reversibly dissociated to the monomer. The OGCHI aggregate has little chiral recognition ability, or has much lower chiral recognition ability than the native OGCHI.

Table 1
Comparison of chiral recognition ability of native OGCHI, associated OGCHI, OGCHI aggregate and dissociated OGCHI aggregate materials[a]

Solute	Column											
	Native OGCHI			Associated OGCHI[b]			OGCHI aggregate[c]			Dissociated OGCHI aggregate[d]		
	k_1^e	α	Rs	k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
Benzoin	5.39	3.16	5.83	3.48	3.11	3.68	1.71	2.68	2.75	4.78	3.11	4.47
Hexobarbital	0.85	1.37	0.65	0.58	1.33	0.40	0.39	1.00	–	0.76	1.38	0.59
Ibuprofen	4.21	1.32	1.42	3.07	1.24	0.80	2.33	1.11	0.25	3.81	1.30	0.97
Ketoprofen	13.95	1.40	1.52	8.57	1.33	1.21	5.21	1.23	0.99	11.54	1.36	1.44
Chlorpheniramine	2.57	2.67	2.82	1.82	2.49	2.44	0.93	2.28	2.20	2.29	2.55	2.75
Tolperisone	0.51	3.31	2.63	0.38	3.18	1.61	0.21	2.64	0.87	0.46	3.30	2.27

[a] HPLC conditions: column, 2.0 mm I.D.×100 mm; eluent, 20 m*M* phosphate buffer (pH 5.1)/ethanol=95/5 (V/V); column temperature, 25°C; flow rate, 0.2 ml/min; detection, 210 nm.
[b] Native OGCHI was incubated at 80°C for 60 min. The weight ratio of the aggregate and monomer of OGCHI was 0.80:1.
[c] The associated OGCHI was subjected to anion-exchange chromatography and the OGCHI aggregate fraction was isolated. The weight ratio of the aggregate and monomer of OGCHI was 1.76:1.
[d] The associated OGCHI was incubated in 20 m*M* phosphate buffer solution (pH 6.6) at 30°C for 30 h. The weight ratio of the aggregate and monomer of OGCHI was 0.30:1.
[e] The k_1 is the retention factor of the first eluted enantiomer.

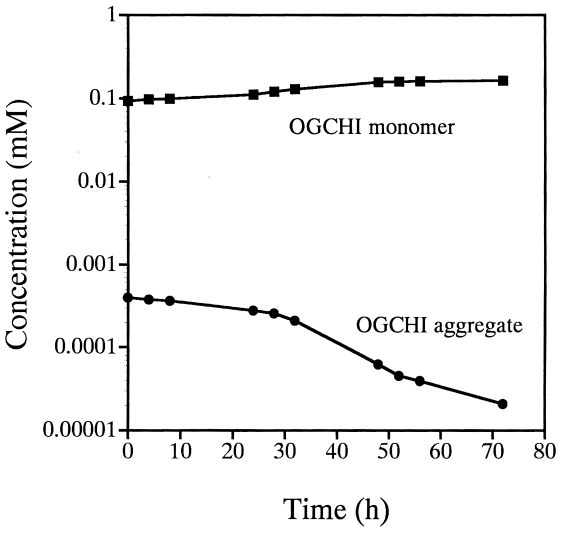

Fig. 5. Time course of dissociation of the OGCHI aggregate. The associated OGCHI was incubated at 30°C in 20 m*M* phosphate buffer solution (pH 6.6). The amount of the monomer of OGCHI was determined by size-exclusion chromatography under the conditions described in Fig. 3.

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