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Countercurrent chromatographic analysis of ovalbumin obtained from various sources using the cross-axis coil planet centrifuge

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Abstract

The present studies have been conducted to investigate the cause of an unusually broad peak of ovalbumin obtained by countercurrent chromatography (CCC) reported earlier [K. Shinomija et al., J. Chromatogr., 644 (1993) 215]. A series of CCC experiments using our prototyte of the cross-axis coil planet centrifuge revealed that commercial ovalbumin products were classified into two groups: group A formed two peaks of ovalbumin at pH 7.0 and 5.8, while group B showed a relatively sharp single peak in a broad range of pH. Electrophoresis indicated that the group A ovalbumin consisted of both natural and denatured products: the natural ovalbumin is a monomer (M_r 45 000) whereas the denatured products form dimers (M_r 90 000). The abnormally broad peak obtained from the group A ovalbumin at pH 9 is apparently caused by the heterogeneity of the sample protein.

Keywords: Counter-current chromatography; Cross-axis coil planet centrifuge; Ovalbumin; Proteins

1. Introduction

The cross-axis coil planet centrifuge (X-axis CPC) has been steadily improved for the performance of countercurrent chromatography (CCC) since it was introduced in the mid-1980s [1,2]. The system produces a unique mode of planetary motion such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge. This motion allows reliable retention of the stationary phase even for aqueous–aqueous polymer phase systems with extremely low interfacial tension.

Our previous studies demonstrated that the improved model of the X-axis CPC holding the columns in the off-center position was useful for the separation of proteins with polyethylene glycol (PEG)–phosphate solvent systems [3]. When the method was used to separate a set of three test proteins, ovalbumin showed an abnormally broad peak compared to the other two. The present studies have been conducted to find the cause of this broad band.

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2. Experimental

2.1. Apparatus

The X-axis CPC used in the present studies was constructed at the machine shop of Nihon University, Chiba, Japan. The basic design of the apparatus was reported earlier [3]. This X-axis CPC is additionally equipped with a cooling fan for controling the ambient temperature during the separation. The motor (750W) mounted at the bottom of the centrifuge directly drives the rotary frame. The stainless-steel miter gear mounted around the central shaft reduces the noise and the system has withstood many months of operation without failure.

2.2. Preparation of columns

Protein separations were performed with a pair of eccentric coil assemblies. Each assembly consists of 20 coil units prepared by winding a 1.00 mm I.D. polytetrafluoroethylene (PTFE) tube (Flon Kogyo, Tokyo, Japan) onto a 7.6 cm×5 mm O.D. nylon pipe making tight left-handed coils. These coil units are symmetrically arranged around the holder with their axes parallel to the holder axis. Two coil assemblies are connected in series on the rotary frame to obtain a total column capacity of 28 ml. The flow tubes are lubricated with grease and individually protected with a short sheath of Viton tubing (2 mm I.D. and 4 mm O.D., Togawa Rubber, Osaka, Japan) to prevent the flow tubes from direct contact with the metal parts.

2.3. Reagent

PEG 1000 (M_r 1000) and ovalbumin (chicken egg, OVA-1) were purchased from Sigma (St. Louis, MO, USA). Chicken egg ovalbumins recrystallized two and five times (OVA-2 and OVA-3, respectively) were purchased from Seikagaku Kogyo (Tokyo, Japan). Another ovalbumin (OVA-4) and KH_2PO_4 and K_2HPO_4 were obtained from Wako Pure Chemicals (Osaka, Japan). All other reagents were of reagent grade.

2.4. Preparation of aqueous two-phase solvent systems and sample solutions

The following three solvent systems were used: system I. 12.5% (w/w) PEG 1000–12.5% (w/w) K_2HPO_4 (pH 9.2); system II. 16% (w/w) PEG 1000–8.33% (w/w) K_2HPO_4, 4.17% (w/w) KH_2PO_4 (pH 7.0); and system III. 24% (w/w) PEG 1000–8% (w/w) KH_2PO_4, 2% (w/w) K_2HPO_4 (pH 5.8). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after the clear two layers were formed.

The sample solutions of commercial ovalbumins were prepared by dissolving each sample in 0.8 ml of the two-phase solvent mixture consisting of equal volumes of each phase.

The natural ovalbumin sample solution was prepared from a fresh chicken egg as follows: About 0.75 ml of the egg white was diluted with 6.25 ml of distilled water in a glass bottle, and 1.6 g of PEG 1000, 0.833 g of K_2HPO_4 and 0.417 g of KH_2PO_4 were added, while the solution was being gently mixed with a magnetic stirrer to avoid foaming. After all additives were completely dissolved and the solution showed a cloudy appearance, the mixing was stopped and the solution was left standing for a few minutes until two clear layers were formed. Then, 1.5 ml of each phase, a total of 3 ml, was mixed and used as the sample solution.

2.5. CCC separation of ovalbumin

In each experiment, the coiled column was completely filled with the PEG-rich upper stationary phase and the sample solution was charged into the column through the sample port. Then, the phosphate-rich lower mobile phase was pumped into the column at a flow rate of 0.2 ml/min, while the column was rotated at 800 rpm in the suitable direction. The effluent was collected into test tubes (0.4 ml per tube) using a fraction collector (Model SF-200, Advantec, Tokyo, Japan).

2.6. Analysis of CCC fractions

Each fraction was diluted with 2.5 ml of distilled water and the absorbance was measured at 280 nm

with a spectrophotometer (Model UV-160, Shimadzu, Kyoto, Japan).

2.7. Evaluation of partition efficiency

Partition efficiencies of each separation was computed from the chromatogram and expressed in terms of both theoretical plate number (N) and peak resolution (R_s). Both values are based on the assumption that each resolved peak represents the distribution of a single component.

2.8. Analysis of CCC fractions by electrophoresis

Ovalbumin in the CCC fractions was precipitated according to the following procedure: 100 μl of trifluoroacetic acid was added to each tube and the contents were mixed and cooled in ice for 1 h and centrifuged at 3500 rpm for 15 min. The precipitate was subjected to analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

SDS–PAGE was carried out with 10% polyacrylamide gel according to Leammli's method [4]. The developed gels were stained with Coomassie brilliant blue.

3. Results and discussion

3.1. Elution profile of commercial ovalbumins by X-axis CPC fractionation

Four different commercial ovalbumin samples (OVA-1, 2, 3 and 4) were subjected to CCC fractionation with PEG 1000–potassium phosphate systems each at three different pHs using the X-axis CPC. Fig. 1 illustrates the elution patterns in group A. At pH 9.2, OVA-1 from Sigma and OVA-3 (five times recrystallized from Seikagaku Kogyo)(group A) formed broad peaks at 22 and 29 TP (theoretical plates), respectively, whereas OVA-2 (two times recrystallized from Seikagaku Kogyo) and OVA-4 from Wako (group B) produced much sharper peaks (Fig. 2) at 80 and 256 TP, respectively.

Both ovalbumins were partially resolved into two peaks at pH 7.0 and 5.8. The peak resolution between the two peaks for OVA-1 is 0.3 at pH 7.0 and 0.5 at pH 5.8 while that for OVA-3 is 0.9 at pH 7.0 and 0.7 at pH 5.8.

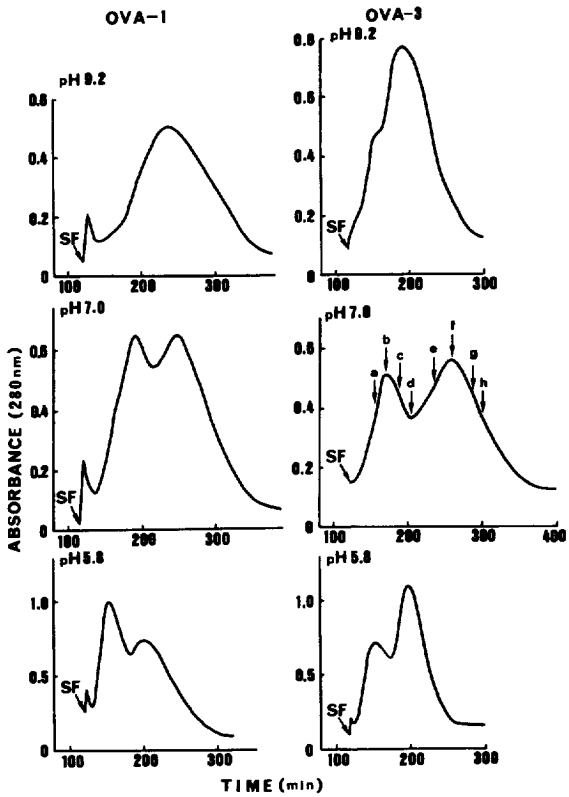

Fig. 1. Elution patterns of commercial ovalbumins in group A (OVA-1 and OVA-3) by X-axis CPC with aqueous two-phase solvent systems at three different pHs. Experimental conditions: solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) K_2HPO_4 (pH 9.2) (top); 16% (w/w) PEG 1000–8.33% (w/w) K_2HPO_4, 4.17% (w/w) KH_2PO_4 (pH 7.0) (middle) and 24% (w/w) PEG 1000–8% (w/w) KH_2PO_4, 2% (w/w) K_2HPO_4 (pH 5.8) (bottom); mobile phase: lower phase; flow-rate: 0.2 ml/min; sample size: 30 mg; revolution: 800 rpm. SF=Solvent front.

Fig. 2 similarly shows the elution patterns of two other ovalbumins (OVA-2 and OVA-4) in group B. Both ovalbumins formed relatively sharp single peaks at all pH values applied. The partition efficiencies computed from the OVA-4 peak falls from 256 TP to 93 TP when the pH drops from 9.2 to 5.8, while the peak retention time is almost unaltered. These findings are summarized in Table 1.

The above results clearly indicate that the group A ovalbumins consist of two or more components,

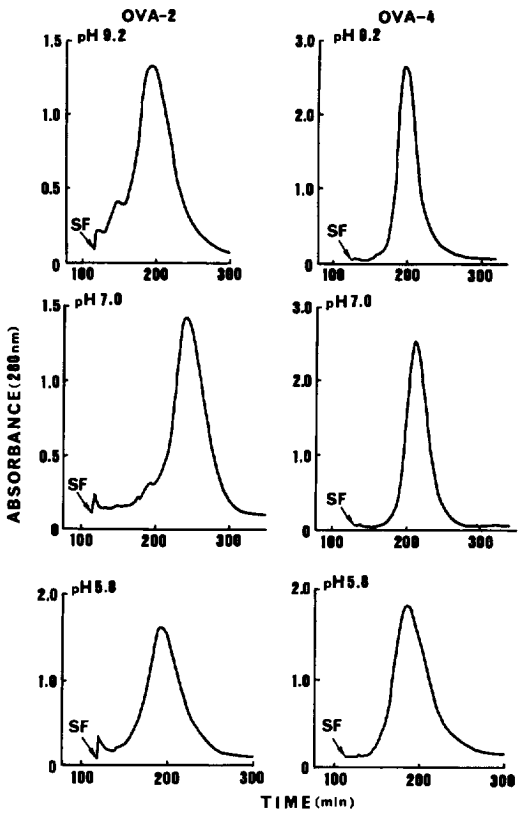

Fig. 2. Elution patterns of commercial ovalbumins in group B (OVA-2 and OVA-4) by X-axis CPC with aqueous two-phase solvent systems at three different pHs. Experimental conditions are same as those described in Fig. 1 caption. SF=Solvent front.

which in turn explains the broad peak when eluted at a basic pH in the previous studies. The above heterogeneity of the ovalbumin may also be attributed to the degree of glycosilation and phosphorylation of the protein molecule.

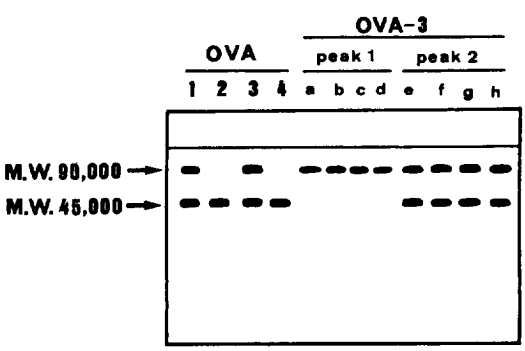

Fig. 3. Electrophoretogram of commercial ovalbumins and CCC fractions by SDS–PAGE. See for experimental conditions.

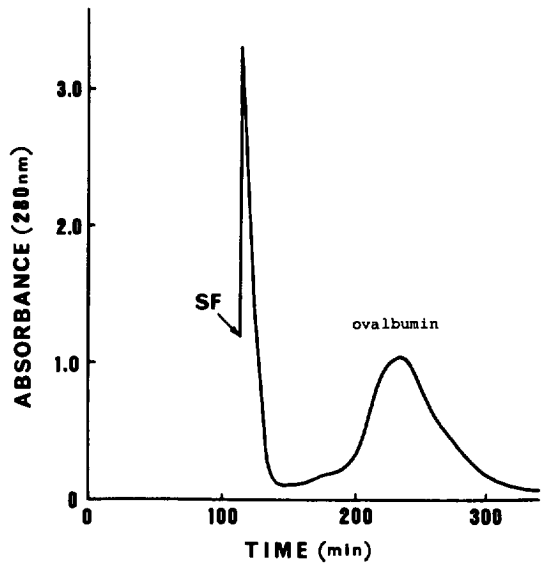

Fig. 4. CCC chromatogram of fresh egg white obtained by X-axis CPC. Experimental conditions: solvent system: 16% (w/w) PEG 1000–8.33% (w/w) K_2HPO_4, 4.17% (w/w) KH_2PO_4 (pH 7.0); mobile phase: lower phase; flow-rate: 0.2 ml/min; revolution: 800 rpm. SF=Solvent front.

3.2. Analyses of CCC fractions by electrophoresis

As described above, the group A ovalbumin formed two peaks below neutral pH suggesting that the sample consists of at least two components. Electrophoretic analyses have been performed on all four ovalbumin samples as well as CCC fractions corresponding to the first and second peaks of OVA-3 eluted at pH 7.0.

Fig. 3 shows the SDS–PAGE electrophoretogram of ovalbumins which are labeled from left to right, OVAs 1, 2, 3, 4, followed by a, b, c, d (the first peak fractions of OVA-3) and e, f, g, h (the second peak fractions of OVA-3) as indicated by arrows on the elution curve in Fig. 1, right. The results clearly indicate that OVA-2 and OVA-4, which produced the single peak in CCC chromatogram, each formed a single band at M_r 45 000 whereas OVA-1 and OVA-3, which produced two peaks in the CCC at lower pH, showed two bands, one at M_r 45 000 and the other at M_r 90 000. Since the molecular weight of

Table 1
Analytical values obtained from elution patterns of commercial and natural ovalbumins

	pH	Retention time (min)		Capacity factor k		Theoretical plate number (N)		Resolution factor (R_s)
Group A								
OVA-1	9.2		234		1.3		22	
	7.0	R_1	193	k_1	1.0	N_1	34	0.3
		R_2	250	k_2	1.7	N_2	26	
	5.8	R_1	151	k_1	0.3	N_1	86	0.5
		R_2	198	k_2	0.8	N_2	47	
OVA-3	9.2		194		1.0		29	
	7.0	R_1	172	k_1	0.7	N_1	137	0.9
		R_2	258	k_2	1.8	N_2	128	
	5.8	R_1	152	k_1	0.4	N_1	122	0.7
		R_2	196	k_2	0.9	N_2	128	
Group B								
OVA-2	9.2		190		0.9		80	
	7.0		242		1.9		130	
	5.8		191		0.9		104	
OVA-4	9.2		196		0.7		256	
	7.0		211		0.9		205	
	5.8		186		0.8		93	
Natural ovalbumin	7.0		230		1.0		104	

R_1, R_2: The value of retention time of peak 1 and peak 2, respectively.
k_1, k_2: The value of capacity factor of peak 1 and peak 2, respectively.
N_1, N_2: The theoretical plate number of peak 1 and peak 2, respectively.

natural ovalbumin is 45 000, the upper bands of OVA-1 and OVA-3 are considered to be the dimer of the natural molecule. The CCC fractions of the first peak (a–d) of OVA-3 consist exclusively of dimers while those of the second peak (e–h) contain a mixture of both dimer and monomer of the ovalbumin molecule.

3.3. CCC elution behavior of natural ovalbumin in fresh egg white

The CCC fractionation of the ovalbumin from fresh egg white was performed with the polymer phase system at pH 7.0 under the identical experimental condition applied to OVA-3 (see Fig. 1 caption).

As shown in Fig. 4, natural ovalbumin eluted at a retention time similar to that of the second peak of OVA-3 (Fig. 1, right). A sharp peak, eluted near the solvent front, contained impurities. There is only a small peak that corresponds to the retention time of the first peak of OVA-3. The SDS–PAGE analysis revealed that this natural ovalbumin yielded a single band at M_r 45 000.

4. Conclusion

The results of the present studies indicate that the molecular weight of natural ovalbumin is 45 000. Therefore, the dimer of the ovalbumin molecule, which was detected in the first peak in the CCC fractionation, is considered to be the denatured products of natural ovalbumin.

We conclude that an extremely broad peak observed in CCC fractionation of the commercial ovalbumin in the previous studies [3] may be caused by the denatured products from natural ovalbumin.

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