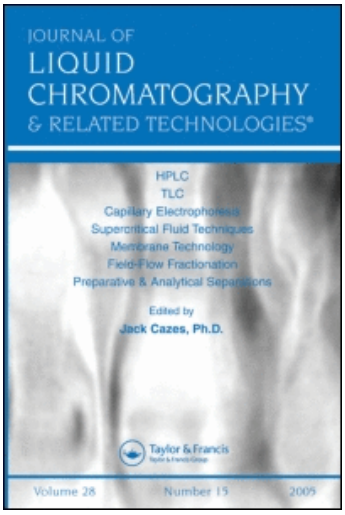

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

SEPARATION OF CHICKEN EGG WHITE PROTEINS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

Yoichi Shibusawa[a]; Satoshi Iino[a]; Heisaburo Shindo[a]; Yoichiro Ito[b]
[a] Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo, Japan [b] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institutes, National Institutes of Health, Bethesda, MD, U.S.A.

Online publication date: 31 August 2001

To cite this Article Shibusawa, Yoichi , Iino, Satoshi , Shindo, Heisaburo and Ito, Yoichiro(2001) 'SEPARATION OF CHICKEN EGG WHITE PROTEINS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 24: 13, 2007 — 2016
To link to this Article: DOI: 10.1081/JLC-100104442
URL: http://dx.doi.org/10.1081/JLC-100104442

J. LIQ. CHROM. & REL. TECHNOL., 24(13), 2007–2016 (2001)

SEPARATION OF CHICKEN EGG WHITE PROTEINS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

Yoichi Shibusawa,[1,]* Satoshi Iino,[1] Heisaburo Shindo,[1] and Yoichiro Ito[2]

[1] Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[2] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institutes, National Institutes of Health, Bethesda, MD 20892-1676, USA

ABSTRACT

Chicken egg white proteins were fractionated by high-speed counter-current chromatography (HSCCC) using a cross-axis coil planet centrifuge (X-axis CPC). The separation was performed with an aqueous polymer two-phase system composed of 16% polyethylene glycol (PEG) 1000 and 12.5% potassium phosphate buffer at different pH of 6.8, 8.0, and 9.2. From 20 g of the crude egg white solution, the best separation was achieved with a polymer phase system at pH 8.0 by eluting the lower phase at 1.0 mL/min. After the sequential elution of ovotransferrin, ovalbumin, and lysozyme from the column, ovomucin still retained in the column was eluted in the reverse direction with upper PEG-rich

*Corresponding author.

phase. All four proteins in the CCC fractions were identified by SDS gel electrophoresis with Coomassie brilliant blue staining.

INTRODUCTION

Aqueous polymer phase systems have been used for partitioning a variety of macromolecules and cell particles, which include proteins, nucleic acids, cell organelles, etc.[1] Using these solvent systems, several different approaches have been made for performing purification of biological samples, such as single step partitioning, repetitive batch extraction, countercurrent distribution (CCD), and countercurrent chromatography (CCC). Among these techniques, CCC is considered to be most the effective in terms of partition efficiency and separation times.

CCC is essentially a form of liquid-liquid partition chromatography. Its unique feature, among other chromatographic systems, is derived from the fact that the method uses no solid support and the stationary phase is retained in the column aided by either gravity or centrifugal force.[2-4] Although, the-multilayer coil planet centrifuge type provides the most efficient CCC separation, it fails to retain viscous, low interfacial tension polymer phase systems. In this connection, several types of cross-axis coil planet centrifuges (X-axis CPCs) have been designed for performing CCC using polymer phase systems.[5,6] The apparatus equipped with column holders mounted in the off-center position on the rotary shaft allows satisfactory retention of the stationary phase of aqueous-aqueous polymer phase systems. In the past, the X-axis CPC has been successfully used for the separation of a variety of protein samples with aqueous-aqueous polymer phase systems.[7-14]

Recently, we reported the separation of ovotransferrin, ovalbumin, and lysozyme from chicken egg white with an aqueous polymer phase system composed of 16% PEG 1000-12.5% potassium phosphate at pH 9.2 using a X-axis CPC.[15] The studies are continued, herein, to isolate and identify the fourth peak of ovomucin, a large molecular weight glycoprotein, at an optimized pH of the solvent system.

EXPERIMENTAL

Apparatus

The CCC fractionation of proteins from chicken egg white was performed using a type-XL cross axis coil planet centrifuge (X-axis CPC) shown in Fig. 1. The apparatus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 10 cm from the central axis of the cen-

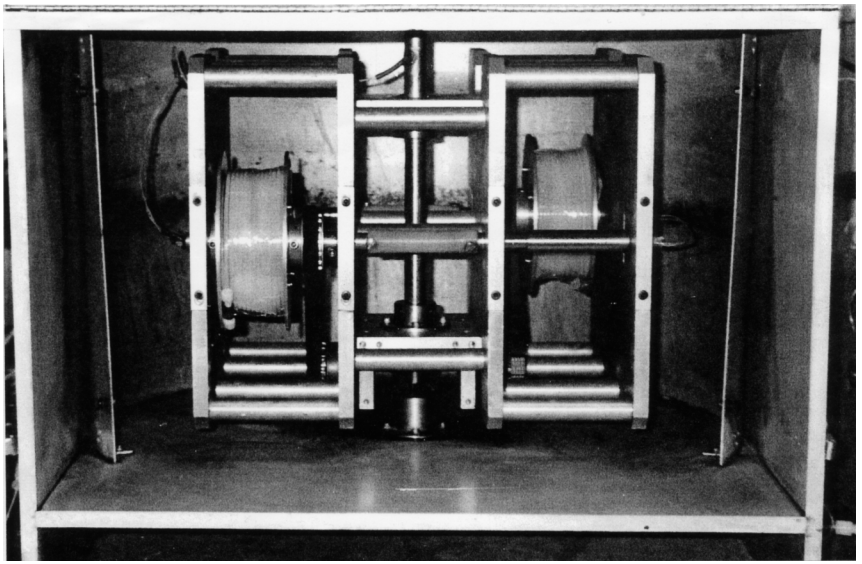

Figure 1. Photograph of type-XL cross-axis coil planet centrifuge.

trifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 10 cm away from the midpoint. A large multilayer coil was prepared from 2.6 mm I. D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 5 cm diameter holder hub, making three layers of left-handed coils between a pair of flanges spaced 5 cm. A pair of columns was serially connected on the rotary frame using a flow tube (PTFE, 0.85 mm I. D.) to give a total column capacity of 165 mL. The rpm was measured with a speed control unit (Bodine Electric, Chicago, IL, USA).

Reagents

Polyethylene glycol (PEG) 1000 (average molecular mass: 950-1050) and potassium phosphate were obtained from Kanto Chemicals (Tokyo, Japan). The protein standards including ovotransferrin, ovalbumin, ovomucin, and lysozyme, were all obtained from Sigma (St. Louis, MO, USA).

Preparation of CCC Sample Solution

The sample solution was prepared from fresh chicken egg white, according to the method described by Awade et al.[16] One volume of fresh chicken white was

mixed with two volumes of 0.05 M Tris-HCl (pH 9.0) containing 0.4 M NaCl and 10 mM β-mercaptoethanol, and the mixture was gently stirred overnight. The sample solution for CCC was then prepared by adding 4.5 g of PEG 1000 and 3.5 g of potassium phosphate to 20 g of the egg white solution, to coincide with the composition of the solvent system used for the separation.

Preparation of PEG-Potassium Phosphate Aqueous Two-Phase Systems

Two-phase solvent systems composed of 16% (w/w) PEG 1000 and 12.5% (w/w) potassium phosphate aqueous solutions were prepared by dissolving 320 g of PEG and 250 g of potassium phosphate (mixture of monobasic and dibasic potassium phosphates) in 1430 g of distilled water. The pH values of the solvent systems were adjusted to 6.8, 8.0, and 9.2 by changing the ratio between monobasic and dibasic potassium phosphates in the two-phase systems.

Measurement of Partition Coefficients of Standard Proteins

The partition coefficient of each protein sample was determined spectrophotometrically by a simple test tube procedure. About 1.0 mL of each phase of the equilibrated two-phase solvent system was delivered in a test tube to which about 1 mg of the standard protein was added. The contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, an aliquot (usually 0.5 mL) of each phase was pipetted and diluted with 1.0 mL of distilled water to determine the absorbance at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). The partition coefficient values ($K_D=C_U/C_L$) were obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Fractionation of Proteins from Egg White

In each experiment, the CCC column was first entirely filled with the PEG 1000-rich upper stationary phase and the CCC sample solution was injected into the column using an Eyela type SV-6010 sample injector (Tokyo Rikakikai, Tokyo, Japan). Then, the potassium phosphate-rich lower mobile phase was eluted through the column at 1.0 mL flow rate while the apparatus was rotated at 400 rpm. The effluent from the outlet of the column was continuously monitored with an Eyela UV-9000 absorbance monitor (Tokyo Rikakikai) at 280 nm and fractionated into a Bio-Rad Model 2110 fraction collector (Bio-Rad, Richmond,

CA, USA). After the desired peaks were collected, the column was eluted with the PEG 1000-rich upper phase (initially used as the stationary phase) in the opposite direction, without stopping the centrifuge run. This reversed elution mode was continued until all retained analytes were eluted from the column.

Analysis of CCC Fractions

An aliquot of each fraction was diluted in distilled water three time its volume, and the absorbance was measured at 280 nm with a Shimadzu UV-1200 spectrophotometer. Eluted proteins in the CCC fractions were also characterized by 7.5% or 12.0% SDS slab gel electrophoresis (SDS-PAGE), according to the method of LaemmLi.[17] Gels containing 3% (w/v) (stacking gel) and 7.5 or 12% (w/v) (separation gel) acrylamide were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N, N'-methylene-bis acrylamide. A 5.5 x 10 cm separation gel and a 1.0 x 10 cm stacking gel, each 0.75 mm thick, were prepared between glass plates. A 5 µL-volume of eluate was mixed with 95 µL of sample solution composed of a mixture of 0.025M tris (hydroxymethyl) aminomethane, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) 2-mercaptoethanol, 4% (w/v) glycerol, and 0.01% (w/v) bromophenol blue (BPB). The aliquot of 10-20 µL of the solution was loaded over the stacking gel.

Electrophoresis proceeded at a current of 10 mA until the BPB marker reached the stacking gel. Thereafter, the current of the apparatus was increased to 20 mA and the electrophoresis was done until the BPB marker reached the bottom of the separation gel. The migrated proteins were stained for 5 min at room temperature with the staining solution composed of 0.25% (w/v) Coomassie brilliant blue, 50% (v/v) methanol, and 10% (v/v) acetic acid. The gel was destained by washing in a solution, which is a mixture of 7.5% (v/v) acetic acid and 2.5% (v/v) methanol.

RESULTS AND DISCUSSION

Partition Coefficients of Standard Proteins

CCC is a two-phase procedure where the separation is based on the difference in partition coefficient of solutes within the two-phase systems. To achieve efficient separation of egg white proteins, it is essential to optimize the partition coefficient of each component by selecting a proper pH of the polymer phase system. Since the PEG-potassium phosphate polymer phase system partitions small molecules unilaterally in either upper or lower phase, purification of proteins can be efficiently performed by selecting their partition coefficient values close to unity. Below, a suitable solvent composition for the separation of egg white pro-

teins, including ovotransferrin, ovalbumin, lysozyme, and ovomucin, is determined by a set of partition data.

In Fig. 2, the partition coefficient values of four standard proteins containing egg white are plotted on a logarithmic scale against the pH of the PEG 1000-potassium phosphate buffer two phase system. The partition coefficient values of ovomucin and lysozyme increase with increasing pH from 6.8 to 8.0 and then slightly decrease at pH 9.2, whereas, the partition coefficient value of ovalbumin steadily increases with increased pH values of the polymer phase system. On the other hand, the partition coefficient value of ovotransferrin decreases as pH value of the solvent systems is increased.

Peak Resolution Between Ovotransferrin and Ovalbumin

In order to evaluate the separation of ovotransferrin and ovalbumin, peak resolution was compared at pH 6.8, 8.0, and 9.2. Partition efficiency was computed from the chromatograms and expressed in terms of peak resolution, R_s, according to a modified conventional fomula

$$R_s = 1.18\ (R_2 - R_1)/(W_{0.5h1} + W_{0.5h2}) \quad (1)$$

where R_1 and R_2 are retention time or volume of two adjacent peaks and $W_{0.5h1}$ and $W_{0.5h2}$, the peak width at the half height of the peaks expressed in the same unit as R_1 and R_2.

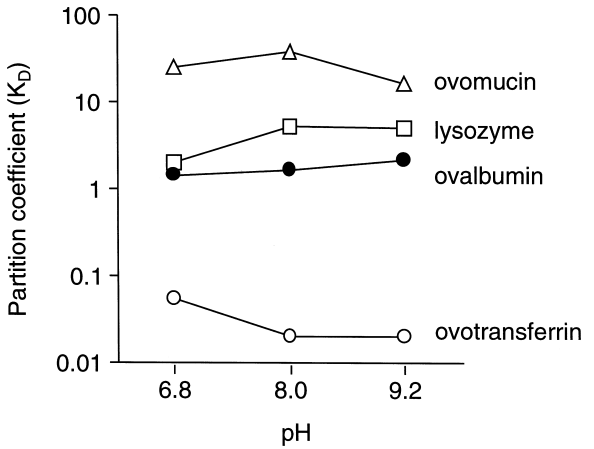

Figure 2. Partition coefficients (K_D) of ovotransferrin (○), ovalbumin (●), lysozyme (□) and ovomucin (△).

In Fig. 3, the peak resolutions between ovotransferrin and ovalbumin are plotted against the pH value of the aqueous-aqueous two solvent systems. The increase of the pH values of the two-phase solvent systems results in higher R_s values between ovotransferrin and ovalbumin. This improvement of the peak resolution may be attributed to a large difference in their partition coefficients value between ovotransferrin and ovalbumin.

CCC of Egg White Proteins at Different pHs of the Solvent System

The CCC separation of egg white proteins were tested using a polymer phase system composed of 16% PEG 1000-12.5% potassium phosphate buffer at different pH values. The separation was performed on 28 g of sample solution under the standard conditions.[5,6] In the solvent system at pH 6.8, the partition coefficient value of ovalbumin was too close to that of lysozyme. Consequently, the lysozyme formed a shoulder on the ovalbumin peak (data not shown). Successful separation of the four proteins was achieved with the solvent systems composed of 16% PEG 1000-12.5% potassium phosphate at higher pH values of 8.0 and pH 9.2, as shown in Fig. 4 A and B. In each chromatogram, UP indicates the point where the direction of elution was reversed with the upper phase, while Ot, Oa, Ly, and Om are abbreviations of ovotransferrin, ovalbumin, lysozyme, and ovomucin, respectively. In the pH 8.0 solvent system, the ovotransferrin, ovoalbumin, and lysozyme were eluted in the order of their partition coefficient

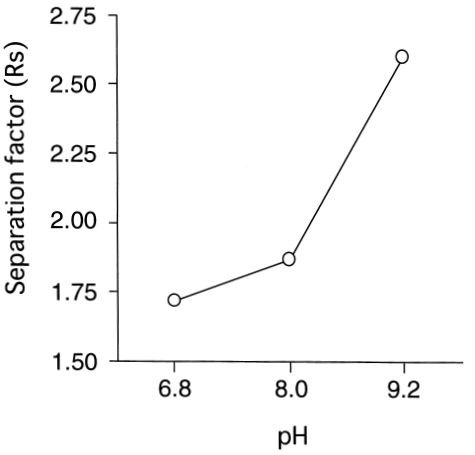

Figure 3. Change of separation factor between ovotransferrin and ovalbumin as a function of pH values of the aqueous-aqueous polymer two phase systems.

values with the lower mobile phase (Fig. 4A). After the elution of the three components, the PEG 1000-rich upper phase was pumped into the column in the reverse direction to facilitate rapid elution of ovomucin still remaining in the column. The upper stationary phase retained in the column was estimated as 56.4% of the total column capacity (165 mL) prior to the application of the reversed elution with the upper phase. The separation was completed within 6 h.

Fig. 4B shows the chromatogram obtained with the solvent system at pH 9.2. Since the difference in the partition coefficient values between lysozyme and ovalbumin is smaller at this pH, the reversed elution was started after the first two peaks eluted, and the lysozyme peak was eluted with the upper phase. The ovomucin became a shoulder on the lysozyme peak. However, the separation between ovotransferrin and ovalbumin was inproved using this solvent system.

Fig. 5 shows the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of CCC fractions corresponding from each peak obtained at pH 8.0 (Fig. 4A). Fractions 25, 45, and 65, collected from the center of the first, second, and third peaks in the chromatogram, contained ovotransfer-

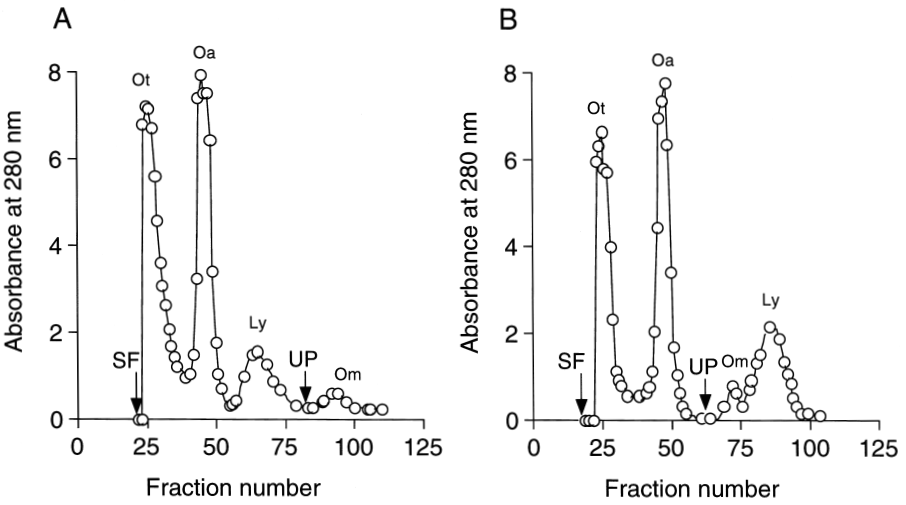

Figure 4. Purification of chicken egg white proteins using PEG 1000-potassium phosphate systems at pH 8.0 (A) and at pH 9.2 (B). Experimental conditions: apparatus: the XL cross-axis coil planet centrifuge with a pair of multilayer coil columns of 2.6 mm ID and a 165 mL total capacity; solvent systems: 16% (w/w) PEG 1000-12.5% (w/w) potassium phosphate at pH 8.0 and pH 9.2; stationary phase: PEG-rich upper phase; mobile phase:phosphate-rich lower phase; sample solution:28 g of chicken egg white solution as described in the text (Experimentals); flow-rate: 1.0 mL/min; revolution: 400 rpm; SF=solvent front; UP=upper phase eluted in the reversed direction.

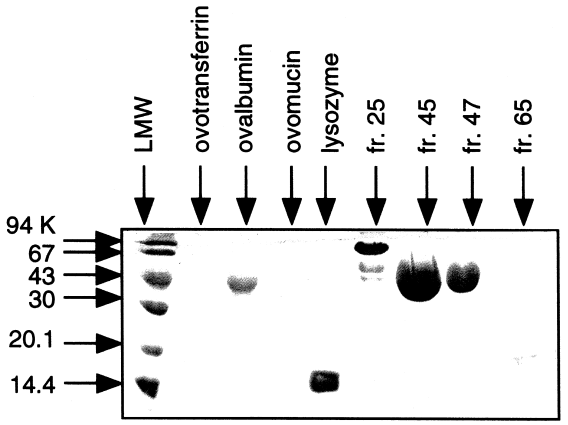

Figure 5. 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of the CCC fractions.

rin, ovalbumin, and lysozyme, which migrated into the respective positions of the standard proteins. The peak eluted by upper phase must be ovomucin containing a small amount (1.5%) of the egg white proteins. Ovomucin was detected at 7.5% of SDS-PAGE due to its large molecular mass 220,000 (data not shown).

Since CCC performs separations without a solid support matrix, adsorptive loss and denaturation of proteins are minimized compared with the conventional column chromatographic methods. The results of our studies indicate, that the CCC is capable of purifying proteins from a crude sample solution prepared from fresh egg white, in a one-step operation in a relatively short elution time.

ACKNOWLEDGMENT

The authors are indebted to Dr. Henry M. Fales for editing the manuscript with valuable suggestions.

REFERENCES

1. Albertsson, P.A. *Partition of Cell Particles and Macromolecule*; Wiley-Interscience: New York, 1986.
2. Ito, Y.; Bowman, R.L. Science (Washington, DC) **1970**, *167*, 281.
3. Conway, W.D. *Countercurrent Chromatography: Apparatus, Theory and Applications*; VCH: New York, 1990.

4. Ito, Y. In *Principle and Instrumentation of Countercurrent Chromatography: Theory and Practice*; Mandava, N.B., Ito, Y., Eds; Marcel Dekker: New York, 1988; Ch. 3, 79.
5. Ito, Y.; Kitazume, E.; Bhatnagar, M. J. Chromatogr. **1991**, *538*, 59.
6. Ito, Y.; Kitazume, E.; Slemp, J.L. J. Chromatogr. **1991**, *538*, 81.
7. Shibusawa, Y.; Ito, Y. J. Chromatogr. **1991**, *550*, 695.
8. Shibusawa, Y.; Ito, Y. J. Liq. Chromatogr. **1992**, *15*, 2787.
9. Lee, Y.W.; Shibusawa, Y.; Chen, F.T.; Myers, J.; Schooler, J.M.; Ito, Y. J. Liq. Chromatogr. **1992**, *15*, 2831.
10. Shibusawa, Y.; Ito, Y.; Ikewaki, K.; Rader, D.J.; Bryan Brewer J. J. Chromatogr. **1992**, *596*, 118.
11. Shibusawa, Y.; Chiba, T.; Matsumoto, U.; Ito, Y. In *Modern Countercurrent Chromatography*; ACS Monographs; American Chemical Society: Washington, DC, 1995; Ch. 13, 385.
12. Shibusawa, Y.; Mugiyama, M.; Matsumoto, U.; Ito, Y. J. Chromatogr. B **1995**, *664*, 295.
13. Shibusawa, Y.; Eriguchi, Y.; Ito, Y. J. Chromatogr. B **1997**, *696*, 25.
14. Shibusawa, Y.; Ito, Y. Am. Biotechnol. Lab. **1997**, *15*, 8.
15. Shibusawa, Y.; Kihira, S.; Ito, Y. J. Chromatogr. B **1998**, *709*, 301.
16. Awade, A.C.; Moreau, S.; Molle, D.; Brule, G.; Maubois, J.-L. J. Chromatogr. A **1994**, *677*, 279.
17. LaemmLi, U.K. Nature **1970**, *227*, 680.

Received December 12, 2000 Manuscript 5468
Accepted December 29, 2000