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Comparison of three liquid chromatographic methods for egg-white protein analysis

Abalo C. Awadé[a], Théo Efstathiou[b,*]

[a]*Institut National de la Recherche Agronomique, Laboratoire de Recherches de Technologie Laitière, 65 Rue de Saint-Brieuc, F-35042 Rennes Cedex, France*

[b]*Nutrinov, 85 rue de Saint-Brieuc, F-35000 Rennes, France*

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Abstract

This paper describes and compares three chromatographic methods for the analysis of egg-white proteins. Gel-permeation chromatography allowed the separation of seven peaks from egg white, with an almost total protein recovery. A clean separation of ovomucin and lysozyme from the bulk of the proteins was obtained with this method. Reversed-phase high-performance liquid chromatography led to the fractionation of at least eight peaks. With this chromatographic method, the recovery was relatively poor. Approximately 30% of the ovalbumin was retained in the column after the elution. Finally, eleven chromatographic peaks were separated from egg white by high-performance liquid chromatography on an anion-exchange column. The recovery of proteins was almost total. The latter method afforded higher resolution.

Keywords: Egg white; Proteins

1. Introduction

Egg white (EW) is a biological medium containing proteins of nutritional, biological and technological interest [1]. Table 1 shows the known EW proteins, as well as some of their physico-chemical and biological properties.

The design of analytical methods for the determination of the composition of biological media, particularly foods, is important from a biological and nutritional standpoint. To our knowledge, despite the increasing number of investigations concerning EW proteins, no method allowing a complete determination of these components has been reported.

This paper describes three new chromatographic methods for rapid qualitative EW analysis, using gel-permeation chromatography, reversed-phase high-performance liquid chromatography (RP-HPLC) and anion-exchange HPLC. The advantages and deficiencies of these techniques are discussed, and they are also compared with those published by other authors.

2. Experimental

2.1. Materials

Protein molecular mass markers were purchased from Pharmacia Biotech (Orsay, France). Reference

*Corresponding author. Fax: +33-2-99-28-53-50.

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Table 1
EW known proteins

Protein	Amount of total protein (%)	M_r	p*I*	Source
Ovalbumin	54	45 000	4.5	[2]
Ovotransferrin (conalbumin)	12–13	77 700	6.0	[2]
Ovomucoid	11	28 000	4.1	[2]
Lysozyme	3.4–3.5	14 300	10.7	[2,3]
Ovomucin	1.5–3.5	$0.22–270\times10^6$	4.5–5.0	[2,4]
G2 ovoglobulin	1.0	47 000	4.9–5.3	[5]
G3 ovoglobulin	1.0	50 000	4.8	[5]
Ovoflavoprotein	0.8	32 000	4.0	[2]
Ovostatin (ovomacroglobulin)	0.5	$7.6–9.0\times10^5$	4.5–4.7	[2]
Cystatin	0.05	12 000	5.1	[2,6]
Avidin	0.05	68 300	10.0	[2]
Thiamin-binding protein	N.D.	38 000	N.D.	[5]
Glutamyl aminopeptidase	N.D.	320 000	4.2	[7]
Minor glycoprotein 1	N.D.	52 000	5.7	[8]
Minor glycoprotein 2	N.D.	52 000	5.7	[8]

N.D.: not determined.

lysozyme, ovotransferrin and ovalbumin were prepared from commercial hen egg white in our laboratory as described [9]. Chicken egg ovomucoid (trypsin inhibitor, type III-0), avidin and ovoglobulins were supplied by Sigma (St. Quentin Fallavier, France). All other reagents were of analytical grade.

2.2. Egg-white preparation

Our whole study was performed using commercial eggs. Initial EW pH was about 9. Total EW protein concentration was close to 100 mg/ml.

For gel-permeation chromatography, EW was diluted 3-fold with 0.05 *M* Tris–HCl buffer (pH 9.0) containing 0.4 *M* NaCl and 10 m*M* β-mercaptoethanol. β-mercaptoethanol was used in order to reduce and separate ovomucin subunits. For RP-HPLC, EW was diluted 11-fold with 0.8% NaCl. For anion-exchange chromatography, egg white was diluted 10-fold with 0.02 *M* Tris–HCl buffer (pH 9.0) containing 10 m*M* β-mercaptoethanol. All samples were gently stirred overnight at 4°C. In all cases, samples were filtered through cellulose-acetate 0.45 μm filters prior to analysis.

Since ovomucin is insoluble at low ionic strength [10], it precipitated during preparation of egg-white samples for anion-exchange chromatography and was eliminated by filtration. Ovomucin remained in solution during sample preparation for gel-permeation chromatography and RP-HPLC, due to the presence of NaCl in the dilution media. However, in the latter case, since no reductant (β-mercaptoethanol) was used, ovomucin remained polymerised and was also retained on the 0.45 μm filters.

2.3. Chromatography

Superose 12 HR 10/30 (30×1 cm I.D.) and Mono Q HR 5/5 (5×0.5 cm I.D.) columns were purchased from Pharmacia Biotech (Orsay, France). The reversed-phase column was a guard column Supelcosil LC-304 (20×4.6 mm I.D., 5 μm particles, 300 Å pores) purchased from Supelco (St. Quentin Fallavier, France).

Two chromatographs were used. RP-HPLC and anion-exchange chromatography were performed with an HPLC system (SpectraSystem P4000, UV detector Spectra 1000). Gel-permeation chromatography was performed using a FPLC system from Pharmacia which consisted of two P 500 pumps, a LCC 500 controller and a UV 1 detector.

The gel-permeation column Superose 12 HR 10/30 was equilibrated with 0.05 *M* Tris–HCl buffer (pH 9.0) containing 0.1 *M* NaCl, using the FPLC system at room temperature. A 50 μl sample of the EW preparation was applied to the column. Proteins were eluted with the same buffer at a flow-rate of 1.0 ml/min and detected at 280 nm.

For RP-HPLC, the C4 Supelcosil LC-304 column was equilibrated with 10% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA), using the HPLC apparatus. A 50 μl volume of the diluted EW was loaded onto the column and elution was carried out by increasing acetonitrile concentration as stated in Table 2. Proteins were detected at 214 nm.

The anion-exchange Mono Q HR 5/5 column was equilibrated with 0.02 *M* Tris–HCl (pH 9.0) using the HPLC system. A 100 μl volume of EW, diluted as described above, was injected into the column. Elution was conducted by increasing NaCl concentration in the same buffer as shown in Table 3. Proteins were detected at 280 nm.

2.4. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed as proposed by Laemmli [11], using a 15% separation gel and a 4% stacking gel containing 0.1% SDS. Samples were heated for 4 min at 95°C in SDS reducing sample buffer (62.5 m*M* Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol). Protein bands were stained with Coomassie Brilliant Blue R 250.

Table 2
Parameters for the elution of EW proteins from the reversed-phase C4 Supelcosil LC-304 column

Time (min)	A (%)[a]	B (%)[b]
0	80	20
20	50	50
22	50	50
23	20	80
35	20	80

[b] B, 90% acetonitrile in water containing 0.05% TFA. Flow-rate: 2.0 ml/min
[a] A, 10% acetonitrile in water containing 0.05% TFA.

Table 3
Parameters for the elution of EW proteins from the anion-exchange Mono Q HR 5/5 column

Time (min)	A (%)[a]	B (%)[b]
0	100	0
5	90	10
6	75	25
14	75	25
14.5	67.5	32.5
17.5	67.5	32.5
18	60	40
24	60	40
25	0	100
30	0	100

[a] A, 0.02 *M* Tris–HCl (pH 9.0).
[b] B, A containing 0.5 *M* NaCl. Flow-rate: 1.0 ml/min.

2.5. Protein determination

Protein concentrations were determined according to the Kjeldahl method [12], using 6.25 as the conversion factor.

3. Results and discussion

3.1. Gel-permeation chromatography

Fig. 1 shows a chromatographic profile at 280 nm of EW, on a Superose 12 HR 10/30 column; seven peaks were detected. The complete elution was achieved within 30 min. In order to identify the peaks, aliquots of reference-protein solutions were added to EW samples before loading onto the column (data not shown). Ovalbumin, ovotransferrin, ovomucoid and ovoglobulins were eluted in peak 2 and lysozyme in peak 5. According to its molecular mass (see Table 1) we assumed that ovomucin was eluted in peak 1.

It is noteworthy that preliminary experiments showed that using buffers containing less than 0.1 *M* NaCl led to retention of lysozyme on the column. When a buffer without NaCl was used, almost all the lysozyme contained in the sample was retained on the column, while other proteins were eluted. The adsorbed lysozyme could be completely eluted with the buffer containing 0.1 *M* NaCl. The high pHi value of lysozyme (10.7) and the operating pH (9.0)

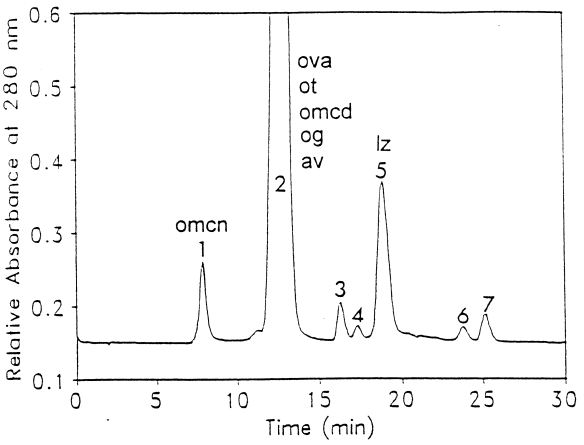

Fig. 1. Gel-permeation chromatography of hen EW proteins on the Superose 12 HR 10/30 column (30×1 cm I.D.). A 50 μl volume of the EW preparation was applied to the column, previously equilibrated with 0.05 *M* Tris–HCl buffer, pH 9.0, containing 0.1 *M* NaCl, using Pharmacia FPLC system at room temperature. Proteins were eluted with the same buffer at a flow-rate of 1.0 ml/min. Abbreviations: omcn, ovomucin; ova, ovalbumin; ot, ovotransferrin; omcd, ovomucoid; og, ovoglobulins; av, avidin; lz, lysozyme.

may explain that at low ionic strength, this protein exhibited ionic interactions with the stationary phase.

SDS–PAGE analysis of protein fractions showed that the main polypeptides present in peak 1 corresponded to ovomucin subunits (data not shown). This analysis also revealed that the bulk of EW proteins were eluted in peak 2 which contained polypeptides with molecular masses between 30 000 and 80 000 Da. Thus, this peak contained ovalbumin, ovotransferrin, avidin, ovomucoid, ovoglobulins and other minor proteins. The band obtained from peak 5 corresponded to lysozyme. The content of peaks 3, 4, 6 and 7 remained undetermined.

Gel-permeation HPLC has been previously proposed as a method for the quantification of lysozyme in EW [13]. In the gel-permeation method we present in this article, peaks were more clearly separated than by the former method. Our method allowed a good separation of ovomucin and lysozyme from the other EW proteins. However, it is not recommended for ovalbumin, ovotransferrin, ovomucoid, avidin and ovoglobulin separation since these proteins were all eluted in the same peak. Thus, other chromatographic techniques are needed for their separation.

3.2. Reversed-phase HPLC

As shown by Itoh et al. [14] and Takeuchi et al. [15], a good recovery of hydrophobic proteins (e.g. ovalbumin) on reversed-phase columns is obtained with less hydrophobic short alkyl-chain and small pore-size stationary phases. Thus, we chose a short C4 guard column (length 20 mm) in order to minimize ovalbumin adsorption.

Fig. 2 shows a typical elution profile at 214 nm of EW proteins on the RP column Supelcosil LC-304. The elution was performed at room temperature within 35 min. At least eight major peaks (2, 3, 6, 7, 9, 11, 12 and 13) and 5 minor ones (1, 4, 5, 8, 10) were detected. Peaks were identified by the addition of reference proteins to the sample prior to chromatography. Peak 2 contained ovomucoid, ovoglobulins

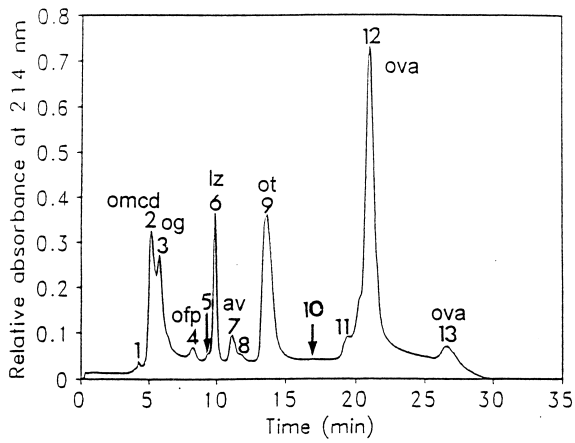

Fig. 2. Reversed-phase high-performance liquid chromatography of EW proteins. RP-HPLC was performed using a C4 Supelcosil LC-304 column (20×4.6 mm I.D., 5 μm particles, 300 Å pores), equilibrated with 10% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA). A 50 μl volume of the diluted EW (see Experimental) was loaded onto the column and elution was carried out as stated in Table 2. Abbreviations: omcd, ovomucoid; og, ovoglobulins; ofp, ovoflavoprotein; lz, lysozyme; av, avidin; ot, ovotransferrin; ova, ovalbumin.

were eluted in peak 3, lysozyme in peak 6, avidin in peak 7, ovotransferrin in peak 9 and ovalbumin in peaks 12 and 13. SDS–PAGE analysis of protein fractions confirmed these identifications (data not shown). Moreover, this analysis showed that peak 4 contained a polypeptide band that may be ovoflavoprotein. Peaks 1, 5, 8, 10 and 11 remained unidentified.

It is noteworthy that the recovery of ovalbumin was not complete; about 30% was retained on the column after the elution gradient. It was necessary to perform the same gradient repeatedly without prior sample loading in order to elute all the adsorbed ovalbumin (data not shown). Such a problem of ovalbumin recovery has been observed by Itoh et al. [14], who used a nonporous C18 spherical silica gel with a particle diameter of 20 μm. However, it seems that Takeuchi et al. [15], by using a 5 μm C4 300 Å silica column, did not observe the same phenomenon.

It should also be noticed that the use of water containing 0.1 rather than 0.05% TFA also decreased ovalbumin recovery. In this concentration range, TFA probably increases protein hydrophobicity and thus favours its binding to the stationary phase. We also observed that the amount of retained ovalbumin increased with temperature during the elution. Raising temperature probably increases the hydrophobic interactions of ovalbumin with the column in such a way that more drastic conditions are needed for its elution. In a similar way, O'Hare et al. [16] studied the separation of proteins using RP-HPLC on short alkyl chain-bonded silica gel and they showed that recoveries could be improved by lowering the temperature during the elution.

As expected, RP-HPLC led to a better separation of EW proteins than gel-permeation chromatography. It allowed the separation of ovalbumin, ovotransferrin, ovomucoid, avidin, ovoglobulins and lysozyme. By comparison with RP-HPLC methods previously reported, the method we present here offered a more complete separation. Although the method proposed by Takeuchi et al. [15] is very similar to ours, the peak corresponding to lysozyme is lacking in their elution profile. However, ovomucin was eliminated by filtration during sample preparation for RP-HPLC, and therefore could not be analysed by our method, whereas it could be separated by gel-permeation chromatography as shown above.

3.3. Anion-exchange HPLC

A typical elution chromatogram of EW proteins by anion-exchange HPLC is shown in Fig. 3. At least 11 peaks were separated. The addition of reference proteins to the samples before loading onto the column indicated that lysozyme was eluted in peaks 1 and 2, avidin in peak 1, ovotransferrin in peak 4, ovoglobulins in peaks 5 and 6, ovalbumin in peaks 7, 8, 9 and ovomucoid in peak 10. This was confirmed by SDS–PAGE analysis of the recovered fractions (data not shown). This analysis also revealed that peak 11 contained a polypeptide that corresponded to ovoflavoprotein. It is noteworthy that peak 3 contained proteins with an approximate mass of 50 000 Da. These proteins may correspond to minor glycoproteins 1 and 2 (Table 1).

Anion-exchange HPLC appeared to be more resolutive than gel-permeation chromatography and RP-HPLC. In addition, as shown by subsequent similar gradient elution without prior sample loading, pro-

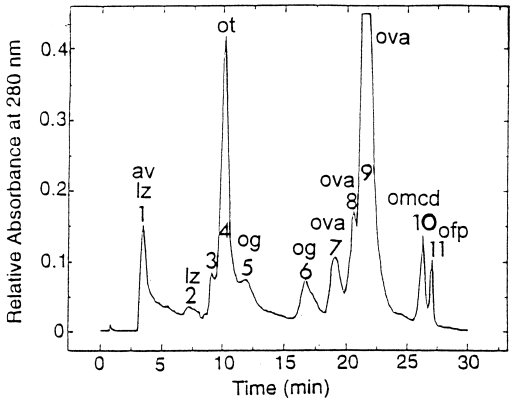

Fig. 3. Anion-exchange chromatography of EW proteins. A 100 μl volume of EW, diluted as described in Experimental, was chromatographed using an HPLC system equipped with a Mono Q HR 5/5 column (50×5 mm I.D.). The column was previously equilibrated with 0.02 *M* Tris–HCl, pH 9.0. Elution was conducted as shown in Table 3. Abbreviations: av, avidin; lz, lysozyme; ot, ovotransferrin; og, ovoglobulins; ova, ovalbumin; omcd, ovomucoid; ofp, ovoflavoprotein.

tein recovery was higher than with RP-HPLC. For example, ovalbumin was almost totally recovered from the anion-exchange column, whereas about 30% was retained on the RP column.

It should also be noticed that the EW proteins elution sequence from the anion-exchange column closely reflected the order of their isoelectric pH values (see Table 1), with the exception of lysozyme, which was eluted in several peaks from the anion exchange column. Due to its pHi value (10.7), lysozyme carries at pH 9.0 an electrical charge opposite to that of most of other EW proteins. Therefore, electrostatic interactions probably occur between lysozyme and other proteins in these conditions, and different populations of lysozyme molecules thus may be eluted together with other proteins and be present in several peaks [9].

Cation-exchange chromatography on CM-cellulose has been used by Rhodes et al. [17] to analyse EW proteins. With their method, elution was accomplished by a stepwise change of the eluting buffer pH from 4 to 10.5, or in certain cases by varying the ionic strength. This method led to the separation of 14 peaks with a recovery higher than 90%. For the moment, to our knowledge, this method allowed the more complete analysis of EW by liquid chromatography. However, due to the procedure being rather cumbersome and time-consuming, it cannot be easily applicable routinely. In view of the pHi of EW proteins (Table 1), anion-exchange chromatography appeared to be better adapted than cation-exchange chromatography for their separation. By our method only two buffers were used and the time needed was smaller than for the method of Rhodes et al. [17]. As regards the time needed for the analysis, the three methods that we present here are almost equivalent.

4. Conclusion

This paper describes the application of three different methods, gel permeation-FPLC, RP-HPLC and anion-exchange HPLC, to rapid EW protein analysis. Anion-exchange chromatography offered the more complete analysis, although gel permeation should be recommended for ovomucin separation, and RP-HPLC would be preferable for an accurate estimation of lysozyme and ovotransferrin since with anion-exchange chromatography these proteins are slightly contaminated with other minor proteins.

In conclusion, we developed three methods that appear to be complementary for rapid EW protein analysis. Each method can be used depending on the protein(s) of interest.

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