ELSEVIER

Journal of Chromatography B, 664 (1995) 201–210

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Isolation of hen egg white lysozyme, ovotransferrin and ovalbumin, using a quaternary ammonium bound to a highly crosslinked agarose matrix

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Abstract

A single-step anion-exchange chromatographic separation of egg white proteins was carried out using a Q Sepharose Fast Flow column. The separation resulted in the isolation of two lysozyme peaks with purities of ca. 99 and 88%, one peak of ovotransferrin purified to ca. 75% and two ovalbumin peaks with purities of ca. 54 and 98%. Recoveries were estimated to be ca. 60, 100 and 83% for lysozyme, ovotransferrin and ovalbumin, respectively. The amino acid compositions of all collected peaks have also been determined. This confirmed the identity of some of the proteins contained in these peaks.

1. Introduction

Egg white is a natural source of proteins of proved and potential nutritional, biological and technological interest. Three major proteins, i.e. lysozyme, ovotransferrin and ovalbumin are of particular significance. Because of its antibacterial activity [1], lysozyme (ca. 3.5% of the total egg white protein) is widely used for food preservation and in pharmaceutical industry [2]. Ovotransferrin (ca. 13%) is an iron-transport glycoprotein which exhibits antimicrobial activity [3]. Ovalbumin (ca. 54% of the total egg white protein) is a glycoprotein that plays a predominant role in egg white gelling [4,5]. Moreover, this protein is assumed to be useful in nutrition.

Several egg white proteins have already been purified on a laboratory scale by procedures using precipitation by salts or solvents, ionic strength reduction or liquid chromatography [6–12]. Disadvantages related to the first two techniques, i.e. protein denaturation and relatively low purity [13,14], explain the success of liquid chromatography, and particularly of ion-exchange chromatography [11,14–23], for the purification of these proteins.

In ion-exchange chromatography, retention depends on the charge of the molecules which can be modified by accurately increasing the ionic strength. Separation is based on the reversible binding of a charged molecule to an oppositely charged ion which is immobilized on a matrix. The fact that molecules of the eluate interact with this ion according to their charge and their charge density allows a very efficient separation, even for very closely related molecules. Moreover, large eluate volumes can be

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0378-4347/95/$09.50
SSDI 0378-4347(94)00411-0

treated, and usually no denaturation of co-products is observed.

In this work, we used a quaternary ammonium ion bound to a highly crosslinked agarose matrix (Sepharose) to separate egg white proteins. The Sepharose matrix is rigid and highly substituted with strong ion-exchange groups and thus has a high capacity. Generally, Sepharose gives a higher degree of purification than soft gels, although it is more expensive. Moreover, the gel used has a good capacity for molecules with a molecular mass (M_r), up to 10^6, it shows excellent flow properties and has stable bed volumes insensitive to changes in ionic strength. We used this matrix for a single-step purification of hen egg white lysozyme, ovatransferrin, and ovalbumin. The activity of the purified lysozyme was determined and the presence of avidin in the lysozyme peaks was checked by Western blotting using rabbit anti-avidin serum. The amino acid compositions of the fractions obtained by this purification has been determined and the compositions of lysozyme, ovotransferrin and ovalbumin peaks were compared with those previously published.

2. Experimental

2.1. Materials and reagents

Dialysis was performed with Spectra/Por MWCO 3500 or 6000–8000 membranes (Spectrum Medical Industries, Houston, TX, USA). Q Sepharose Fast Flow and protein molecular mass markers were supplied by Pharmacia Biotechnology (St. Quentin-en-Yvelines, France). *Micrococcus lysodeikticus* cells, avidin, rabbit anti-avidin serum came from Sigma (L'Isle d'Abeau Chenes, France). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Interchim (Montluçon, France).

2.2. Egg white preparation

Egg white was diluted with 2 volumes of 0.05 *M* Tris-HCl buffer (pH 9) containing 10 m*M* β-mercaptoethanol and gently stirred overnight at 4°C. Prior to the anion-exchange chromatography, the precipitate was removed by centrifugation at 27 000 *g* for 15 min.

2.3. Analytical methods and lysozyme activity assay

Protein determination

Protein concentrations were determined according to the method of Bradford [24] using the Bio-Rad dye reagent (Bio-Rad, Ivry-sur-Seine, France) with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) was performed as described by Laemmli [25] using a 15% resolving gel and a 4% stacking gel containing 0.1% SDS. Protein bands were stained with the silver staining kit from Bio-Rad. Immunoblots were basically performed, according to the method of Towbin et al. [26] using a Hybond C nitrocellulose membrane from Amersham (Les Ulis, France). Serological reactions were detected by the coloric method described by Blake et al. [27] using the nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate kit from Sigma.

Analytical chromatography

Proteins obtained by egg white fractionation on the anion-exchange column were re-chromatographed on a size exclusion TSK G 3000 SW column (30 × 0.75 cm I.D., Supelco, St. Germain-en-Laye, France), using a Spectra-Physics (Fremont, CA, USA) HPLC system. Sodium phosphate buffer (0.05 *M*, pH 2.4) containing 0.2 *M* NaCl was used as eluent at a flow-rate of 0.4 ml/min. Fractions assumed to contain ovomucin were chromatographed with the same buffer but at pH 7.0.

Lysozyme activity

Lysozyme activity on *Micrococcus lysodeikticus* cells was determined with a turbidimetric method based on that proposed by Weaver et al. [28].

2.4. Amino acid composition

The amino acid composition was determined as previously described by Spackman et al. [29] using a Pharmacia-LKB (Alpha Plus) analyser. Protein samples (0.5–1 mg) were dried and hydrolysed under vacuum in 6 *M* HCl for 24 h at 110°C. To quantify the cysteine and methionine residues, samples were first oxidized by performic acid and then hydrolysed at 110°C in 6 *M* HCl, according to Moore [30].

3. Results and discussion

Preliminary experiments were carried out to determine the capacity of the Q Sepharose Fast Flow resin for egg white proteins. For this purpose, egg white preparations (see Experimental) containing 0.5–30 g of protein were applied at a flow-rate of 7.5 ml/min onto the column (10 × 5 cm I.D.) previously equilibrated with 0.05 *M* Tris · HCl buffer (pH 9). Non-retained proteins were recovered and after thoroughly washing the column, proteins that were retained on the column were eluted with the same buffer containing 1 *M* NaCl. The capacity of the resin was estimated as ca. 40 mg/ml packed column, with ca. 87% recovery of retained proteins. It is noteworthy that by overloading the column, lysozyme and ovotransferrin were not retained on the column (data not shown).

In order to obtain a good resolution, experiments were performed below the capacity of the resin. However, if no high purity of the proteins is needed, the quantity of protein loaded onto the column may be increased. Thus, an egg white preparation containing ca. 800 mg of protein was applied to the same Q Sepharose Fast Flow column. Proteins were eluted by stepwise and linear gradients using 0.05 *M* Tris-HCl buffer (pH 9) and the same buffer containing 0.3 *M* NaCl as described in Table 1. The elution profile showed 10 peaks (Fig. 1). Proteins contained in collected peaks were dialyzed against water and freeze-dried. They were then dissolved in deionized water prior to re-chromatography on a size exclusion column and analysis by SDS-PAGE.

Table 1
Parameters for the elution of egg white proteins from Q Sepharose Fast Flow column

Time (min)	A (%)	B (%)
0	100	0
35	100	0
50	60	40
105	60	40
115	55	45
135	55	45
145	50	50
195	50	50
205	45	55
270	45	55
295	20	80
315	20	80
335	0	100
370	0	100

Two buffers, (A) 0.05 *M* Tris HCl (pH 9) and (B) buffer A containing 0.3 *M* NaCl, were used for stepwise increments and gradients from 100% A to 100%. Flow-rate: 7.5 ml/min.

Size exclusion chromatograms of all collected peaks are shown in Fig. 2. The ratios of the

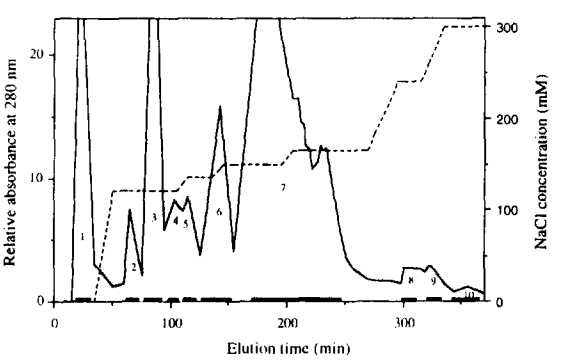

Fig. 1. Anion-exchange chromatography of diluted egg white on a Experimental Q Sepharose Fast Flow column (10 × 5 cm I.D.). Egg white preparation (see Experimental) containing ca. 775 mg of proteins was applied to the column previously equilibrated with 0.05 *M* Tris-HCl buffer (pH 9). After thoroughly washing the column with the same buffer, proteins were eluted as shown in Table 1. Collected fractions are indicated by solid bars.

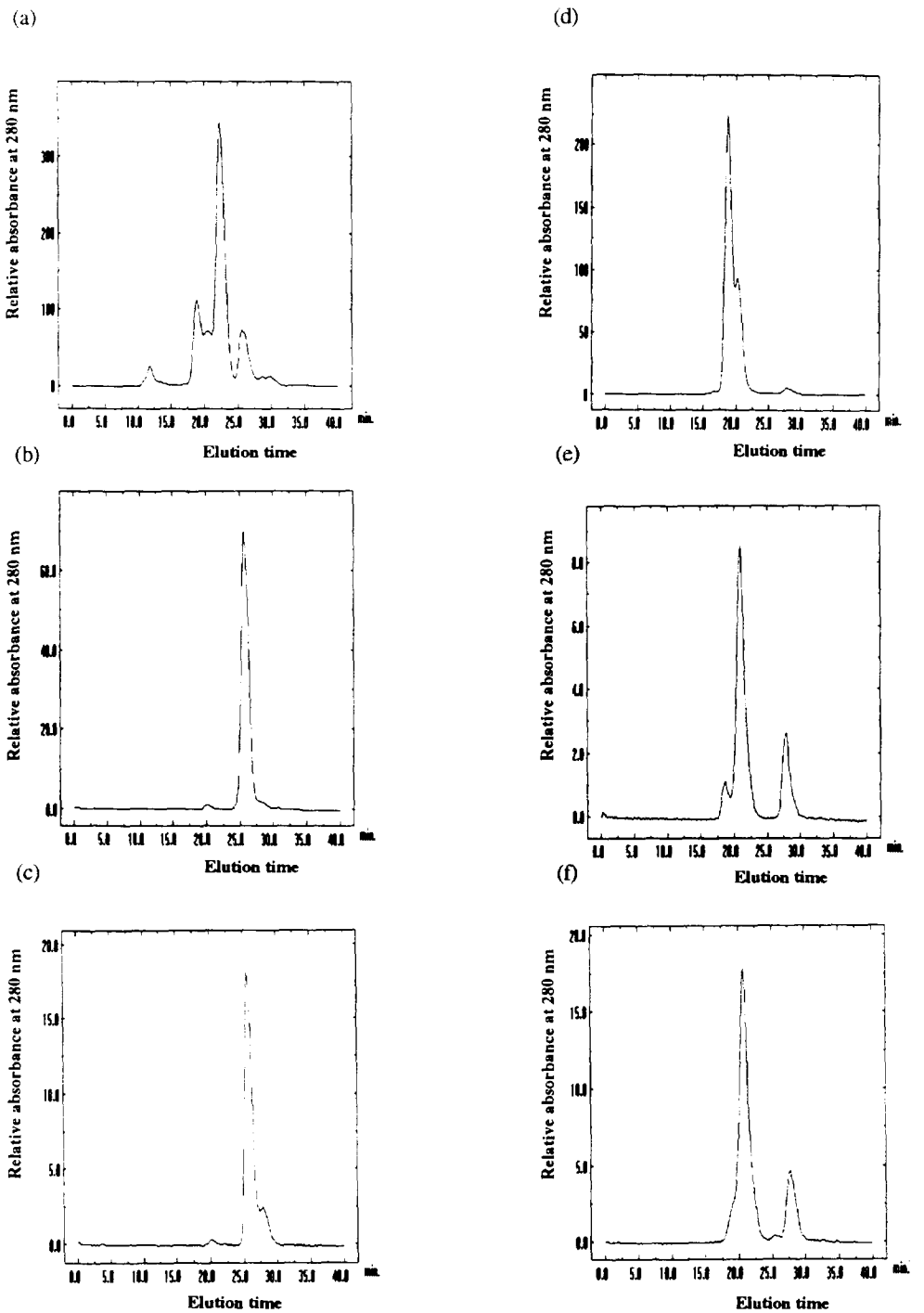

Fig. 2. Re-chromatography, using a size exclusion column (TSK G 3000 SW), of proteins eluted from the anion-exchange column (see Fig. 1). (a) Whole egg white, (b) peak 1, (c) peak 2, (d) peak 3, (e) peak 4, (f) peak 5, (g) peak 6, (h) peak 7, (i) peak 8, (j) peak 9 and (k) peak 10.

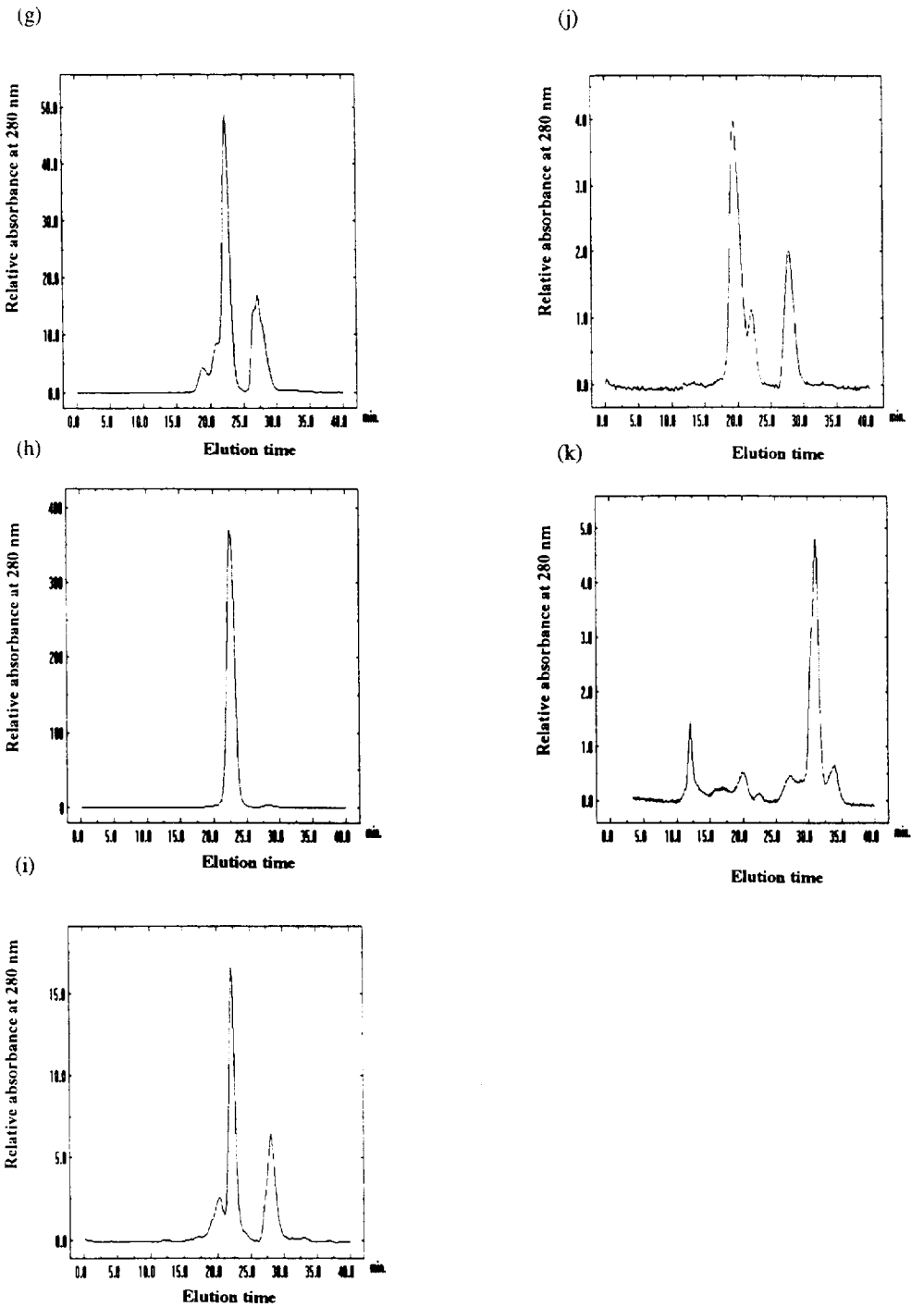

Fig. 2. *(continued)*

different peaks obtained by re-chromatgraphy and their retention times are shown in Table 2.

Protein peaks have also been analysed by SDS-PAGE (Fig. 3). Analysis of peaks 1 and 2 showed a single protein band at ca. 14.5 kDa. Peak 3 corresponded to a major protein band at ca. 79 kDa and a minor one at ca. 50.5 kDa. Peak 4 showed two major polypeptides at ca. 47.5 and 34.5 kDa and at least four minor polypeptide bands at ca. 79, 43, 37 and 36.5 kDa. Peak 5 exhibited a similar profile except for the band at 79 kDa which is replaced by a band at ca. 51.5 kDa. Analysis of peak 6 revealed a major polypeptide band at ca. 44 kDa and minor protein bands at ca. 51.5, 32.5 and 31 kDa. Peak 7 contained polypeptides at ca. 44 kDa. Peak 8 exhibited the 44 kDa polypeptides and a smear between 34 and 43 kDa. Analysis of peak 9 also revealed the band at ca. 44 kDa and a smear between 31 and 43 kDa. In peak 10, two major protein bands, one at ca. 170 kDa and the other near 14.5 kDa, and a smear between 30 and 43 kDa were detected.

Table 2
Ratios of peaks obtained by size exclusion re-chromatography of proteins contained in peaks from Q Sepharose Fast Flow column (Fig. 1), and respective retention times (t_R)

Peak	Chromatogram (see Fig. 2)	t_R (min)	% of area
1	b	19.99	1.13
		25.63	98.87
2	c	20.07	1.17
		25.59	88.94
		27.43	9.89
3	d	18.86	75.50
		20.22	22.93
		27.83	1.57
4	e	19.09	4.20
		20.72	76.33
		25.47	0.84
		27.77	18.63
5	f	18.54	8.17
		20.90	71.14
		27.80	20.69
6	g	19.27	4.49
		20.88	8.74
		22.32	54.79
		26.79	31.98
7	h	22.39	98.76
		27.99	1.24
8	i	20.22	12.19
		22.18	57.42
		28.01	30.39
9	j	19.34	57.88
		21.98	13.74
		27.75	28.38
10	k	12.08	12.42
		19.95	5.67
		22.39	1.32
		27.17	8.82
		31.09	61.29
		33.89	10.48

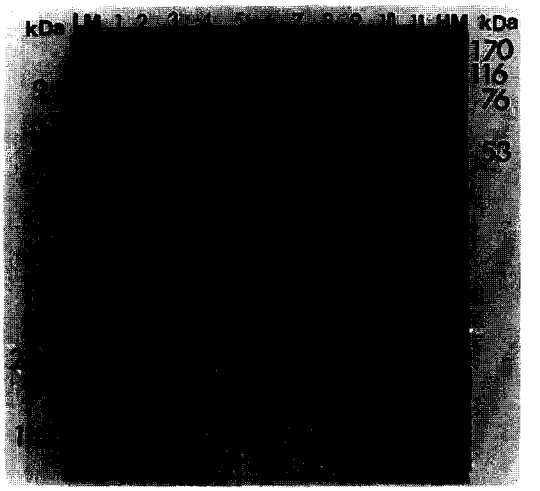

Fig. 3. SDS 15% PAGE of whole egg white proteins (11 μg) (lane 1), proteins contained in peak 1 from the anion-exchange chromatography (5 μg) (lane 2), peak 2 (2 μg) (lane 3), peak 3 (2 μg) (lane 4), peak 4 (7.5 μg) (lane 5), peak 5 (5 μg) (lane 6), peak 6 (5 μg) (lane 7), peak 7 (2 μg) (lane 8), peak 8 (5 μg) (lane 9), peak 9 (4 μg) (lane 10) and peak 10 (7 μg) (lane 11). LM and HM denote low and high M_r markers, respectively.

The amino acid composition of the collected peaks is shown in Table 3. The composition of the lysozyme, ovotransferrin and ovalbumin peaks was compared with those previously determined [13,14,31–33].

SDS-PAGE analysis of proteins contained in peaks 1 and 2 revealed a protein band with an approximate M_r which corresponds to the reported lysozyme M_r value of 14.3 kDa [16].

Determination of the lysozyme activity showed an activity of ca. 41 900 U/mg of protein and 36 400 U/mg of protein in peaks 1 and 2, respectively. These activities are similar to those stated by the manufacturers. However, they are slightly lower; this may be explained by the fact that the lysozyme preparation was dialysed against water rather than against an acidic buffer after purification. In fact, it has been shown that lysozyme loses its activity in distilled water [34]. It is less active than the lysozyme purified in our laboratory by size exclusion [35]. The native conformation of the enzyme may be better conserved in the size exclusion procedure than in procedures like ion-exchange chromatography or precipitation by salts, which may involve more physico-chemical changes.

The amino acid composition of peak 1 and peak 2 (Table 3) corresponded to that obtained for lysozyme by Canfield [31] and Guérin and Brulé [14].

As indicated by size exclusion rechromatography (Fig. 2) the peak 1 lysozyme appears to be purer (ca. 99%) than the lysozyme of peak 2 (ca. 88%). However, as shown in Fig. 3, the two preparations are electrophoretically identical.

Mandeles [21] separated 3 peaks of lysozyme, one major and two minor, using anion-exchange chromatography of DEAE-cellulose. He suggested that the lysozyme of the minor peaks might represent a molecular species different from the lysozyme of the major peak or that it might be the result of lysozyme interacting with other proteins or even with the cellulose. Cotterill and Winter [36] previously reported an interaction between ovomucin and lysozyme at low pH and ionic strength. They showed that an increase with ionic strength or pH caused dissociation of the complex. According to this observation, we suggest that the first lysozyme peak obtained in our procedure contained free lysozyme and that the second one contained lysozyme involved in the interaction with ovomucin. Indeed, at the pH used in this work, free lysozyme was not retained on the column and was eluted in the void volume. Lysozyme involved in the complex with ovomucin was eluted later, when the ionic strength was high enough to break up the complex. It is noteworthy that peak 2 lysozyme was not contaminated with ovomucin. The second lysozyme peak may also contain lysozyme which interacts with the Sepharose. Our hypotheses are strengthened by the fact that by diluting egg white with 0.05 *M* Tris-HCl (pH 9) containing 0.05 *M* NaCl instead of the same buffer without NaCl, and by beginning the gradient with the buffer containing NaCl, peak 2 lysozyme disappeared (data not shown).

The smaller peak (ca. 1.13%), revealed by size exclusion rechromatography of lysozyme peak 1 (Fig. 2c) may correspond to avidin. However, due to its low amount in the preparation, this protein is not clearly detected by SDS-PAGE analysis. Analysis by Western blot using anti-avidin serum revealed a reaction with a protein band corresponding to avidin polypeptide in peaks 1 and 2 (data not shown).

Analysis of peak 3 using SDS-PAGE (Fig. 3) showed that it contained principally a polypeptide corresponding to ovotransferrin. This was confirmed by amino acid analysis (Table 3) which indicated that the amino acid composition of peak 3 was similar to that obtained for ovotransferrin by Guérin and Brulé [14] and Jeltsch and Chambon [32], except for a phenylalanine (Phe) residue. The difference in the Phe content may be due to contamination with a polypeptide that showed an approximate M_r value of 50.5 kDa by SDS-PAGE analysis (Fig. 3, lane 4).

In view of their major protein band M_r (ca. 48 kDa), peaks 4 and 5 may include globulins as previously reported by Jacobs et al. [23]. These proteins are ca. 76 and 71% pure, respectively, as indicated by re-chromatography (Fig. 2 f and g, Table 2).

SDS-PAGE analysis of peaks 6–9 revealed principally two very close protein bands of ca. 44 kDa, which correspond to ovalbumin. The amino acid compositions of these peaks (Table 3) were similar to that determined by Nisbet et al. [33] for ovalbumin. With regard to the elution profile on Q Sepharose Fast Flow column (Fig. 1) and SDS-PAGE analysis, ovalbumin seems to be a contaminant protein in peaks 8 and 9.

Table 3
Amino acid composition of protein peaks obtained by Q Sepharose Fast Flow anion-exchange chromatography. Comparison with previously published results for lysozyme, ovotransferrin and ovalbumin

Amino acid	Lyso A[a]	Peak 1	Peak 2	Lyso B[a]	Ovotrf A[a]	Peak 3	Ovotrf B[a]	Peak 4	Peak 5	Peak 6	Ovalb[a]	Peak 7	Peak 8	Peak 9	Peak 10
Asp	16.3	17.8	17.1	17.9	11.4	12.5	11.4	13.0	14.7	12.6	8.0	9.3	11.5	10.3	9.0
Thr	5.4	5.9	5.9	6.2	5.5	5.7	5.4	6.5	6.7	5.2	3.9	3.7	4.7	4.5	5.1
Ser	7.8	8.8	9.0	8.1	7.1	6.9	6.9	7.5	6.7	8.1	9.9	8.9	8.4	7.7	6.8
Glu	3.9	5.1	4.4	3.6	10.3	10.5	9.8	8.8	8.9	12.3	12.5	13.4	13.7	13.2	14.3
Pro	1.6	0.0	0.0	1.5	4.5	3.9	4.1	4.2	3.7	3.7	3.6	3.6	3.8	6.8	6.3
Gly	9.3	10.5	10.4	9.8	7.4	7.4	7.6	6.4	7.2	5.9	4.9	5.3	5.5	6.5	9.8
Ala	9.3	9.7	9.5	9.0	7.7	7.7	7.7	6.8	6.5	7.7	9.1	9.9	9.4	9.0	7.9
Cys	6.2	5.1	5.2	5.1	4.6	4.2	4.4	5.4	6.7	3.8	1.6	1.9	1.9	1.9	2.4
Val	4.6	4.4	5.2	4.4	7.0	6.6	7.1	7.5	7.2	6.7	8.0	7.1	7.1	6.3	5.4
Met	1.5	1.3	1.1	0.7	1.3	2.0	1.6	1.3	1.2	2.1	4.2	3.7	2.0	2.3	1.9
Ile	4.7	3.8	4.0	4.7	3.9	3.5	3.9	2.2	2.0	3.9	6.5	5.5	4.4	4.3	3.8
Leu	6.2	5.8	6.0	6.7	7.7	6.8	7.3	7.4	7.1	7.6	8.3	8.2	7.3	7.3	8.1
Tyr	2.3	2.6	2.9	3.2	3.1	2.9	3.1	2.6	2.9	2.5	2.6	2.6	2.0	2.1	2.3
Phe	2.3	3.0	4.3	2.6	4.0	6.2	3.7	8.8	7.5	6.9	5.2	5.9	7.0	7.0	5.3
His	0.8	1.7	1.0	0.8	1.8	1.8	1.7	2.1	2.2	1.9	1.8	1.7	2.2	2.4	2.6
Lys	4.7	5.0	5.6	5.3	8.5	7.5	8.5	5.6	5.7	5.5	5.2	5.5	5.3	4.8	5.9
Arg	8.5	9.5	8.4	10.4	4.2	3.9	4.2	3.8	3.1	3.6	3.9	3.7	3.8	3.6	3.1
Trp	4.7	ND	ND	ND	ND	ND	1.6	ND	ND	ND	0.8	ND	ND	ND	ND

[a] Values are in mol/100 mol of residues. Lyso A: lysozyme amino acid composition according to Canfield [31]; Lyso B: lysozyme amino acid composition according to Guérin and Brulé [14]; Ovotrf A: ovotransferrin amino acid composition according to Guérin and Brulé [14]; Ovotrf B: ovotransferrin amino acid composition according to Jeltsch and Chambon [32]; Ovalb: ovalbumin amino acid composition according to Nisbet et al. [33].

It is noteworthy that our procedure led to the separation of two distinct peaks for ovalbumin (Fig. 1, peaks 6 and 7). Some differences in amino acid composition were observed, especially for aspartic acid, threonine, cysteine and methionine; this is certainly due to contamination of ovalbumin in peak 6. According to Awadé et al. [35], the ovalbumin contained in peak 6 may correspond to the S-ovalbumin that comes from the conversion of ovalbumin during storage [37]. Since S-ovalbumin has been reported to be more heat-stable than ovalbumin [38], heat stability studies will allow the verification of this assumption.

Analysis of peak 10 using SDS-PAGE showed 2 major protein bands, one at ca. 14.5 kDa and the other at ca. 170 kDa. According to the M_r values, the corresponding proteins may be lysozyme for the first one band and ovomacroglobulin sub-unit or/and α-ovomucin sub-unit for the second one band.

The fact that lysozyme was found in this peak seemed surprising, since lysozyme was expected to be eluted in the exclusion volume. It has been shown that lysozyme forms complexes with ovomucin, particularly with α-ovomucin, and that this interaction is ionic strength dependent [12,36]. Thus, we suggested that the lysozyme detected in peak 10 may be that which was very strongly tied up to α-ovomucin sub-units.

4. Conclusions

Some studies using anion-exchange chromatography for egg white fractionation have already been published. Thus, the use of DEAE-cellulose has been reported by Mandeles [21]. A non-linear gradient of phosphate buffers with increased NaCl concentrations was used for elution of egg white proteins. Lysozyme, ovotransferrin, ovomucoid and ovalbumin were isolated with good yields (over 75%). However, from the standpoint of the time required and the number of buffers involved, this method was of limited use because of the complexity of the elution schedule. Whatman DE52 and DE92 anion-exchange cellulose have been used by Levison et al. [22,39] who carried out some studies to determine the capacity of these resins. They used Tris-HCl buffer (pH 7.5) containing different salt concentrations as eluents. They found that DE 92 has a capacity of 100 mg/ml packed column volume with 89% recovery of bound proteins, whereas for DE52 they found a capacity of 158 mg/ml with 98% recovery. However, proteins were not well separated by these resins in comparison with the method published by Mandeles [21] who used DEAE-cellulose, though the elution schedule proposed by Mandeles was rather cumbersome. In addition, ovalbumin was the major protein retained on the column, whereas lysozyme and ovotransferrin were not bound to the column.

The low cost and ability to regenerate these softer gels make them attractive for fractionation. However, their swelling and shrinking properties make them difficult to use. More recently, in order to characterize selenium distribution in egg white proteins, Jacobs et al. [23] carried out a purification with Q Sepharose Fast Flow which is a rigid matrix highly substituted with strong ion-exchange groups. Elution using a linear gradient of 0.02 *M* bis-Tris propane-HCl (pH 6.9) and the same buffer containing 0.5 *M* NaCl led to the separation of 9 protein peaks (lysozyme, ovotransferrin, globulin 1, globulin 2, ovalbumin 1, ovalbumin 2, ovomucoid and flavoprotein). Our procedure that used the same resin gave of 10 chromatographic peaks. Even though no protein purity and recovery was reported by Jacob et al. [23], we think that, in view of the chromatographic profiles, protein separation reported in the present paper is better.

Our procedure allow a one-step isolation of lysozyme, ovotransferrin and ovalbumin from egg white on a laboratory scale. From ca. 775 mg of the egg white proteins applied to the Q Sepharose Fast Flow column (10 ×5 cm I.D.), ca. 17, 174 and 388 mg were recovered in the lysozyme, ovotransferrin and ovalbumin peaks, respectively. The respective recoveries, according to the theoretical ratios of these proteins in egg white, were estimated to be ca. 60, 100 and 83%. Re-chromatography of protein peaks indicated that lysozyme peak 1 was purified to ca.

99%, lysozyme peak 2 to ca. 88%, ovotransferrin to ca. 75%, ovalbumin 1 (peak 6) to ca. 54% and ovalbumin 2 (peak 7) to ca. 98%. The overall protein recovery was estimated as 86%. Although the column capacity and recoveries appear to be lower than those of what DE52 and DE92 [22,39], protein separation is better on Q Sepharose Fast Flow. Moreover, the capacity of this resin may be improved using less basic eluents (e.g. with a pH range between 7 and 8). In this work, we used a pH 9 buffer with the objective of being near the pH of the egg white. On the basis of these results, purification of these proteins on a process scale is possible.

Acknowledgements

The authors wish to thank K. Raulot for her efficient technical assistance. M.C. Vachier was funded by the Ministère de l'Agriculture et de la Pêche.

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