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Two-step chromatographic procedure for the purification of hen egg white ovomucin, lysozyme, ovotransferrin and ovalbumin and characterization of purified proteins

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Abstract

An improved procedure is described involving gel permeation and anion-exchange chromatography for the purification of four major hen egg white proteins. The procedure involves a first-step purification of ovomucin and lysozyme by gel permeation on a Superose 6 Prep Grade column. In the second step, anion-exchange chromatography on Q Sepharose Fast Flow led to the isolation of ovotransferrin and ovalbumin from a gel permeation chromatographic peak. The purities were estimated as *ca.* 80, 100, 80 and 100% for ovomucin, lysozyme, ovotransferrin and ovalbumin, respectively. The purification yield was over 60% for each protein. Further characterization of purified lysozyme revealed that it was fully active and homogeneous in relation to the electrospray ionization mass spectrum. The electrospray ionization mass spectrum showed different ovotransferrin species. The amino acid composition of purified ovomucin was compared to those published previously.

1. Introduction

In the last 10 years, egg production in industrialized countries has remained almost constant. The value of egg components is becoming increasingly necessary for the poultry product industry. With this in view, the isolation and purification of such components, particularly proteins, appears to be promising.

Over two decades, different methods [1] including protein precipitation by salts or by ionic strength reduction and liquid chromatography have been developed for purifying major egg white proteins. Almost all of these procedures were affected by the lack of emphasis on the importance of the co-product resulting from each step.

In this paper, we discuss a serial procedure that allows the purification of four major egg white proteins: ovalbumin (*ca.* 54% of egg white), ovotransferrin (*ca.* 13%), lysozyme (*ca.* 3.5%) and ovomucin (*ca.* 1.5%). Ovomucin is a highly polymerized glycoprotein with a molecular mass (M_r) ranging between *ca.* $0.22 \cdot 10^6$ and $270 \cdot 10^6$, depending on the sample preparation conditions [2]. This protein is assumed to play a

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preponderant role in the structure of the egg white, and is largely implicated in its foaming properties [3]. It has been proposed by Van Boeckel [4] as a possible source of glycopeptides and oligosaccharides for antibiotic preparations. The function of ovotransferrin is generally accepted to be that of iron transport. This glycoprotein with $M_r \approx 78\,000$ can also exhibit antimicrobial activity [5,6]. Lysozyme ($M_r = 14\,300$) is an enzyme (EC 3.2.1.17) that is used in the food and pharmaceutical industries [7] on account of its antimicrobial activity. Ovalbumin, which constitutes over half of the egg white proteins by mass, is a glycoprotein with $M_r \approx 45\,000$. This protein presents functional properties in egg white that are related to foaming and gelling. Although no biological function has been proposed for ovalbumin, its role in the immunological and allergenic properties of egg white has been investigated [1]. Moreover, this protein can be assumed to be useful in nutrition.

The object of our work is to purify a maximum number of proteins from the same sample. The procedure presented here includes gel permeation and anion-exchange chromatography and it was tried out on a laboratory scale. The activity of the purified lysozyme was determined and the amino acid composition of the ovomucin was compared with those published previously. Moreover, purified lysozyme and ovotransferrin were characterized by electrospray mass-spectrometry.

2. Experimental

2.1. Materials and reagents

The system for sample concentration, Amicon 8200, Centricon-3 and Centriprep-3, was obtained from Amicon (Epernon, France). A Superose 6 HR 10/30 column was purchased from Pharmacia Biotechnology (St.-Quentin-Yvelines, France). Reference lysozyme, ovotransferrin, ovomucoid, ovalbumin and *Micrococcus lysodeikticus* cells were obtained from Sigma (L'Isle d'Abeau Chenes, France). Superose 6 Prep Grade, Q Sepharose Fast Flow and protein M_r markers were supplied by Pharmacia Biotechnology. All other reagents were of analytical-reagent grade.

2.2. Egg white preparation

Egg white was diluted with 2 volumes of 0.05 *M* Tris–HCl (pH 9) containing 0.4 *M* NaCl and 10 m*M* β-mercaptoethanol and gently stirred overnight.

2.3. Lysozyme activity assay

Lysozyme activity was determined by the turbidimetric method as proposed by Weaver *et al.* [8].

2.4. Amino acid composition

Amino acid composition was determined as described by Spackman *et al.* [9] using a Pharmacia–LKB (Alpha Plus) analyser. Protein samples (1–2 mg) were dried and hydrolysed under vacuum in 6 *M* HCl for 24, 48 or 96 h at 110°C. In order to determine cysteine and methionine residues, protein samples were first subjected to performic acid oxidation and then hydrolysed at 110°C in 6 *M* HCl according to Moore [10].

2.5. Other methods

Protein determination

Protein concentrations were determined according to the method of Bradford [11], using the Bio-Rad protein assay with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [12], using 7.5% or 15% separation gels and 4% stacking gel containing 0.1% SDS. Protein bands were stained with Coomassie Brilliant Blue.

Analytical chromatography

Isolated fractions were rechromatographed on a TSK-G3000 SW gel permeation column

(Supelco, St.-Germain-en-Laye, France) using 0.01 *M* sodium phosphate buffer (pH 2.4) containing 0.2 *M* NaCl as the eluent. The ovomucin fraction was analysed at pH 7 instead of pH 2.4. The flow-rate was 0.4 ml/min.

Electrospray mass spectrometry

Electrospray mass spectrometry was conducted with a single-quadripole mass spectrometer (API I; Sciex, Toronto, Canada) equipped with an atmospheric-pressure ionization ion source (ion spray). Sample solutions (diluted in water containing 0.1% trifluoroacetic acid), delivered to the sprayer by a syringe infusion pump (generally at a flow-rate of 5 μl/min), were sprayed through a stainless-steel capillary held at a high voltage between +5 and +5.2 kV. The concentrations of the samples were *ca.* 56 μM for lysozyme and 20 μM for ovotransferrin. Solutions were sprayed at 55°C. The liquid nebulization was aided by a coaxial air flow along the sprayer, the nebulizer pressure being adjusted between 0.3 and 0.4 MPa. The interface between the sprayer and the mass analyser consisted of a conical orifice of 100 μm I.D.; the potential on the orifice was 100 V. A gas curtain formed of a continuous nitrogen flow was used in the interface to break up the ion cluster formation. The instrument was calibrated with ammonium adduct ions of polypropylene glycols (PPG) [13]. The unit resolution was maintained across the entire mass range for singly charged PPG calibrant ions, according to the 55% valley definition.

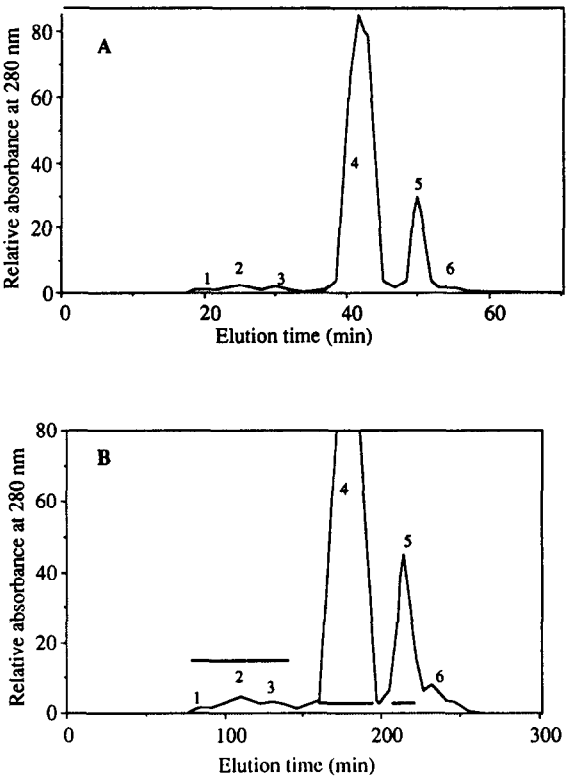

Fig. 1. (A) Chromatography of hen egg white proteins on a Superose 6 HR column (30 × 1 cm I.D.): 100 μl of the egg white preparation containing *ca.* 6 mg of protein were applied to the column previously adjusted with 0.05 *M* Tris–HCl buffer (pH 9) containing 0.2 *M* NaCl, using the Pharmacia FPLC system at room temperature. Proteins were eluted with the same buffer at a flow-rate of 0.4 ml/min. (B) Chromatography of hen egg white proteins on a Superose 6 Prep Grade column (90 × 2.6 cm I.D.), using 0.05 *M* Tris–HCl buffer (pH 9) containing 0.02 *M* NaCl: 10 ml of egg white preparation containing *ca.* 615 mg of proteins were loaded on the column and protein elution was performed at a flow-rate of 2 ml/min.

3. Results and discussion

3.1. Gel permeation chromatography allowing simultaneous isolation of ovomucin and lysozyme

Preliminary experiments on the fractionation of egg white proteins were carried out using a Superose 6 HR column (30 × 1 cm I.D.). As shown in Fig. 1A, six major chromatographic peaks (1–6) were obtained from the whole egg white sample. Aliquots of preparations containing reference proteins from Sigma were subsequently added to egg white sample before loading on the column. This indicated that peak 4 may contain ovalbumin, ovotransferrin and ovomucoid as major proteins, while lysozyme may be present in peak 5. According to its M_r (see above), we assumed that ovomucin was eluted in either peak 1, 2 or 3. Subsequent preparative-scale experiments were performed on a 90 × 2.6 cm I.D. Superose 6 Prep Grade column using the same fast protein liquid chro-

matographic (FPLC) system at room temperature. As shown in Fig. 1B, the chromatographic protein profile was similar to that obtained with the smaller column. Each peak was recovered and concentrated by gel filtration. SDS-PAGE analysis of peaks 1, 2 and 3, taken separately (data not shown), revealed that the principal polypeptides present corresponding to those previously reported for ovomucin [14]. Therefore, in further experiments, the three fractions were pooled. However, for other experiments it may be interesting to study these fractions separately, e.g., in order to determine their actual composition. Thus, SDS-PAGE analysis (Fig. 2) of different fractions revealed that the pool of proteins contained in peaks 1, 2 and 3 essentially correspond to ovomucin that was contaminated with small amounts of polypeptides corresponding to ovalbumin and ovotransferrin. This analysis also showed that peak 4, which contains the largest amount of proteins, is mainly composed of polypeptides corresponding to ovalbumin and ovotransferrin. The polypeptide band obtained from the peak 5 corresponded to that of lysozyme. The recovery of ovomucin in three different adjacent peaks may be explained by the different degrees of polymerization of this protein. Indeed, it has been shown by gel filtration that native ovomucin exhibited different apparent M_r in relation to its elution profile on Sepharose 4B [15].

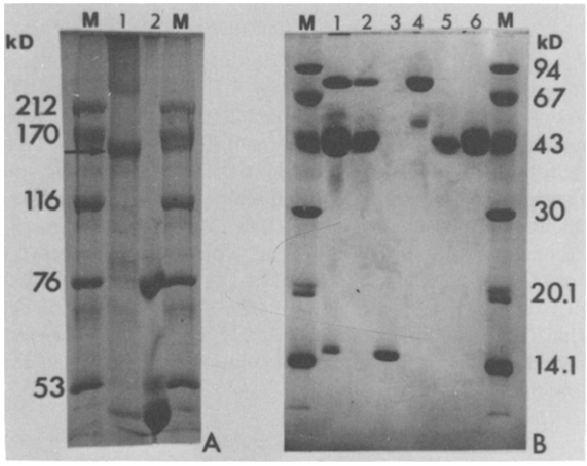

Fig. 2. (A) SDS-PAGE (7.5%) of egg white proteins (100 μg) (lane 2) and proteins (40 μg) contained in the pooled fractions from gel permeation peaks 1, 2 and 3 (see Fig. 1). The arrow indicates a protein band that may contain ovomucin and ovostatin subunits. (B) SDS-PAGE (15%) of egg white proteins (100 μg) (lane 1), and proteins contained in peak 4 from gel permeation (40 μg) (lane 2), peak 5 from gel permeation (5 μg) (lane 3), peak A from Q Sepharose Fast Flow (see Fig. 4) (10 μg) (lane 4), peak B from Q Sepharose Fast Flow (10 μg) (lane 5) and peak C from Q Sepharose Fast Flow (40 μg) (lane 6). M denotes M_r markers kD = kilodalton.

The purity of the fractions corresponding to ovomucin and lysozyme was further investigated with an analytical gel permeation column (TSK-G 3000 SW) using an HPLC system. As shown in Fig. 3, lysozyme was purified to homogeneity. However, the ovomucin fraction was purified only to *ca.* 80% if we assume that it was contaminated with ovostatin (see below). Other workers have isolated ovomucin from egg white by adding egg white slowly to three volumes of water and adjusting the pH to 6 [15,16]. This procedure had the advantage of being rapid and applicable in industry. However, it was difficult to resolubilize the ovomucin obtained. In addition, the ovomucin obtained by this procedure was contaminated with lysozyme, which interacts with ovomucin at low pH and ionic strength [15,16]. Conversely, no contamination with lysozyme was observed in the ovomucin prepared in our laboratory by gel permeation. However, the ovomucin preparation may be contaminated with ovostatin if we refer to the M_r of this protein (780 000) [17]. Our supposition is strengthened by the studies of Nagase *et al.* [17] on the purification of ovostatin by a procedure including gel filtration. This may be confirmed unambiguously, for example, by Western blot experiments, using antibodies raised against ovostatin. The ovomucin we prepared can be used more easily for further studies as it is soluble. Gel permeation was previously used by Young and Gardner [18] to purify soluble ovomucin from egg white. However, their fractions were less separated than ours. They showed that another peak was included in the ovomucin peak from total egg white, using chromatographic analysis of ovomucin-depleted egg white; in our opinion, this peak may correspond to ovostatin. Gel permeation has also

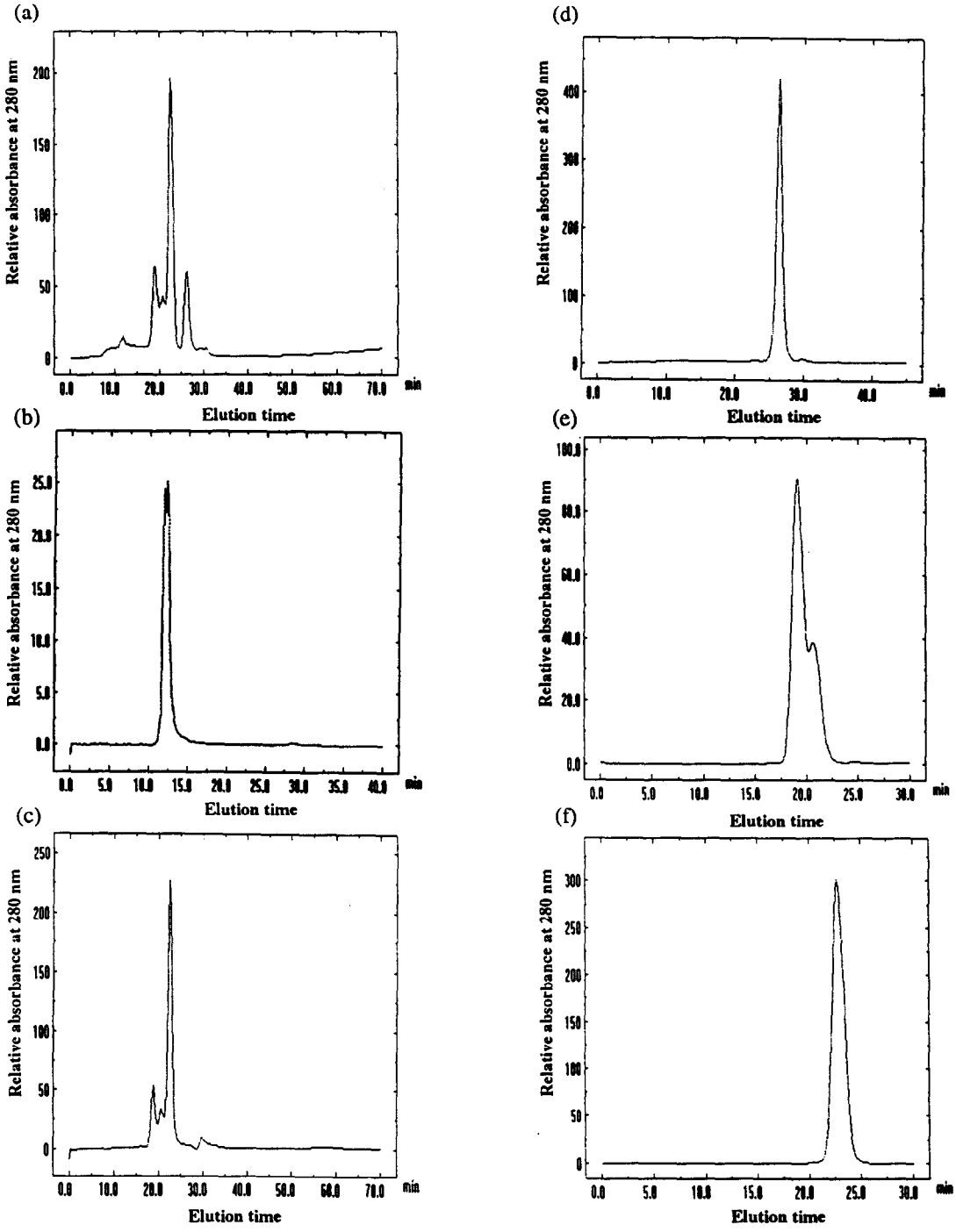

Fig. 3. Rechromatography using a TSK-G3000 SW gel filtration column (see Experimental) of fractions eluted from Superose 6 Prep Grade gel permeation column (see Fig. 1) or fractions eluted from Q Sepharose Fast Flow (see Fig. 2). (a) Whole egg white; (b) ovomucin fractions (peaks 1, 2 and 3 from gel permeation); (c) proteins contained in peak 4 from gel permeation; (d) lysozyme fraction (peak 5 from gel permeation); (e) ovotransferrin fraction (peak A from the anion-exchange column); (f) ovalbumin fractions (peak B or C from the anion-exchange column).

been used for the isolation of lysozyme [19]. However, to our knowledge, this is the first report on the simultaneous purification of ovomucin and lysozyme by gel permeation chromatography.

Protein quantification assay showed that from *ca.* 615 mg of total egg white protein, *ca.* 9 mg of ovomucin and 14 mg of lysozyme were isolated in one step; *ca.* 520 mg of protein were recovered in peak 4. The purification yields were estimated to be *ca.* 80% for ovomucin and 70% for lysozyme.

3.2. Purification of ovotransferrin and ovalbumin by using an anion-exchange column

Following the serial purification procedure, the proteins present in gel filtration peak 4 (see above) were pooled, concentrated, dialysed against 0.05 *M* Tris–HCl buffer (pH 9) and chromatographed on a Q-Sepharose Fast Flow column (Table 1). As shown in Fig. 4, the elution profile revealed three major peaks whose aspect suggests that they contain several proteins. SDS-PAGE analysis of proteins contained in these peaks indicated that the ovotransferrin protein band ($M_r \approx 78\,000$) (in peak A) was contaminated by a protein band with $M_r \approx$ 50 000. This protein band may contain at least one of the two minor glycoproteins with $M_r =$ 52 000, recently isolated from hen egg white by heparin affinity chromatography by Itoh *et al.* [20]. Peaks B and C contain only ovalbumin. Hence it is noteworthy that two distinct peaks of ovalbumin were separated in our procedure. The form contained in peak B may correspond to the S-ovalbumin that comes from a conversion of ovalbumin during storage [21,22]. This may be verified by heat-stability studies on ovalbumins recovered in the two peaks. It has been shown that S-ovalbumin was more heat stable than ovalbumin [23]. Kurisakai *et al.* [24] concluded that as there was no difference in the electrophoretic and ion-exchange chromatographic profiles of fresh and stored ovalbumin, the increased heat stability of S-ovalbumin could not be explained by an increase in net negative charge. The fact that two chromatographic peaks of ovalbumin (one probably corresponding to S-ovalbumin) were obtained in our procedure by anion-exchange chromatography may lead to a revision of this statement. Rechromatography of fractions from Q Sepharose Fast Flow as shown in Fig. 4 indicated that ovalbumin fractions were purified near to homogeneity, whereas ovotransferrin was purified to only *ca.* 80% The aspect of the peaks may be due to the heterogeneity in

Table 1
Parameters for the elution of gel permeation peak 4 proteins from Q Sepharose Fast Flow column

Time (min)	A (%)	B (%)
0	100	0
25	100	0
40	60	40
85	60	40
95	55	45
115	55	45
125	50	50
175	50	50
185	45	55
235	45	55
280	0	100

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

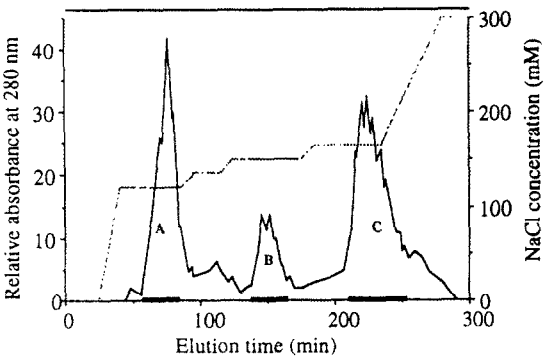

Fig. 4. Chromatography of proteins contained in peak 4 from gel permeation (see Fig. 1) on a Q Sepharose Fast Flow column (10 × 5 cm I.D.). Protein sample was dialysed in 0.05 *M* Tris–HCl buffer (pH 9) (A) and applied to the column previously adjusted with the same buffer. After thoroughly washing the column (25 min) with buffer A the proteins were eluted as presented in Table 1.

ovotransferrin and ovalbumin. That may be related in part to the heterogeneity in glycans bound to the proteins, in the case of ovotransferrin. For ovalbumin, this could be explained by the presence of components that differ in phosphorus content [25–27].

From *ca.* 500 mg of the proteins present in the gel permeation peak 4, *ca.* 91 and 220 mg of proteins were recovered in the ovotransferrin and the ovalbumin peak, respectively. Hence, the final recovery of proteins, according to the theoretical ratios of these proteins in egg white, was estimated to be *ca.* 90% and 66% for ovotransferrin and ovalbumin, respectively.

An anion-exchange column (DE 92) has been used for egg white fractionation, principally to separate ovalbumin [28]. However, proteins were better separated and certainly purer in the present work. Q Sepharose Fast Flow has recently been used by Jacobs *et al.* [29], who studied the selenium contents of the fractions obtained by chromatography of the whole diluted egg white on a column made of this resin. With regard to the chromatographic profiles, their fractions may be less pure than those obtained in this work. For instance, the two ovalbumin peaks were not separated.

3.3. Further characterization of purified proteins

The M_r of purified lysozyme and ovotransferrin were determined by electrospray ionization mass spectrometry (ESI-MS). The mass spectra for lysozyme and ovotransferrin are shown in Figs. 5 and 6, respectively. The average M_r for lysozyme was calculated from five multiply charged molecular ions (Fig. 5, Table 2). The M_r for lysozyme, 14 303.4 (±0.6), is consistent with that expected from the polypeptide chain (14 304.2). The corresponding spectrum revealed that the molecule was homogeneous, although the presence of sodium adducts (mass 14 325.7 ± 0.9) could be observed. Three major species were detected for ovotransferrin. The major population, average M_r 77 513 ± 8.3, calculated from fifteen multiply charged molecular ions, is presented in Fig. 6A, Table 3. The other M_r values of 77 659.4 ± 8.7 and 77 324.2 ± 9.1 were

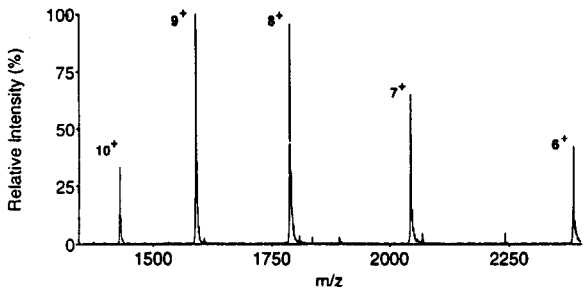

Fig. 5. Electrospray ionization mass spectrum of purified lysozyme; 326 pmol were consumed in acquiring the spectrum. Scan accumulation and mass calculation were performed with Tune 2.3 and Macspec 3.2 programs, respectively. The protonation states are indicated above the peaks. Average M_r: 14 303.4 ± 0.6.

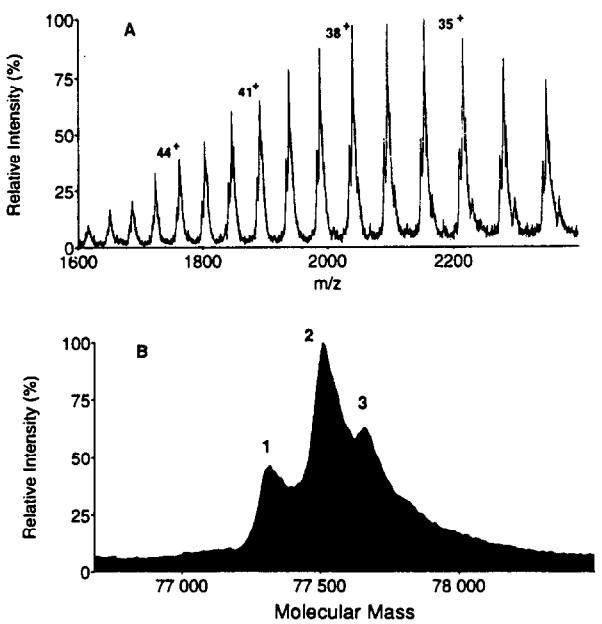

Fig. 6. (A) Electrospray ionization mass spectrum of purified ovotransferrin. The spectrum was acquired by using 384 pmol on the basis of ovotransferrin M_r = 78 000. Scan accumulation and mass calculation were performed with Tune 2.3 and Macspec 3.2 programs respectively. Protonation states of the three major species are indicated above some peaks. The fourth less important form of ovotransferrin belongs to another protonation states series. (B) M_r spectrum reconstructed from the mass spectrum by using the Macspec 3.2 program. Peak 1, 2 and 3 correspond to calculated M_r values of 77 324.2 ± 9.1, 77 513.9 ± 8.3 and 77 659.4 ± 8.7, respectively.

Table 2
ESI-MS determination of lysozyme M_r by HyperMass method

Actual peak (m/z)	Intensity	Predicted peak (m/z)	Charge	Compound mass
1431.40	735 000	1433.40	10	14 303.92
1590.20	2 228 000	1590.33	9	14 302.73
1789.00	2 131 000	1789.00	8	14 303.94
2044.40	1 445 000	2044.43	7	14 303.74
2384.80	929 000	2385.00	6	14 302.75

The primary charge agent was H, mass 1.0079. The average compound M_r calculated from five estimates was 14 303.42 with a standard deviation of 0.62.

determined from fourteen and twelve multiply charged molecular ions, respectively. Another less important species with average M_r = 75 795.3 was also detected. The multiply charged ions for this last species belongs to a series different from that of the three major forms which belong to the same series. M_r values of 76 000 or 77 500 have been determined previously by ESI-MS for ovotransferrin [30,31]; we assume that only one species of the ovotransferrin population was characterized in these cases. The mass reconstruct obtained from the mass spectrum and presented in Fig. 6B illustrates the heterogeneity in the glycans bound to this protein. The different ovotransferrin forms are not evidenced by polyacrylamide gel electrophoresis even though they were slightly separated by chromatography on a Q Sepharose Fast Flow column (see Fig. 4).

The amino acid composition of the purified ovomucin was compared to that of the protein purified by gel permeation, reported by Young and Gardner [18], and by precipitation, reported by Guérin and Brulé [32] (Table 4). Ovomucin

Table 3
ESI-MS determination of the ovotransferrin major population M_r by HyperMass method

Actual peak (m/z)	Intensity	Predicted peak (m/z)	Charge	Compound mass
1650.40	64 000	1650.07	47	77 521.43
1686.20	78 000	1685.92	46	77 518.84
1723.60	125 000	1723.37	45	77 516.64
1762.80	149 000	1762.51	44	77 518.85
1803.60	179 000	1803.48	43	77 511.46
1846.80	229 000	1846.39	42	77 523.27
1891.40	247 000	1891.40	41	77 506.08
1938.80	299 000	1938.66	40	77 511.69
1988.80	334 000	1988.34	39	77 523.89
2041.00	371 000	2040.64	38	77 519.70
2095.80	374 000	2095.77	37	77 507.31
2154.40	381 000	2153.96	36	77 522.12
2215.40	366 000	2215.47	35	77 503.73
2280.60	317 000	2280.60	34	77 506.14
2349.40	282 000	2349.68	33	77 496.94

The primary charge agent was H, mass 1.0079. The average M_r of the compound from fifteen estimates was 77 513.87 with a standard deviation of 8.32.

Table 4
Amino acid composition of ovomucins from different purification procedures

Amino acid	Ovo A[a]	Ovo B[a]	Ovo C[a]
Asx	12.3	10.2	9.3
Thr	7.1	7.9	8.5
Ser	8.5	8.4	7.8
Glx	8.8	8.1	12.0
Pro	5.1	5.9	5.8
Gly	7.2	5.8	3.7
Ala	6.2	3.9	4.0
Cys	5.9	6.0	7.7
Val	6.2	6.4	6.0
Met	0.9	1.3	0.8
Ile	4.7	4.9	4.7
Leu	7.0	6.9	7.5
Tyr	2.9	4.4	4.1
Phe	4.9	7.6	4.9
His	1.7	2.3	2.4
Lys	5.6	6.4	6.4
Arg	4.9	3.7	4.5
Trp	ND	ND	ND

[a] Values are mol per 100 mol of residues. Ovo A = ovomucin prepared by the method of Guérin and Brulé [24]; Ovo B = ovomucin presented in this work; Ovo C = ovomucin isolated by Young and Gardner [17].

obtained by the two gel permeation procedures showed similar compositions, even though some striking differences were observed for the Phe, Glx and Gly composition. This might be due to the differences in contamination.

The specific activity of the purified lysozyme was estimated at *ca.* 60 000 U/mg of protein. This value is slightly higher than those stated by manufacturers or, for example, that obtained by Guérin and Brulé [32]. This indicates that the lysozyme preparation obtained by filtration on Superose 6 Prep Grade was purer and more active than the latter preparations. In fact, even though lysozyme is presented as a relatively stable protein, the native conformation of the enzyme might be better conserved with the gel permeation procedure than with the other procedures such as precipitation by salt addition or ion-exchange chromatography. Determination of the lysozyme activity from original egg white indicated that the enzyme was purified with a yield of *ca.* 60%. This value is in line with the yield deduced from protein quantification (see above) and confirms the quasi-absence of enzyme degradation during purification.

4. Conclusions

Several procedures have been developed for the separation of major egg albumen protein, usually on a laboratory scale and, to a lesser extent, on a process scale [33–43]. It is noteworthy that ovomucin is generally isolated as a precipitate by adding to egg white three volumes of water at an acidic pH [15,16]. The resolubilization of this precipitate in non-denaturing conditions is not evident. Soluble ovomucin has been prepared from egg white diluted in a saline solution at a relatively basic pH, by using gel permeation chromatography [18].

The past procedures used for egg white protein isolation have usually suffered from a lack of importance being attributed to the co-product. In keeping with the idea of maximum value of the egg white, we set up a procedure that allows the purification of several egg white proteins from the same sample. This has been tried previously by Guérin and Brulé [32], who developed a method involving ovomucin precipitation and separation of lysozyme and ovotransferrin from the co-product, by using cation-exchange chromatography. This procedure has the advantage of probably being applicable in industry. However, as mentioned above, the ovomucin obtained by this method was insoluble. In addition, as this protein coprecipitates with lysozyme, this led to a substantial decrease in the yields with lysozyme preparations. The two-step purification procedure proposed here allows the isolation of ovomucin purified to *ca.* 80% with a yield of 80%, lysozyme purified to homogeneity with a yield of *ca.* 65%, ovotransferrin purified to *ca.* 80% with a yield of 90% and ovalbumin purified to homogeneity with a yield of *ca.* 66%. Moreover, gel permeation chromatography allowed the separation of three peaks containing ovomucin. It will be interesting

to investigate the actual composition of proteins present in these peaks.

Although the gel permeation chromatography may not be easily transferrable to industry, the procedure we have developed constitutes a good means of preparing highly purified proteins with relatively good yields, especially for laboratory use. One advantage is that our purification scheme takes into account the maximum value of the egg white, by purifying several proteins from the same sample. The combination of this method with that of Guérin and Brulé [32] may allow the setting up of a procedure more easily adaptable to industry.

Acknowledgements

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