Simple Rapid Procedure for Preparation of Large Quantities of Ovalbumin

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A simple rapid procedure for preparation of large quantities of highly purified homogeneous ovalbumin from egg white by using an anion exchanger is described. It is based on the principle of frontal chromatography. The volume of "mucin-free" egg white loaded onto the column was determined in order to exceed resin capacity. Thus, competition between proteins for resin sites was created. Owing to its high negative charge density, ovalbumin drives other egg white proteins from the column progressively. Two hundred and fifty milliliters of Q-Sepharose FF gel eluted isocratically with 0.5 M NaCl extracted 9.55 g of ovalbumin with a purity rate of 83%. A 6.75 g amount of ovalbumin, with a purity rate of 94%, was recovered with an isocratic elution program using 0.14 M NaCl. Purified ovalbumins were compared by electrophoresis and analytical chromatography with other ovalbumin preparations.

Keywords: *Ovalbumin; extraction; anion exchanger; frontal chromatography*

INTRODUCTION

Hen egg white is widely used in food processing. However, a better valorization of egg white proteins would contribute to widening the egg-product market. Improvements on the biochemistry and structure-function relationship knowledge of the isolated proteins are required for a potential use. Thus, methods for their extraction are extensively studied. These include procedures such as precipitation using salts or solvents, precipitation by reducing ionic strength and/or pH, and isolation by using liquid chromatography (Vachier et al., 1995). The disadvantage of the first two procedures is the more or less reversible degradation of proteins. Liquid chromatography supplants these precipitation methods for protein purification. Ovalbumin extraction is a perfect illustration of the transfer from precipitation to chromatographic methods.

Ovalbumin, the main egg white protein (54% of egg white proteins), is a phosphoglycoprotein constituted of 385 amino acids (Mw 45 000 Da) of which half are hydrophobic and a third are charged, the majority acidic, conferring to the protein an isoelectric point (pI) of 4.5 (Li-Chan and Nakai, 1989). Ovalbumin is a predominant protein contributing to the functional properties of egg white (Mine et al., 1991; Kitabatake et al., 1989; Hatta et al., 1986).

Usually, ovalbumin purification from egg white is realized using precipitation at a specific salt concentration, pH, and temperature. Ammonium sulfate (Sorensen and Hoyrup, 1915-1917) or sodium sulfate (Kekwick and Cannan, 1936) are salts commonly used for ovalbumin precipitation. These methods of purification give rise to the separation of large quantities of ovalbumin but lead to a byproduct with a high salt concentration. Moreover, high purity is reached by multiplication of the precipitation/solubilization cycles. Ovalbumin is generally crystallized four or five times. Some reports have shown heterogeneity in such preparations (Doi and Kitabatake, 1997). These disadvantages have contributed to the modification of the ovalbumin extraction process.

For more than 30 years, ion exchange chromatography (IEC) has been the most popular method for the fractionation and purification of proteins. Several procedures for ovalbumin extraction have already been studied either with cation exchangers, CM cellulose (Rhodes et al., 1958), or with anion exchangers, DEAE cellulose (Mandeles, 1960), DE92 cellulose (Levison et al., 1992), and Q-Sepharose FF (Jacobs et al., 1993; Awade et al., 1994; Vachier et al., 1995). Purity rates for IEC are better than for other extraction methods, and the absence of protein degradation either for extracted proteins or for byproducts are incomparable assets (Awade, 1996). However, IEC leads to low yields of ovalbumin.

In this study, we developed a simple, rapid, laboratory-scale method for preparation of large quantities of highly purified homogeneous ovalbumin by using a Q-Sepharose FF gel. Ovalbumin was extracted according to the principle of frontal chromatography, also called "displacement chromatography". Frontal chromatography use the competitive binding between components for resin sites during saturation loading of the column. Finally, only the protein more tightly bound to the resin is retained (Janson and Jönsson, 1989).

MATERIALS AND METHODS

Preparation of "Mucin Free" Egg White Solution. Hen eggs (Isabrown) were purchased from a local market (10-day-old refrigerated chicken eggs). Albumen from each egg was manually separated from the yolk and pooled. Batches of 400 mL of egg white were firstly diluted with 2 volumes of 20 mM Tris-HCl and the mixture was adjusted to pH 6.0 with 1 M HCl. The solution was gently stirred and kept at 2 °C for 3 h,

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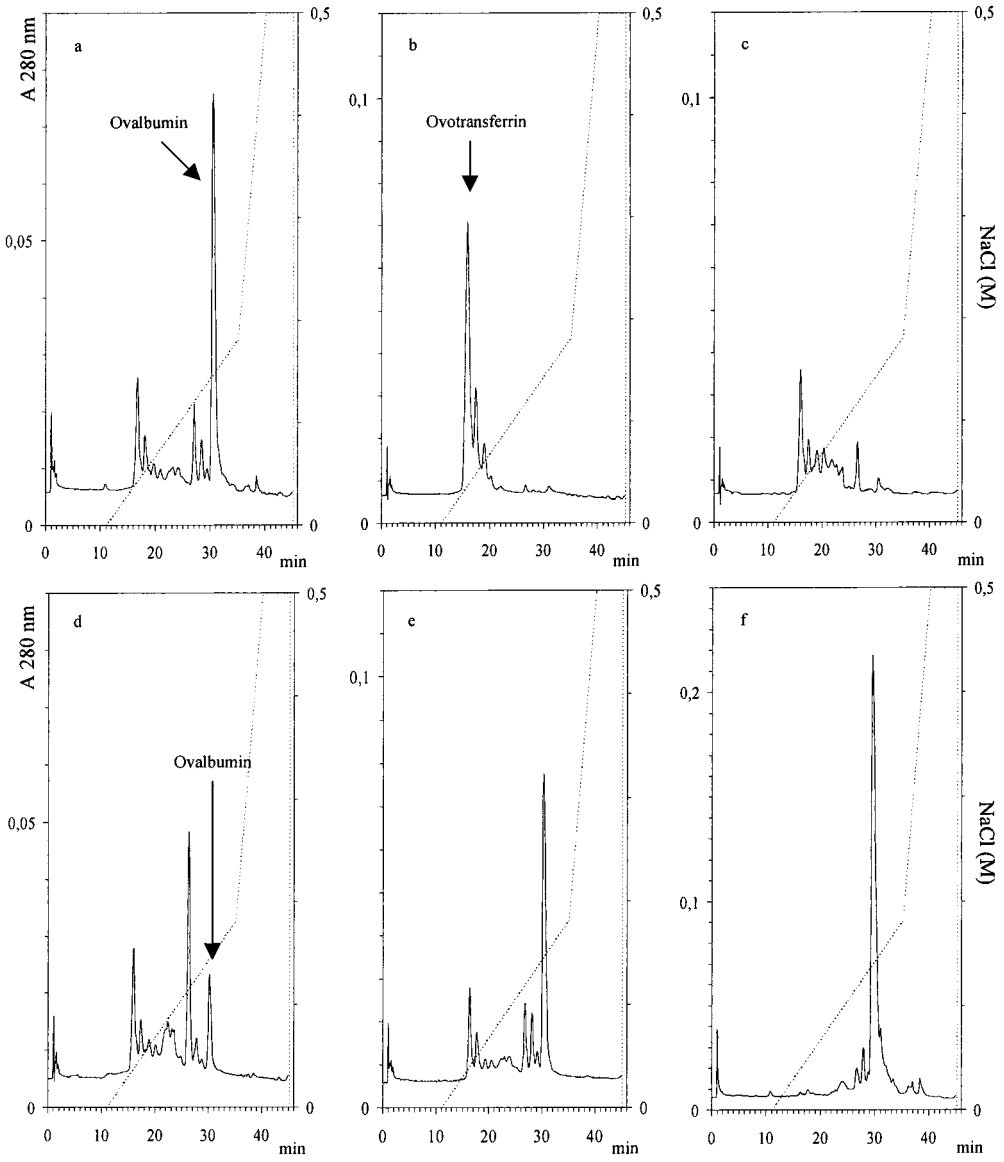

Figure 1. Anion-exchange high-performance liquid chromatograms of displaced fractions during mucin-free EW saturation loading. (a) Mucin-free EW; displaced fractions for (b) 360 mL of mucin-free EW loaded onto the Q-sepharose FF column, (c) 435 mL, (d) 510 mL, (e) 660 mL, and (f) bound fraction.

enabling ovomucin precipitation. The mixture developed a white, gelatinous precipitate, which was removed by 3 min of centrifugation at 2000*g*. Prior to the anion exchange chromatography, the Tris−egg white mixture was adjusted to pH 8.2 with 5 M NaOH, then centrifuged at 25000*g* for 20 min at 4 °C in order to remove insoluble material. The "mucin-free" egg white (EW) supernatant was used to avoid plugging the resin.

Ovalbumin Extraction According to the Principle of Frontal Chromatography. Preparative chromatography was performed with 250 mL of Q-Sepharose fast flow anion exchanger purchased from Pharmacia Biotech AB (Saclay, France). The preparation and the packing of the Q-Sepharose fast flow gel in a XK 50/20 column (13 x 5 cm i.d.) (Pharmacia Biotech AB, Saclay, France) was carried out following the manufacturer's recommendations. The column was connected to a BiopilotTM system (Pharmacia Biotech AB) equipped with 280-nm UV, conductivity and pH detectors.

Mucin-free EW (1125 mL) was applied to the column pre-equilibrated with Tris-HCl 20 mM, pH 8.2 (dilution solution). Nonbound material was removed by washing the column with the dilution solution until the absorbance reached the baseline. Bound material was recovered by using an isocratic elution program with the dilution solution containing 0.5 or 0.14 M NaCl. All steps were carried out at a flow rate of 30 mL min^{-1}.

Similar experiments were carried out at pH 7.5 or 9 with 40 mM Tris-HCl or water as dilution solutions. All experiments were run with the same cleaned column. The cleaning sequence was as follows: water rinse (600 mL, 30 mL min^{-1}; 0.1 M NaOH (600 mL, 10 mL min^{-1}); water rinse (600 mL, 30 mL min^{-1}); 0.5 M NaCl (600 mL, 30 mL min^{-1}).

Ovalbumin Desalting. Ovalbumin fractions were desalted by diafiltration with deionized water on a DC 10LA system (Millipore, St. Quentin en Yvelines, France) equipped with a spiral-wound ultrafiltration cartridge (S1OY10, nominal cut off 10 kDa, Millipore, St. Quentin en Yvelines, France) and freeze-dried. Freeze-dried ovalbumin contained less than 0.5% salt on a dry weight basis.

Other Ovalbumin Preparations. Purified ovalbumins according to the principle of frontal chromatogra-

phy were compared to ovalbumin preparations, crystallized, and lyophilized, essentially salt-free, grade V, minimum 98% purity, lot No. 14H7035 (Ovc1) and No. 76H7045 (Ovc2) from Sigma (St. Quentin Fallavier, France) and an ovalbumin prepared by classical gradient elution chromatography (Ova). For the latter, the albumin from a single egg was diluted ten-fold in 50 mM Tris-HCl, pH 8.0 (buffer A) and gently stirred overnight at 4 °C. Precipitated material was discarded by centrifugation (25 000*g*, 75 min, 4 °C) and filtration (filter paper Whatman 541). Ovalbumin from diluted, buffered egg white was purified by anion-exchange chromatography on a Q-Sepharose FF (13 × 5 cm i.d.) column equilibrated with buffer A. After injection of a 200 mL sample of diluted egg white, the column was washed with buffer A and proteins were eluted by linear gradients of NaCl concentration in buffer A, from 0 to 0.18 M in 95 min and from 0.18 to 0.5 M in 20 min. The fraction corresponding to the top of the ovalbumin peak was collected, extensively dialyzed against water, and lyophilized. Five hundred milligrams of ovalbumin were recovered and kept at 4 °C until use (Pezennec et al., in press).

Analytical Chromatographies. Anion exchange chromatography (IEC) was performed on a Q-Hyper D 10 (10 × 0.46 cm i.d.) column (BioSepra, Villeneuve la Garenne, France). Proteins were eluted by linear gradients of NaCl concentration in a 50 mM Tris-HCl, pH 8.0 buffer, from 0 to 0.18 M in 24 min and from 0.18 to 0.5 M in 5 min. Separation was carried out at a flow-rate of 1 mL min^{-1} and proteins were detected by absorption at 280 nm.

Reverse-phase (RP) chromatography was performed on a Vydac C4 214 TP (5 × 0.21 cm i.d.) column (Touzart et Matignon, Vitry S/Seine, France). HPLC-grade acetonitrile (ACN) (Carlo Erba, Nanterre, France) containing 0.025% trifluoroacetic acid was used as eluent under linear gradient elution conditions. The linear gradient elution increased from 7% ACN to 70% in 25 min, at a flow-rate of 0.8 mL min^{-1}. Detection was carried out at 214 nm with a UV/visible detector. The chromatograms were processed with Winner on Windows software on a Compaq Prolinea 3−25 microcomputer. Ovalbumin purity rate ((ovalbumin peak area/total peak area) × 100) was automatically calculated from the integrated chromatograms.

Polyacrylamide Gel Electrophoresis. SDS-slab polyacrylamide gel electrophoresis was conducted according to Laemmli (1970) using a 12% acrylamide separating gel and a 4% stacking gel containing 0.1% SDS. Protein samples were heated at 95 °C for 4 min. Electrophoresis was carried out at constant current (155 V) for 40 min in a Tris−glycine buffer pH 8.3 containing 0.1% SDS. The gel sheet was stained for proteins by using the Coomassie Brilliant Blue R250 or the silver staining method (Tunon and Johansson, 1984).

RESULTS AND DISCUSSION

Loading. Previous studies showed the high resolution of Q-sepharose FF gel for egg white protein fractionation with 10 peaks being identified (Awade et al., 1994). Among the egg white proteins, ovalbumin is one of the most strongly retained. Only three minor proteins have a retention time higher than that of ovalbumin. The chromatogram of "mucin-free" EW, analyzed using Q-Hyper D10 anion exchange column, is shown in Figure 1a. Ovalbumin was eluted with a retention time of 30 min.

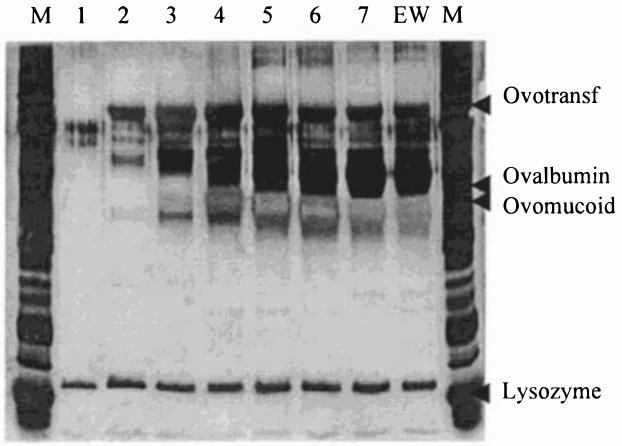

Figure 2. Silver-stained SDS-PAGE of displaced fractions during mucin-free EW saturation loading. Displaced fractions for (1) 185 mL of mucin-free egg white loaded onto the Q-sepharose FF column, (2) 310 mL, (3) 360 mL, (4) 410 mL, (5) 460 mL, (6) 510 mL, (7) 660 mL, (8) mucin-free EW.

The fact that ovalbumin had a high affinity for the resin was used in the following experiments. The Q-Sepharose FF column was intentionally overloaded and proteins displaced by competitive binding for resin sites were followed by anion-exchange HPLC (Figure 1b−e).

In this experiment, Tris−EW mixture at pH 8.2 was applied to the column. At pH 8.2, proteins were negatively charged, except lysozyme and avidin (pI of 10.7 and 10 respectively; Li-Chan and Nakai, 1989). Lysozyme was not retained by the resin and its concentration remained constant in excluded fractions during saturation loading, as shown by SDS PAGE (Figure 2). Other proteins were retained on the column and were not detected at the beginning of loading (the volume loaded was lower than 300 mL, which was equivalent to 10 g of protein loaded onto the column).

As soon as resin capacity was exceeded, negative proteins were selectively displaced according to their pI, i.e., their retention time on an analytical profile (Figure 1a). Ovotransferrin (pI 6.5) was the first protein eluted from the column (retention time of 16 min). Its concentration increased to much higher than in the initial solution (Figure 1b). Then, the amount of ovotransferrin eluted from the column decreased (Figure 1c) and reached the concentration in the mucin-free EW (Figures 1d,e). At that stage, for one molecule of ovotransferrin entering the column, another was eluted. It seems probable that the same kinetics applied to the other proteins present in mucin-free EW. These observations were confirmed by electrophoresis (Figure 2). Thus, for a given loaded volume of mucin-free EW higher than 400 mL, quantity of protein displaced was about equal to the quantity of protein loaded onto the column. Nevertheless, competition for resin binding sites modified the relative proportion of protein in the eluted fractions.

In the eluted fractions, ovalbumin was detected by the Q-Hyper D10 column when about 500 mL of mucin-free egg white had passed through the column, which is equivalent to 8.5 g of ovalbumin (Figure 1d). Mucin-free EW loading was maintained until ovalbumin concentration in the eluted fractions was equal to its concentration in mucin-free EW (volume loaded higher than 700 mL). Chromatograms in Figure 1a and e were

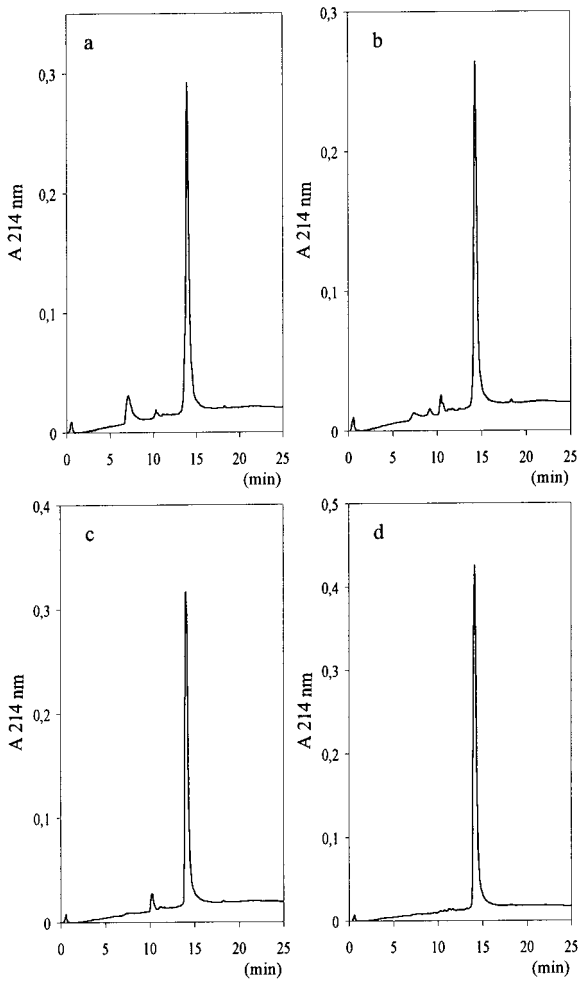

Figure 3. Reverse-phase high-performance liquid chromatograms of ovalbumin fractions. (a) Ovalbumin Sigma, grade V, lot 14H7035 (Ovcl); (b) ovalbumin eluted using an isocratic elution program with 0.5 M NaCl (OvaA); (c) ovalbumin eluted using an isocratic elution program with 0.1−5 M NaCl (OvaB); (d) ovalbumin purified by gradient elution chromatography (Ova).

different only for proteins with a retention time higher than that of ovalbumin.

After the loading, the column was washed with 20 mM Tris-HCl, pH 8.2. Bound material was eluted isocratically with the same buffer containing 0.5 M NaCl (400 mL). IEC analysis of bound material (Figure 1f) showed that the proportion of ovalbumin was much higher than in mucin-free EW. In one step, the quantity of ovalbumin extracted reached 9.7 g and its purity rate was 83% (Figure 3b). Contaminants were compounds with pI close to that of ovalbumin (Figure 1f). Ovotransferrin and the initial compounds eluted in Figure 1a were missing in profile 1f showing a displacement attributable to the competitive binding of proteins for resin sites.

Extraction Optimization. For a potential scale-up it is important to determine the influence of extraction parameters on process performance. The optimization was conducted through an experimental design strategy. Factors studied were pH (from 7.5 to 9), Tris concentrations (from 0 to 40 mM), and amount of protein loaded onto the column (from 25 to 50 g, i.e., from 750 to 1500 mL of mucin-free EW). As we didn't know the variable model, we chose a complete factorial design for the three quantitative factors (pH, Tris, loading) with two levels

Table 1. Matrix of Trials and Results of the Experimental Design

matrix of trials				results		
trial	pH	[Tris], mM	loading, mL	quantity, g	purity, %	recovery rate, %
1	1	1	1	8.5	87	27.4
2	1	−1	1	8.9	82	27
3	−1	1	1	9	80	26.7
4	−1	−1	1	10.7	82	32.5
5	1	1	−1	10.9	82	66.2
6	**1**	**−1**	**−1**	**11.3**	**83**	**69.5**
7	−1	1	−1	8.6	75	47.8
8	−1	−1	−1	8.4	77	47.9
9	0	0	0	8.8	81.5	35.4
10	0	0	0	10.6	84.5	44.2
11	0	0	0	9.7	82	39.3
12	0	0	0	10.7	81	42.8

level	factor 1 pH	factor 2 [Tris], mM	factor 3 loading, mL
−1	7.5	0	750
0	8.2	20	1125
1	9	40	1500

for each factor. This design was completed with four central points, necessary to test the linearity and the variability of the model. Two of these four trials were carried out at the beginning and the two others at the end of the experiment to test for any potential drift. The matrix design is presented in Table 1. Quantity, purity rate, and recovery yield were the three measurements we selected in order to characterize ovalbumin extraction. Regarding ovalbumin, the recovery yield didn't seem to be of major importance because of the low cost of the raw material. However, for some applications, obtaining a byproduct with low quantities of ovalbumin could be interesting. Purity rate of ovalbumin fractions was quantified by RP-HPLC. Results are presented in the Table 1.

For each response (quantity, purity rate, and recovery yield), an analysis of variance was carried out with the eight trials of the experimental design. The complete set of coefficients of the model, as well as standard deviations, are presented in Table 2. Only significant coefficients (Student's t test, $p < 0.05$) were taken into account. The equations of the model, obtained by analysis of variance, are presented below.

$$\text{quantity} = 9.55 \text{ g} \quad (1)$$

$$\text{purity} = 81 + 2.5\,(\text{pH}) + 1.75\,(\text{loading})\ (\%) \quad (2)$$

$$\text{recovery} = 43.1 + 4.4\,(\text{pH}) - 14.7\,(\text{loading}) - 5.6\,(\text{pH})(\text{loading})\ (\%) \quad (3)$$

In these equations, the different factors (pH, Tris, loading) can be equal to +1, 0, −1, according to the trial conducted (see Table 1).

Results indicated that Tris concentration didn't influence any responses. Mucin-free EW proteins acted as a buffer and kept the experimental conditions constant during the loading. The quantity of ovalbumin were independent of factor levels: it reached 9.55 g in our experiment, which was equivalent to 38.2 g L^{-1} of resin. Thus, the recovery yield was negatively correlated with loading and was affected by the pH level. Ovalbumin purity rate was positively correlated with pH and the volume of mucin-free egg white loaded onto the column. The purity rates range from 76.7% to 85.3%.

Table 2. Coefficients Matrix of the Equations Derived by Variance Analysis (the Standard Deviation Is Indicated in italic under Each Coefficient)

effect	quantity	purity	recovery rate
intercept	9.55	81	43.1
pH	0.35	2.5	4.4
	0.31	*0.55*	*1.39*
Tris	−0.3	0	−1.1
	0.31	*0.55*	*1.39*
loading	−0.27	1.75	−14.72
	0.31	*0.55*	*1.39*
pH ×	0.1	1	0.38
Tris	*0.31*	*0.55*	*1.39*
pH ×	−0.92	−0.75	−5.6
loading	*0.31*	*0.55*	*1.39*
Tris ×	−0.22	0.75	−0.25
loading	*0.31*	*0.55*	*1.39*
pH × Tris ×	0.22	0.75	1.18
loading	*0.31*	*0.55*	*1.39*

In a second step, the process was developed in order to increase purity. Experimental conditions were kept as simple as possible. Optimization of experimental trial no. 6 in Table 1 was chosen. It enabled an extraction without Tris, giving rise to a byproduct with low quantity of ovalbumin and one of the best ovalbumin purity rates. Elution with 470 mL of 0.14 M NaCl, which was selected after several trials (data not shown), enabled a purity rate increase to 94%. A 27 g amount of ovalbumin per liter of resin was extracted, which was equivalent to a recovery rate of 49%. In these two cases, ovalbumin quantities recovered were much higher than quantities extracted by classical gradient elution chromatography. Moreover, the elution program was simplier. The cumbersome program proposed by Vachier et al. (1995) enabled the extraction of about 2 g of ovalbumin per liter of resin. Except for the removal of ovomucin, which contributed to plugging the column, ovalbumin extraction did not need any particular precautions (large experimental field studied), indicating the possibility of larger scale utilization.

Analysis of Ovalbumin Fractions. As methods for determining purity rate are often controversial, the best way to evaluate the quality of our preparations was to compare them with ovalbumin extracted according to conventional methods. Ovalbumin (experimental trial no. 6) eluted with 0.5 M NaCl (ovaA) and ovalbumin (experimental trial no. 6) eluted with 0.14 M NaCl (ovaB) were compared to Ova, Ovc1, and Ovc2 (see Material and Methods). The latter are extensively used in research. Comparisons were made by electrophoretic (SDS−PAGE, Figure 4) and chromatographic (anion exchange, Figure 5, and reverse phase, Figure 3) methods.

RP chromatograms of Ovc1 and Ovc2 were similar (data not shown). The ovalbumin purity rate was found to be at 84% (Figure 3a), which was close to the purity rate of OvaA (83%)(Figure 3b). However, differences in contaminants were shown. On the basis of the work of Nau et al. (1999), Ovc1 and Ovc2 had ovomucoid as main contaminant (13%) with a retention time of 7.2 min, whereas OvaA had several contaminants in lower quantities (ovomucoid, ovoglobulin, cystatin, ovoflavoprotein). All these proteins have pI values close to that of ovalbumin. OvaB contained lower quantities of ovoflavoprotein and ovomucoid which were likely more strongly attached to the resin than ovalbumin. OvaB purity rate was calculated at 94% (Figure 3c), close to the Ova purity rate of 98% (Figure 3d).

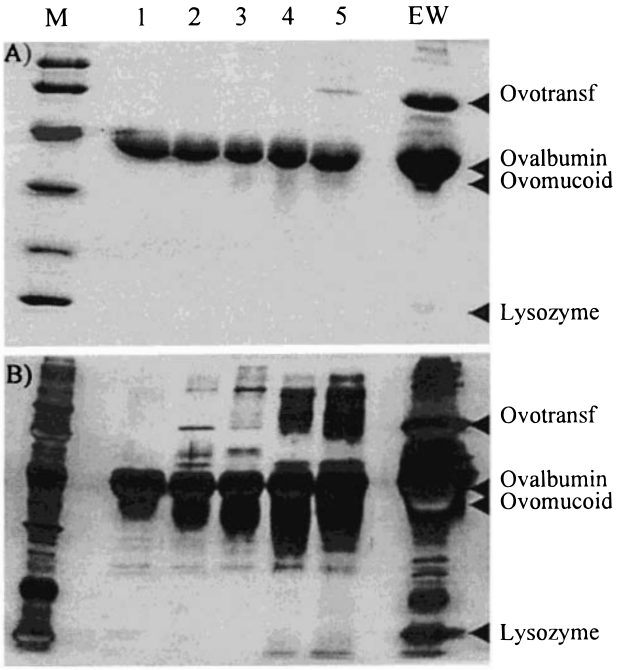

Figure 4. SDS-PAGE of ovalbumin fractions. (A) Coomassie Brillant Blue staining method. (B) Silver nitrate staining method. (M) Markers; (1) ovalbumin purified by gradient elution chromatography (Ova); (2) ovalbumin eluted using an isocratic elution program with 0.15 M NaCI (OvaB); (3) ovalbumin eluted using an isocratic elution program with 0.5 M NaCl (OvaA); (4) ovalbumin Sigma, grade V, lot 14H7035 (Ovc1); (5) ovalbumin Sigma, grade V, lot 76H7045 (Ovc2); (EW) mucin-free egg white.

Analysis of the same samples by SDS−PAGE is presented in Figure 4. Ovalbumin fractions were compared together and with the mucin-free EW. The Coomassie brilliant blue staining method was not sensitive enough to characterize impurities. On the egg white lane, lysozyme, which accounted for 4% of egg white proteins, was hardly detectable. Whatever the preparation, ovalbumin seemed to be pure, in particular due to the low ovomucoid coloration. The silver staining method (about 10-fold more sensitive than Coomassie brilliant blue) gave a more precise characterization of contaminants. Commercial ovalbumin was highly contaminated with ovomucoid as already shown by RP-HPLC. SDS−PAGE also highlighted the presence of high molecular weight contaminants, which were absent in frontal chromatography preparations and gradient elution chromatography preparation. Results showed a purity rate increase for ovalbumin in the order Ovc2 < Ovc1 < OvaA < OvaB < Ova.

IEC analysis highlighted the differences among ovalbumin preparations (Figure 5). When ovalbumin fractions extracted from frontal chromatography and classical gradient elution chromatography were analyzed on the Q-hyper D10 column, ovalbumin was eluted in one main large peak. Chromatograms differed only by the intensity of peaks on both side of the ovalbumin peak. The main ovalbumin peak in egg white and the ovalbumin peak from chromatographic extraction were superimposable. Compared with these preparations, the commercially available ovalbumin was characterized by a high heterogeneity (Figure 5a), despite the same reverse phase chromatograms. Therefore, IEC separates ovalbumin isoforms with respect of charge density. Ovalbumin is a phosphoglycoprotein, which differs in the phosphorylation and/or glycosylation states. Three

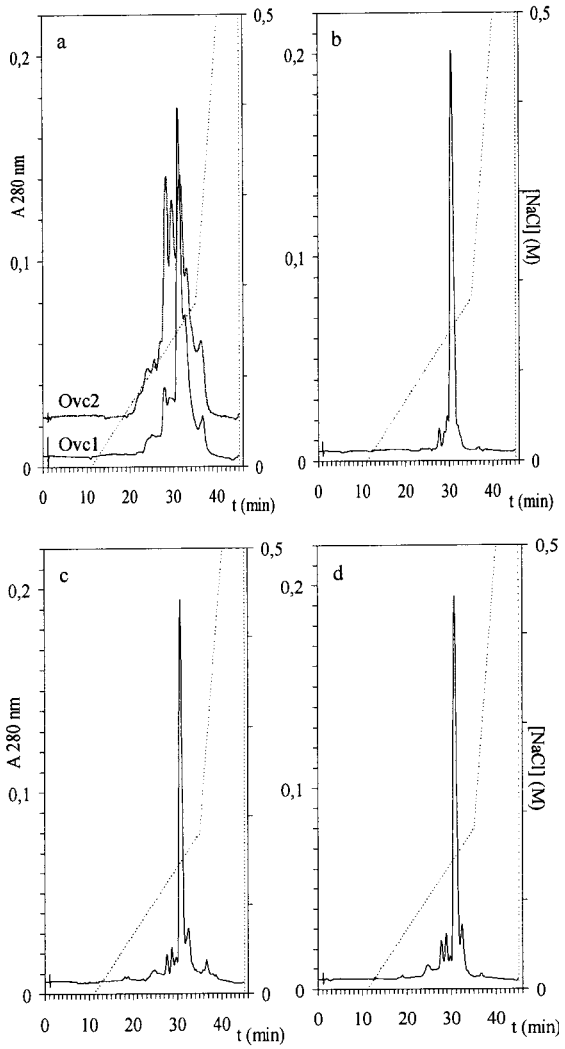

Figure 5. Anion-exchange high-performance liquid chromatograms of ovalbumin fractions. (a) Ovalbumin Sigma, grade V, lot 14H7035 (Ovc1) and 76H7045 (Ovc2); (b) ovalbumin purified by gradient elution chromatography (Ova); (c) ovalbumin eluted using an isocratic elution program with 0.5M NaCl (OvaA); (d) ovalbumin eluted using an isocratic elution program with 0.15M NaCl (OvaB).

degrees of phosphorylation A3:A2:A1, with no, one, or two phosphate groups per molecule, and the proportion 2:13:85, respectively, and several glycoforms have already been identified (Stein et al., 1991; Rago et al., 1992). IEC show that several isoforms coexisted in commercially available ovalbumin, but their relative proportion was different from that in egg white.

CONCLUSION

Presently, ovalbumin application is limited to research. The extraction procedure developed is aimed at researchers from various disciplines, including food science and biochemistry, who need large quantities of homogeneous ovalbumin.

Recent studies showed that a rapid preparation of ovalbumin without buffer is interesting. A protein mixture including 80% of ovalbumin with better functional properties than egg white was defined for a potential use in food technology (Baniel et al., 1996). Moreover, a vasorelaxing peptide isolated from a pepsin digest of ovalbumin has been identified (Fujita et al., 1995).

This method also led to a nonaltered byproduct, consequently usable. Ovalbumin extraction could, for example, be a supplementary step in the ovomucin, lysozyme, and ovotransferrin extraction in series from egg white (Guerin and Brule, 1992). Trials, conducted in our laboratory, showed that the ovalbumin extraction step could precede or follow the lysozyme extraction step. By modifying the protein proportion of mucin-free EW in the eluted fractions, displacement chromatography offered the possibility of obtaining fractions with an enrichment in one or several proteins, as shown by electrophoresis. Fractions with partial or total absence of ovalbumin could be exploited for their biological properties.

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