Batch Purification of Ovomucoid and Characterization of the Purified Product*

John Gorton Davis, Carol J. Mapes, and John W. Donovan

ABSTRACT: Commercial ovomucoid has been freed from the usual contaminants, lysozyme, ovoinhibitor, and ovalbumin, by successive batch treatment with anion and cation exchangers. Only a few hours working time are required to obtain highly purified material. Purity and homogeneity have been demonstrated by gel electrophoresis, fluorescence, sedimentation–diffusion, sedimentation equilibrium, and absence of chymotrypsin inhibition.

Although ovomucoid has been separated from egg white by a number of methods, the Lineweaver and Murray (1947) trichloroacetic acid–acetone procedure has probably been the most widely used in recent years. It is the method used commonly for commercial production (see Melamed, 1966, and Montreuil *et al.*, 1965, for reviews). Preparations of ovomucoid obtained by this method, including those from commercial sources, may contain up to 20% of other egg-white proteins as impurities. Among those identified are lysozyme (Tomimatsu *et al.*, 1966; Donovan, 1967), ovoinhibitor (Feeney *et al.*, 1963; Tomimatsu *et al.*, 1966; Ketterer's, 1965), glycoprotein (Montreuil *et al.*, 1965), ovalbumins, and probably flavoprotein (see below).

Melamed (1966) has summarized the methods used to purify Lineweaver–Murray (1947) ovomucoid. Chromatography on ion-exchange celluloses is the generally accepted procedure even though doubt has been raised concerning the stability of the sialic acid residues of ovomucoid at the low pH encountered during chromatography on carboxymethylcellulose (Rhodes *et al.*, 1960; Chatterjee and Montgomery, 1962). The method of purification described here, a batchwise treatment of ovomucoid solutions with ion exchangers, is simple, rapid, and minimizes time of exposure to low pH. In view of the charge differences of the various components of ovomucoid (Bier *et al.*, 1953; Melamed, 1966; Feeney *et al.*, 1967; Beeley, 1970), it is unlikely that ovomucoid purified by ion exchange represents exactly the complete mixture of ovomucoid components originally present in the egg white, since the fraction of some components may be enriched on purification. However, ovomucoid prepared as follows can be considered highly purified in terms of activity and in freedom from contaminants.

Materials and Methods[1]

The ovomucoid used here was Worthington OI 575-6. Ovomucoid prepared in this laboratory by ammonium sulfate fractionation (between 0.6 and 1.0 saturation) of fresh egg white has also been purified by this procedure. DEAE-cellulose (0.97 mequiv/g) and carboxylmethyl Bio-Gel CM-P-30 (6 mequiv/g) were obtained from Bio-Rad Laboratories.

Hydrodynamic measurements were made between 18 and 20° with a Beckman-Spinco Model E analytical ultracentrifuge as described previously (Donovan *et al.*, 1969). Calculations were made with the program system for boundary analysis of Trautman (1969) and Trautman *et al.* (1969) for the Underwood-Olivetti Programma 101.

Optical measurements were made with a Cary Model 15 or Beckman DB spectrophotometer, a Turner Model 210 spectrofluorometer, and a Perkin-Elmer Model 141 polarimeter, as described previously (Donovan, 1967; Donovan *et al.*, 1969). Horizontal starch gel electrophoresis was carried out by the method of Poulik (1957) as described by Garibaldi *et al.* (1968). Amino acid analyses and chymotrypsin assays were performed as described by Davis *et al.* (1969).

Ovomucoid Purification. Details of a specific experiment are given. Since both amount and nature of impurities may vary, modifications may be necessary for other preparations of ovomucoid.

Commercial ovomucoid (750 mg) was dissolved in 250 ml of 0.5% NaCl and adjusted to pH 9.4 with 1 M NH_4OH. Wet Bio-Gel CM P-30, freshly regenerated by washing with 0.1 M HCl, then with water to neutrality, was added to bring the pH to 4.5. (Carboxymethylcellulose can be added instead, but Bio-Gel CM-P-30 gave superior results.) After 1-hr stirring, the Bio-Gel was filtered off. The filtrate was adjusted to pH 3.5 with 1 M H_3PO_4. Wet DEAE-cellulose, freshly regenerated by washing with 0.1 M NaOH and then to neutrality with water, was immediately added, with stirring, to bring the pH to 4.5. Stirring was continued for 1 hr. The DEAE-cellulose was filtered off, the filtrate concentrated by ultrafiltration, dialyzed, and lyophilized.

Results and Discussion

Principle of Method. The differently charged components of ovomucoid are close to their isoionic points at pH 4.5 (Bier *et al.*, 1953), and therefore should have little affinity for either anion or cation exchangers. The pH chosen, 4.5, is somewhat higher than the isoelectric points determined for five electrophoretically separated components by Bier *et al.* (1953). In practice, if the pH was below 4.5 with CM Bio-Gel, an excessive amount of ovomucoid was lost by absorption. More acidic contaminants, particularly ovalbumin, were not completely removed unless the pH was raised to 4.5 in the DEAE-cellulose treatment.

* From the Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California 94710. *Received July 22, 1970.*

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

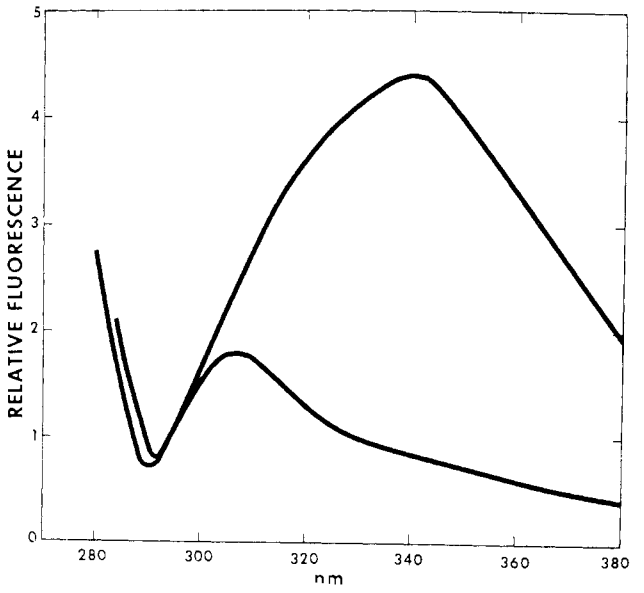

FIGURE 1: Fluorescence emission spectra for purified ovomucoid (lower curve) and for starting material (upper curve). Excitation at 278 nm, excitation and emission bandwidths 100 Å. Both samples had an absorbancy of 0.12 at 280 nm.

Yield. On a weight basis, the yield of purified ovomucoid was 75%. On the basis of absorbancy at 280 nm, only 55% of absorbing material was recovered. This indicates that the contaminants removed had a greater absorbancy at 280 nm/unit weight.

Purity of Material. The absence of tryptophan in ovomucoid (Lineweaver and Murray, 1947; Osuga and Feeney, 1968) and the low quantum yield of fluorescence from the phenolic chromophores (Teale, 1960; Donovan, 1967) suggested fluorescence as a sensitive means for detecting the presence of contaminating proteins. The pronounced difference in the spectra for commercial and purified ovomucoids is evident in Figure 1. Absorbancy of the purified ovomucoid at 278 nm is $E^{1\%}_{1\,cm}$ 4.10 and of the commercial product, $E^{1}_{1\,cm}$ 5.7. Since the solutions used for fluorescence measurements were of equal absorbancy, the protein concentration in the purified ovomucoid solution was proportionally higher. When a portion of the purified preparation was treated a second time with both DEAE-cellulose and CM Bio-Gel, there was no detectable change in the fluorescence spectrum.

TABLE I: Inhibition of Chymotrypsin by Purified Ovomucoid.[a]

Expt	Amount Tested (μg)	Slope (Division/min)	Inhibn (%)	Ovoinhibitor Content (%)
Blank		0.5		
Chymotrypsin		22.4		
control[b]		22.3		
Ovoinhibitor[c]	4.2	14.2	37.3	100
Worthington ovomucoid	200	7.2	69.4	3.9
Purified ovomucoid	250	22.2	0.8	0.04

[a] See Davis *et al.* (1969) for assay procedure. [b] 6.7 μg/assay of Worthington CDI, three-times crystallized, lot 6JF. [c] Prepared according to Davis *et al.* (1969).

TABLE II: Composition of Ovomucoid.

Component	Moles/10,000 g 20 hr	40 hr	70 hr	Best Value[b]	Residues/Mole (27,300 g)[a]
Lys	4.66	4.67	4.73	4.69	12.8
His	1.62	1.64	1.56	1.61	4.4
Arg	2.18	2.17	2.18	2.18	6.0
Asp	11.05	11.07	11.03	11.05	30.1
Thr	4.89	4.69	4.49	5.05[c]	13.8
Ser	3.93	3.72	3.34	4.21[c]	11.5
Glu	5.04	5.06	5.08	5.06	13.8
Pro	2.55	2.65	2.65	2.65[d]	7.2
Gly	5.39	5.39	5.37	5.39	14.7
Ala	3.94	3.98	3.95	3.96	10.8
½-Cys	5.68	5.32	4.92	5.99	16.3
Val	5.09	5.26	5.44	5.44[d]	14.9
Met	0.67	0.66	0.67	0.67	1.8
Ile	1.03	1.05	1.06	1.06[d]	2.9
Leu	3.93	3.97	3.95	3.96	10.8
Tyr	2.14	2.08	2.06	2.09	5.7
Phe	1.83	1.81	1.79	1.85[c]	5.1
NH_3	9.99	12.11	13.73	8.24[e]	22.5
Glucosamine	5.34	3.57	1.71	8.75[c]	23.9

[a] Molecular weight giving best approach to integral numbers of residues. [b] Averages except as otherwise noted. [c] Corrected for hydrolysis losses by extrapolation to zero time. [d] Maximum value. [e] Extrapolated to zero time. Corrected for both HCl and buffer blanks.

Ovoinhibitor is a major contaminant of Lineweaver and Murray (1947) ovomucoid (Feeney *et al.*, 1963; Tomimatsu *et al.*, 1966). It also contains no tryptophan so that fluorescence cannot be used to detect its presence. However, since ovoinhibitor inhibits chymotrypsin while ovomucoid does not (Rhodes *et al.*, 1960), assay of chymotrypsin inhibitory activity was employed to detect ovoinhibitor (Table I). The ovoinhibitor content shown for Worthington ovomucoid may be somewhat low because the inhibition found in this experiment is in the range in which the inhibition curve starts to depart from linearity (Tomimatsu *et al.*, 1966). The small amount of ovoinhibitor in the purified ovomucoid could well be zero, within experimental error.

The composition of the purified ovomucoid (Table II) is in good agreement with that reported by Osuga and Feeney (1968), with the exception of the glucosamine content. The value for glucosamine reported here was extrapolated to zero hydrolysis time, whereas that of Osuga and Feeney (1968) was not corrected for hydrolysis losses. Nitrogen content of the purified ovomucoid was 13.3%, and calculated recovery of nitrogen (Table II) was 13.7%. The agreement with the amino acid analysis reported by Kanamori and Kawabata (1969) is less good, but the presence of tryptophan in their trypsin inhibiting fractions I and II indicates their fractions were contaminated with other proteins.

TABLE III: Physical Constants of Ovomucoid.[a]

Molecular weight ($\bar{M}_w$)	
Sedimentation–diffusion[b] at 60,000 rpm	28,300 ± 3000
Sedimentation–diffusion[b] at 48,000 rpm	26,100 ± 600
Sedimentation equilibrium[c] at 16,000 rpm	27,100 ± 350
Sedimentation equilibrium[c] at 18,000 rpm	26,800 ± 1000
Sedimentation equilibrium[c] 20,000 rpm	27,000 ± 800
From amino acid analysis (Table II)	27,300
Partial specific volume, $\bar{v}$ (ml/g) (from Table II)	0.697
$s^0_{20,w}$ (S)	2.72 ± 0.06
$D_{20} \times 10^7$ (cm²/sec) at 2 mg/ml	8.28 ± 0.09
Intrinsic viscosity,[d] [η] (ml/g) (pH 3.8–4.6)	5.5 ± 0.05
Scheraga–Mandelkern parameter β	2.26 ± 0.11 × 10^6
Frictional ratio (f/f_{min})	1.33 ± 0.05
Concentration dependence parameter, g[e]	0.031
Specific extinction, $E^{1\%}_{1\,cm}$(278 nm)[d]	4.10

[a] For previous summaries of physical data, see Melamed (1966). Errors given are standard deviations, unless otherwise noted. [b] From boundary position and spreading with time (Trautman, 1969). [c] Errors are either standard deviation in slope of ln A *vs.* r^2 plot or one-half the maximum difference between molecular weight measured in H_2O and D_2O, whichever was greater. [d] From Donovan (1967). [e] $s = s^0(1 - gc)$, where c is in A_{280}/ml (see Trautman *et al.*, 1969, eq 24).

Starch gel electrophoresis of purified ovomucoid and starting material is shown in Figure 2. Lysozyme, ovoinhibitor or conalbumin, or both, and ovalbumins are evident as impurities in the starting material. A faint band in the flavoprotein position was visible in the starting material on the original gel, but did not photograph.

Physical Characterization. A summary of physical measurements is given in Table III. The optical rotatory parameters a_0 and b_0 of the Moffitt–Yang equation (Urnes and Doty, 1961) were calculated with the mean residue weight 149, obtained from the results presented in Table II. Unweighted least-squares fit to a straight line gave $a_0 = -599.8 \pm 0.8°$ and $b_0 = 63.9 \pm 1.6°$. Tomimatsu and Gaffield (1965) reported $a_0 = -524°$ and $b_0 = -86°$ for an unpurified commercial preparation of ovomucoid on the basis of a mean residue weight of 170. The a_0 and b_0 values of -410 and $-70°$ previously reported by Donovan (1967) were erroneously based on a mean residue weight of 110, the carbohydrate content not having been taken into account in that calculation.

Figure 3A shows results of a typical sedimentation equilibrium experiment. Figure 3B shows the boundary spreading during a velocity run, from which the diffusion coefficient was calculated. Both Figures 2 and 3 indicate that the purified ovomucoid appears homogeneous with respect to molecular weight. Attempts to determine $\bar{v}$ by hydrodynamic methods were frustrating. Calculations from sedimentation velocity

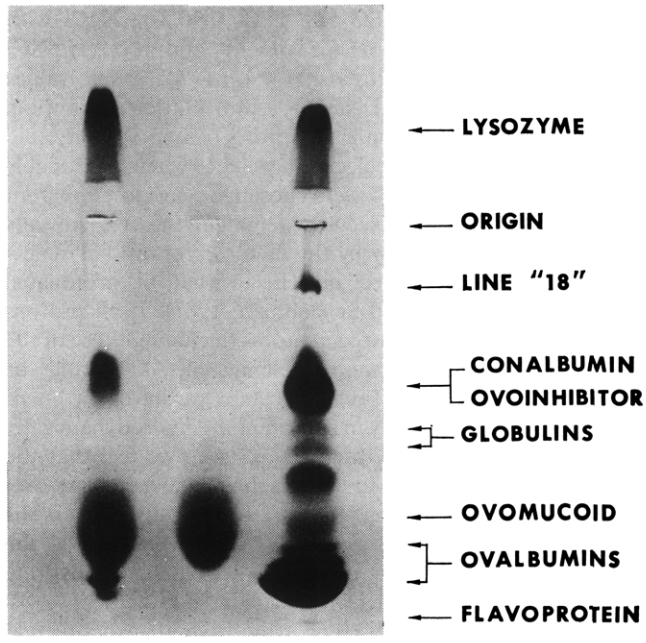

FIGURE 2: Starch gel electrophoretograms. From left to right: 4 mg of commercial ovomucoid, 4 mg of purified ovomucoid, and egg white.

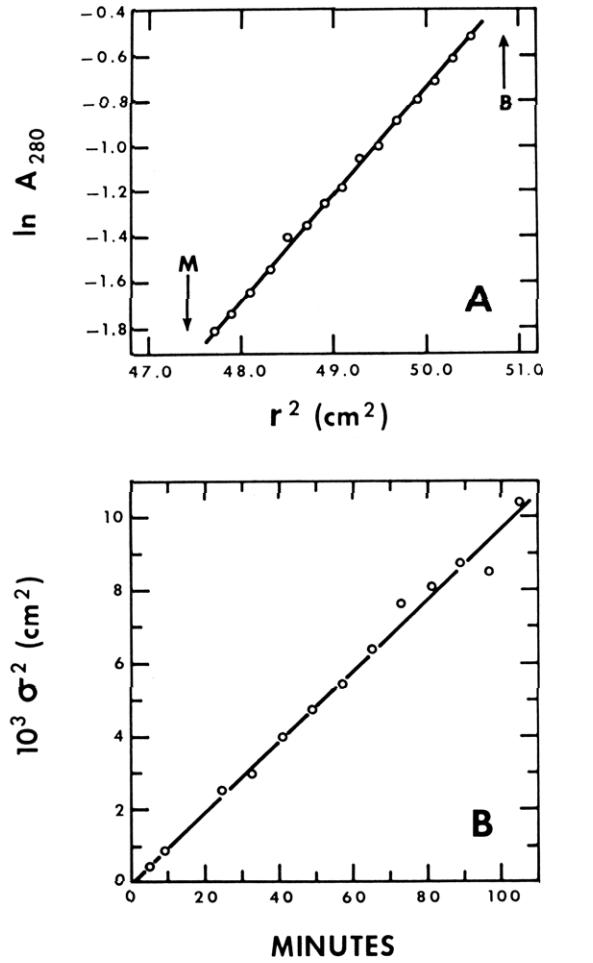

FIGURE 3: Sedimentation of purified ovomucoid. (A) Low-speed equilibrium, 16,000 rpm, 0.2 M NaCl plus 1.5% sucrose in water solution, pH 4.3 at 18.0°. M and B indicate positions of meniscus and bottom of solution, respectively. (B) Square of boundary width *vs.* time–velocity run at 48,000 rpm in 0.2 M NaCl at pH 4.3, 20.0°. Slope of line is twice the diffusion coefficient (see Trautman *et al.*, 1969, eq 28 and 29).

runs (Gagen, 1966) in H_2O and D_2O, in 0.2 M NaCl with 1.5% sucrose present at pH 6.5, pD 7.9, and in 0.2 M NaCl plus 0.01 M sodium acetate at pH 4.4, pD 4.5 gave $\bar{v}$ values in the range of 0.63–0.64. Likewise, sedimentation equilibrium runs in H_2O and D_2O, while indicative of homogeneity, did not always agree in molecular weight when reasonable values of $\bar{v}$ in H_2O and D_2O were chosen, or gave low apparent values of $\bar{v}$ when the molecular weight in the two solvents was assumed to differ only by the factor k (for ratio of molecular weight of deuterated protein to that of protonated protein, see Edelstein and Schachman, 1967). If all protons attached to oxygen and nitrogen atoms (including those of the carbohydrate residues) were exchangeable, k should be 1.0190 (calculated from Table II). Since we have not previously experienced difficulty in determining $\bar{v}$ hydrodynamically (Davis *et al.*, 1969; Donovan *et al.*, 1969), we feel that the large amount of carbohydrate attached to ovomucoid may make D_2O an "interacting" solvent, when compared to the reference solvent H_2O. Accordingly, we have employed the value of $\bar{v}$ calculated from the amino acid and carbohydrate composition, 0.697.

We have observed discrepancies beyond experimental error in values of $s^0_{20,w}$ for different purified preparations. We have reported the values of 2.72 S in Table III because several fresh preparations sedimented at this rate (±0.02 S), but have observed values as low as 2.5 S for older preparations (stored freeze-dried). The large range in sedimentation coefficients previously reported has been discussed earlier (Donovan, 1967), and may be related to presence or absence of carbohydrate side chains, or the configuration of the carbohydrate under different solvent conditions. However, the molecular weight as determined by sedimentation equilibrium appeared constant, within our limits of experimental error, and approximately equal to the molecular weight reported by Fredericq and Deutsch (1949).

Acknowledgments

We thank Dr. James C. Zahnley for chymotrypsin inhibition assays and Mrs. Mary B. Wiele for starch gel electrophoresis. Dr. Rodes Trautman of the Plum Island Animal Disease Laboratory, Agricultural Research Service, U. S. Department of Agriculture, kindly provided us with copies of his programs for sedimentation calculations. We also thank Bio-Rad Laboratories for a gift of Bio-Gel CM P-30.

References

Beeley, J. G. (1970), *Biochem. J. 117*, 70P.

Bier, M., Terminiello, L., Duke, J. A., Gibbs, P. J., and Nord, F. F. (1953), *Arch. Biochem. Biophys. 47*, 465.

Chatterjee, A. K., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 99*, 426.

Davis, J. G., Zahnley, J. C., and Donovan, J. W. (1969), *Biochemistry 8*, 2044.

Donovan, J. W. (1967), *Biochemistry 6*, 3918.

Donovan, J. W., Mapes, C. J., Davis, J. G., and Hamburg, R. D. (1969), *Biochemistry 8*, 4190.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Feeney, R. E., Osuga, D. T., and Maeda, H. (1967) *Arch. Biochem. Biophys. 119*, 124.

Feeney, R. E., Stevens, F. C., and Osuga, D. T. (1963), *J. Biol. Chem. 238*, 1415.

Fredericq, E., and Deutsch, H. F. (1949), *J. Biol. Chem. 181*, 499.

Gagen, W. L. (1966), *Biochemistry 5*, 2553.

Garibaldi, J. A., Donovan J. W., Davis, J. G., and Cimino, S. L. (1968), *J. Food Sci. 33*, 514.

Kanamori, M., and Kawabata, M. (1969), *Agr. Biol. Chem. (Tokyo) 33*, 75.

Ketterer, B. (1965), *Biochem. J. 96*, 372.

Lineweaver, H., and Murray, C. W. (1947), *J. Biol. Chem. 171*, 565.

Melamed, M. D. (1966), *in* The Glycoproteins, Gottschalk, A., Ed., Amsterdam, Elsevier, pp 317–334.

Montreuil, J., Castiglioni, B., Adam-Chosson, A., Caner, F., and Queval, J. (1965), *J. Biochem. (Tokyo) 57*, 514.

Osuga, D. T., and Feeney, R. E. (1968), *Arch. Biochem. Biophys. 124*, 560.

Poulik, M. D. (1957), *Nature (London) 180*, 1477.

Rhodes, M. B., Bennett, N., and Feeney, R. E. (1960), *J. Biol. Chem. 235*, 1686.

Teale, F. W. J. (1960), *Biochem. J. 76*, 381.

Tomimatsu, Y., Clary, J. J., and Bartulovitch, J. J. (1966), *Arch. Biochem. Biophys. 115*, 536.

Tomimatsu, Y., and Gaffield, W. (1965), *Biopolymers 3*, 509.

Trautman, R. (1969), *Ann. N. Y. Acad. Sci. 164*, 52.

Trautman, R., Spragg, S. P., and Halsall, H. B. (1969), *Anal. Biochem. 28*, 396.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.