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Isolation and Characterization of a Variant of Ovomucoid

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A simple procedure, which can be used on a preparative scale, for the isolation and purification of a major variant of ovomucoid from egg white is described. Ovomucoid was precipitated by salt, and further fractionated by chromatography on sulphoethyl-Sephadex. It showed size homogeneity as revealed by gel chromatography and sodium dodecyl sulphate–polyacrylamide-gel electrophoresis where the mobility was consistent with a molecular weight of 28300 ± 2300. The inhibitor showed full antitryptic but no antichymotryptic activity. The u.v.-absorption and fluorescence characteristics indicated the absence of tryptophan. Polyacrylamide-gel electrophoresis in the presence of 9M-urea demonstrated absence of charge heterogeneity. The intrinsic viscosity of ovomucoid was 5.36ml/g which yielded an equivalent hydrodynamic radius (2.9nm), axial ratio (6.0) and frictional ratio (1.31) of the molecule. The Stokes radius (3.5nm), diffusion coefficient ($7.8 \times 10^{-7}\,cm^2/s$) and frictional ratio (1.35) were calculated from gel-filtration data. These results suggest that ovomucoid exists in non-globular conformation under native conditions and that the deviation from the behaviour of a typical globular protein seems to be due both to asymmetry and hydration.

Ovomucoid has generally been isolated from egg white by (i) precipitation with organic solvents (Lineweaver & Murray, 1947; Fredericq & Deutsch, 1949), by (ii) ion-exchange chromatography (Rhodes *et al.*, 1958, 1960), or by a combination of the two (Beeley, 1971). A procedure consisting of successive batch treatments with cellulose anion and cation exchangers has been used in the purification of commercial ovomucoid (Davis *et al.*, 1971). An attempt has also been made (Melamed, 1967) to achieve fractionation of ovomucoid by starch-gel electrophoresis. However, the latter cannot be conveniently used on a preparative scale.

The ethanol- or acetone-precipitated ovomucoid is usually the starting material for the purification procedure described above. The organic solvents are known to disrupt, sometimes irreversibly, the native protein conformation (Singer, 1962; Herskovits & Jaillet, 1969). Further, the purified material is often heterogeneous (see Melamed, 1966) and wherever it is claimed to be pure (Davis *et al.*, 1971), more sensitive techniques such as polyacrylamide-gel electrophoresis have shown it to be otherwise (Kay *et al.*, 1974).

The present paper describes a preparative procedure which is more convenient and gave better separation than that of Beeley (1971) for the isolation and purification of a variant of ovomucoid. The crude ovomucoid was precipitated by salt instead of organic solvents and was further purified in one step (cf. Beeley, 1971) by sulphoethyl-Sephadex chromatography by using a linear salt gradient. Ovomucoid has been systematically characterized in terms of its activity, molecular weight, optical and hydrodynamic properties.

Experimental

Materials

Proteins and reagents for polyacrylamide-gel electrophoresis were the same as used previously (Ansari & Salahuddin, 1973). Papain (V.P. Chest Institute, Delhi, India) was homogeneous in the ultracentrifuge. Chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was converted into chymotrypsin by using trypsin (Sigma) by the procedure of Wilcox (1970). Carboxypeptidase A, α-*N*-benzoyl-DL-arginine *p*-nitroanilide, *N*-acetylneuraminic acid, D-mannose and D-glucosamine were from Sigma. *N*-Acetylglucosamine (Fluka A.G., Buchs, Switzerland) and D-galactose (E. Merck, Darmstadt, Germany) were used. Sulphoethyl(SE)-Sephadex C-50 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Other reagents were analytical grade.

Methods

Isolation and purification of ovomucoid. To 1 litre of egg-white homogenate, obtained from eggs of White Leghorn hens, was added an equal volume of 10% trichloroacetic acid (adjusted to pH3.5 with 1M-NaOH) (Fredericq & Deutsch, 1949). The precipitate

which was recovered by filtration contained extraneous proteins devoid of antitryptic activity. The filtrate, containing 13g of protein, was brought to 90% $(NH_4)_2SO_4$ saturation, pH4.6; 12g of protein was thus precipitated. The salt was removed by dialysis and the globulin precipitate discarded leaving 10g of 'crude ovomucoid' in solution. The latter was fractionated on an SE-Sephadex column, by using a linear salt gradient, into three ovomucoid fractions; the most 'basic' fraction was pooled and used throughout.

Determination of protein concentration. The protein concentration was determined either by Lowry's (1951) method or by the dry-weight method. In the latter an isoionic preparation of ovomucoid was prepared by passing it through a Dintzis mixed-bed ion-exchange column (Dintzis, 1952) with Dowex 50W-X8 and Dowex 1-X4 being used in appropriate cycles. The isoionic preparation was heated in pre-weighed bottles to constant weight at about 107°C and the protein concentration determined from the difference. The specific extinction coefficient, $E_{1cm}^{1\%}$, of ovomucoid was estimated by determining the absorbance on a Carl Zeiss Jena spectrophotometer, VSU 2-P, of the isoionic protein in sodium phosphate buffer, pH7.0, ionic strength 0.15 at 278nm. Fluorescence measurements were made on Aminco–Bowman spectrophotofluorimeter. pH measurements were made on an Elico pH-meter.

Measurement of antitryptic activity. Trypsin (0.56mg in phosphate buffer, pH7.0, ionic strength 0.15) was incubated with different concentrations (0.1–1.96mg) of ovomucoid in the same buffer for 5min and then 1.0mg of *α-N*-benzyl-DL-arginine *p*-n itroanilide in phosphate buffer, pH7.0, ionic strength 0.15, was added and the mixture incubated at 37°C for 20min. The total volume of assay mixture was 2.75ml. The control was prepared without ovomucoid under strictly identical conditions. The specific activity was expressed as the change in absorbance at 420nm/min per mg of the enzyme. The antichymotryptic activity was determined by incubating 160μg of chymotrypsin in sodium phosphate buffer, pH7.5, ionic strength 0.15, with 180μg of ovomucoid in the same buffer (molar ratio, 1:1) for 15min. To this was added 360μg of acid-denatured haemoglobin which was prepared by titrating freshly isolated sheep haemoglobin to pH2.0 with 1M-HCl and keeping the solution for 12h. The assay mixture (1.2ml) was incubated for 1h at 37°C. The degraded products in the trichloroacetic acid soluble supernatant were determined by Lowry's (1951) method.

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out essentially by the method of Davis (1964); ovomucoid solution containing glycerol was applied directly instead of adding the protein to the large-pore gel. Both the buffer and the gel contained 9M-urea in experiments on polyacrylamide-gel electrophoresis of ovomucoid in the presence of 9M-urea (Parish & Marchalonis, 1970). For sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, the procedure described earlier (Weber & Osborn, 1969; Ansari & Salahuddin, 1973) was followed and the relative mobility, R_m, of the marker proteins was calculated by dividing the mobility of the marker protein by that of cytochrome *c*.

Viscosity measurements. The intrinsic viscosity of ovomucoid was determined in sodium acetate buffer, pH4.7, ionic strength 0.15, by the procedure described by Ahmad & Salahuddin (1974), by using a calibrated Cannon–Fenske viscometer with a flow time for distilled water at 25°C of 400s. The intrinsic viscosity, $[\eta]$, was calculated by the equation (Tanford, 1955),

$$[\eta] = \lim_{c \to o} \frac{(\eta - \eta_0)}{\eta_0 c} = \lim_{c \to o} \frac{(t - t_0)}{t_0 c} + \frac{(1 - \bar{v}_2 \rho_0)}{\rho_0} \quad (1)$$

where c is the protein concentration in g/ml, η_0 and η refer to the viscosities of solvent and that of solution; $\bar{v}_2$ is the partial specific volume and was taken to be 0.697ml/g (Davis *et al.*, 1971). The density of the solvent, ρ_0, was determined to be 1.0003g/ml. The time of fall of solvent, t_0, and that of protein solution, t, were measured at 25±0.05°C.

Analytical methods

Hexose. The total neutral hexose content was determined by Winzler's orcinol–H_2SO_4 method as used by Francois *et al.* (1962).

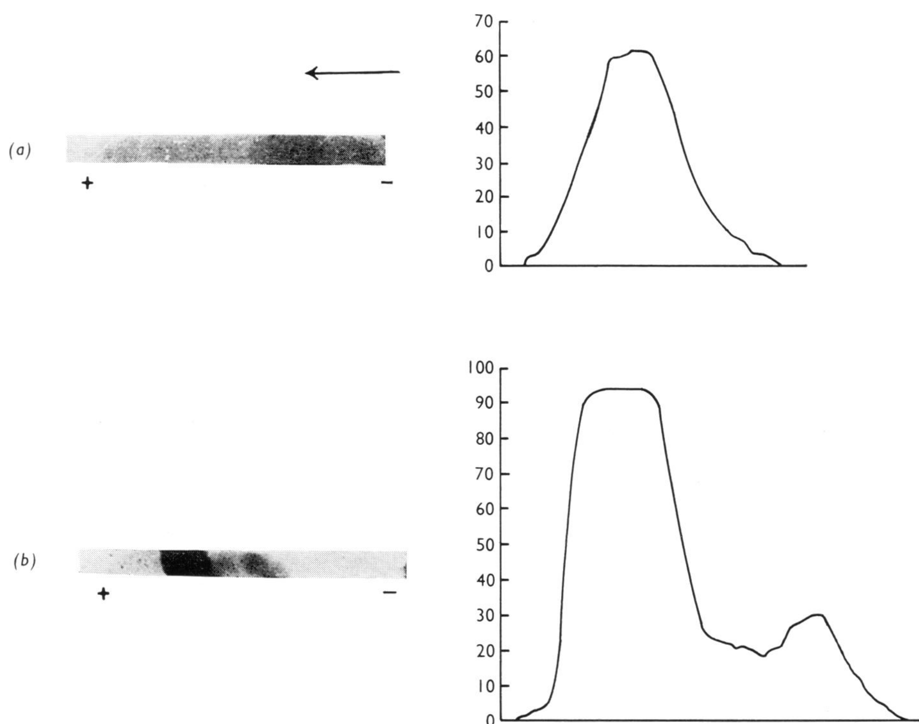
Glucosamine and *sialic acid.* These were released by treating ovomucoid with 5.5M-HCl at 100°C for 4h and with 0.1M-H_2SO_4 at 80°C for 30min respectively. The contents of glucosamine and sialic acid were then determined respectively by the methods of Reissig *et al.* (1955) and Svennerholm (1957).

Mannose galactose ratio. This was determined by the method of Eylar & Jeanloz (1962).

Acetyl groups. The method of Duncombe (1963) was used, with *N*-acetylglucosamine as standard. Acetyl groups were released by acid hydrolysis of ovomucoid with 2M-HCl at 100°C per 2h.

N-Terminal residue. Ovomucoid was dansylated (Gray, 1967) and the dansyl derivative of the amino acid was identified by silica-gel t.l.c. in benzene–acetic acid–pyridine (80:20:2, by vol.) as the solvent system.

C-terminal residue. The *C*-terminal residue of ovomucoid was identified by the standard procedure (Ambler, 1967). However, ovomucoid was first fully reduced and alkylated and then treated with 6M-urea for 24h. The denatured ovomucoid was treated with carboxypeptidase A as described by Narita (1970). The released amino acid residues were identified by dansylation and by subsequent t.l.c.



EXPLANATION OF PLATE I

Electrophoresis of ovomucoid in polyacrylamide gel

The polyacrylamide gel contained 7% (w/v) acrylamide, 0.5% (v/v) *NNN′N′*-tetramethylethylenediamine, 0.1% (w/v) ammonium persulphate and 0.18% *NN′*-methylenebisacrylamide. About 100μg of the protein in glycerol was applied on the gel tubes (0.5cm × 7cm) and electrophoresis performed for 30min with a current of 8mA/gel. The gels were stained in Amido Schwartz and destained in aq. 7.5% (v/v) acetic acid. The letters represent electrophoresis at ionic strength 0.012 and under different conditions: (*a*) acetate buffer, pH5.5; 9M-urea; (*b*) acetate buffer, pH5.5. The gels obtained under conditions (*a*) and (*b*) were scanned on a Photovolt Densicord.

Results

Chromatography of the salt-precipitated ovomucoid on SE-Sephadex C-50 column in sodium acetate buffer, pH4.6, by using a gradient of ionic strength from 0.01 to 0.21 gave three major peaks at 0.01, 0.04 and 0.15 mol/litre as shown in Fig. 1; one minor peak and two minor shoulders are also detectable. About 70% of the total protein which was applied to the column showed antitryptic activity and was recovered in the three peaks. The remaining 30% proteins were devoid of antitryptic activity and were eluted with higher ionic strength or pH. The protein under the peak III was the major one forming about 50% of total ovomucoid. Obviously this variant of ovomucoid was most 'basic'. It should be recalled that Beeley (1971) using SE-Sephadex column chromatography without salt gradient, could only fractionate ethanol-precipitated ovomucoid into two fractions, I and II. The relative concentrations of the two fractions were roughly calculated from the chromatographic profile (*a*) of Fig. 1 obtained by Beeley (1971) and were 43% (fraction I) and 57% (fraction II) respectively. On further chromatography of fraction II on DEAE-cellulose column, Beeley (1971) found two peaks and a hardly visible shoulder corresponding to fractions IIRA, IIRB and IIRC. As we shall see below, it is the last fraction (IIRC) which is identical with our preparation in chemical properties.

Polyacrylamide-gel electrophoresis of the ovomucoid preparation gave one major and one minor band containing 97 and 3% of the protein respectively, in sodium acetate buffer, pH5.5 (see Plate 1). Only one band was obtained in 9M-urea, pH5.5, and the mobility was about 14% of that of the major band obtained in aqueous buffer (see Plate 1). The preparation of ovomucoid gave a single symmetrical peak on a column of Sephadex G-100 and a single band on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

The inhibition of trypsin was determined at different molar ratios of enzyme/inhibitor and at a molar ratio of 1:1 it was found to be 96% (see Table 1). Ovomucoid did not show any antichymotryptic activity. In contrast, the salt-precipitated ovomucoid obtained in this study as well as Calbiochem (Los Angeles, Calif., U.S.A.) ovomucoid (lot 000142) produced 44% inhibition in the chymotryptic activity. The percentage inhibition of chymotrypsin with Sigma ovomucoid (lot 27B-8500) was only 30%.

The results of the chemical analysis of ovomucoid are summarized in Table 1. The data of Beeley (1971) for the fraction IIRC have been included for comparison. The contents of glucosamine, hexose, mannose, sialic acid and galactose of our preparation are identical, within experimental error, with those found by Beeley (1971) for fraction IIRC. Similarly, *N*-terminal residue, tyrosine content and the number of basic nitrogens (which is equal to the sum of α-amino, histidine, lysine and arginine residues of the protein) are also the same for the two preparations. The number of acetyl residues, hitherto uninvestigated on a pure preparation, was 16 per molecule.

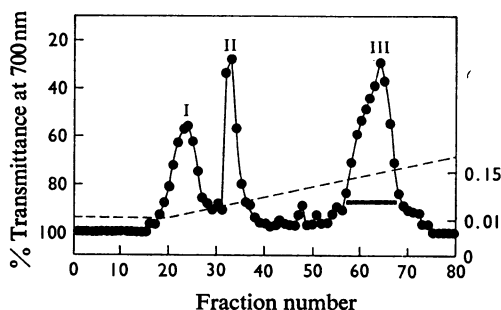


Fig. 1. *Chromatography of the salt-precipitated ovomucoid*

About 1 g of crude ovomucoid in 25 ml was applied to an SE-Sephadex C-50 column (3.8 cm × 51–26 cm) which was previously equilibrated with acetate buffer, pH4.6, ionic strength 0.012. The proteins were eluted in 15 ml fractions with a flow rate of 40 ml/h, with the same buffer by using a linear salt gradient from 0.012 to 0.212 mol/litre (----). The fractions under the thick line were used throughout.

Table 1. *Chemical characterization of ovomucoid*

The values in column 1 were calculated by using the data of Beeley (1971) and a molecular weight of 28300.

	Content (mol/mol)	
Component	IIRC fraction of Beeley (1971)	This study
Glucosamine	15.0	15.0
Hexose	12.2	12.0
Mannose	11.3	10.0
Sialic acid	0.03	0.05
Galactose	0.84	1.0
Acetyl residues	—	16
N-Terminal residue	Alanine	Alanine
C-Terminal residue	—	Phenylalanine
Total basic nitrogens	22	22†
Tryptophan	0	0
Tyrosine	5	5†
Trypsin inhibition (%)	94*	96

* Percentage inhibition calculated relative to the inhibitory activity of the most active fraction, IB (Beeley, 1971).

† Results taken from Waheed & Salahuddin (1974).

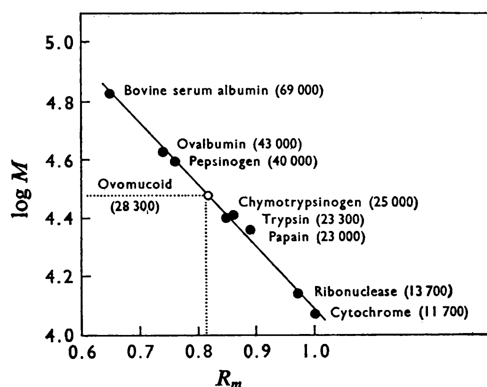


Fig. 2. *Calibration curve for sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of proteins*

Proteins were reduced and denatured in 1% sodium dodecyl sulphate and 0.1M-2-mercaptoethanol and then taken with a few drops of glycerol and Bromophenol Blue. After incubation for 10min at 105°C and overnight at room temperature, about 50μg of protein was applied to a polyacrylamide gel (7%, 0.5cm×7cm) containing 0.5% sodium dodecyl sulphate, and electrophoresed in sodium phosphate buffer, pH7.0, ionic strength 0.007, for 1h with a current of 3mA/gel; the gels were stained with Coomassie Blue and destained mechanically. Molecular weight of a protein is given in parenthesis. The linear plot between log M and R_m was obtained by the method of least-squares and fits the equation:

$$\log M = 6.233 - 2.146 R_m$$

The C-terminal residue of ovomucoid was identified to be phenylalanine which was demonstrated previously in other ovomucoid preparations by chemical methods (see Melamed, 1966). It is noteworthy that native ovomucoid is refractory to carboxypeptidase attack (see Melamed, 1966) presumably owing to the inaccessibility of the C-terminal residue. The latter became exposed by treating carboxymethylated ovomucoid with 6M-urea for 24h and was therefore available for the enzymic attack. Carboxypeptidase is not inactivated by 6M-urea (see Narita, 1970).

The u.v.-absorption spectrum of ovomucoid in phosphate buffer, pH7.0, ionic strength 0.15, showed a minimum at 255nm and two maxima at 278 and 280nm. The specific extinction coefficient, $E_{1\,\mathrm{cm}}^{1\%}$, at 278nm, in the same buffer was determined to be 5.13cm^2·g^{-1} which decreased to 5.10 at 280nm. The fluorescence spectra of ovomucoid solution showed an excitation maximum at 278nm and an emission maximum at 309nm. Similar spectral studies were also done with ribonuclease A which is devoid of tryptophan. Both the proteins showed qualitatively similar features in u.v.-absorbance and in fluorescence spectra. These results suggest absence of tryptophan in our preparation. It is noteworthy that fluorescence measurements, indicating absence of tryptophan, have been used by Davis *et al.* (1971) in establishing the purity of their ovomucoid preparation.

From the data obtained by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Fig. 2) it is clear that the plot of logM against R_m is linear over the entire range of molecular weight, 11700–68000. The relative mobility, R_m, of ovomucoid was determined to be 0.83 which would correspond to a molecular weight of 28300. The maximum experimental uncertainty in the estimation of R_m was 2% which would introduce an error of 8% in the determination of molecular weight.

The intrinsic viscosity of ovomucoid preparation in acetate buffer, pH4.7, ionic strength 0.15 was 5.36ml/g at 25±0.05°C (see Table 2). This is comparable with the value 5.5±0.05ml/g reported by Donovan (1967) for his ovomucoid preparation but is higher than that (4.7ml/g) computed from the reduced viscosity for the Lineweaver & Murray (1947) preparation of ovomucoid. The equivalent hydrodynamic radius, R_e, axial ratio, a/b and frictional ratio, f/f_0, for ovomucoid were computed from viscosity data by using the relations (Tanford, 1961):

$$[\eta] = \nu(\bar{v}_2 + \Sigma m_i \bar{v}_i^\circ) \quad (2)$$

$$R_e^3 = 3M[\eta]/10\pi N \quad (3)$$

where ν is the shape factor and m_i is the weight of solvent components with partial specific volume $\bar{v}_i^\circ$ bound to unit weight of dry protein, N is Avogadro's number and M is the molecular weight. If ovomucoid is globular ν will be 2.5 and $\Sigma m_i \bar{v}_i^\circ$ from eqn. (2) will be 1.45ml. Assuming that water is the only component that was bound to ovomucoid, this calculation suggests that 1g of ovomucoid binds 1.45g of water. This hydration is too high even for a markedly hydrophilic glycoprotein such as ovomucoid. On the other hand, in the absence of solvation, eqn. (2) would yield a value of 7.7 for ν indicating high asymmetry. By using 0.2 for $\Sigma m_i \bar{v}_i^\circ$ (Tanford, 1961), the shape factor reduces to 7.1 which would correspond to an axial ratio of 6 and to a frictional ratio of 1.31. The latter agrees well with that (1.33±0.05) calculated from sedimentation-diffusion data by Davis *et al.* (1971). This overestimation (1.5%) in frictional ratio is trivial and even if it is real, it can very well be ascribed to the use of a lower value for the molecular weight (27300) in their calculation (Davis *et al.*, 1971), since a decrease in molecular weight would slightly decrease the f_0 and increase the axial ratio. The value of R_e for ovomucoid was calculated from $[\eta]$ by using eqn. (3) and was 2.9nm (see Table 2).

Table 2. *Physical constants of ovomucoid*

The proteins used, with their Stokes radii in parentheses, were cytochrome *c* (1.64nm), ribonuclease (1.92nm), chymotrypsinogen A (2.24nm), ovalbumin (2.73nm) and bovine serum albumin (3.55nm).

	Range of values for ovomucoid	This study
Molecular weight	27000–32600†‡	28300 ± 2300
10^7 × Diffusion coefficient (cm^2/s)	6.01–8.28†‡	7.8
Intrinsic viscosity	5.50 ± 0.05	5.36
Equivalent hydrodynamic radius (nm)	—	2.9*
Axial ratio	6.3–14†	5.98*
Frictional ratio	1.33‡	1.31*, 1.35§
Stokes radius (nm)	—	3.15
Specific extinction coefficient, $E_{1cm}^{1\%}$ at 280nm	4.13–6.1†‡	5.10

* Computed from the viscosity data.
† See Melamed (1966).
‡ See Davis *et al.* (1971).
§ Calculated from the gel-filtration data obtained on Sephadex G-100 prepared and calibrated by Ansari *et al.* (1975).

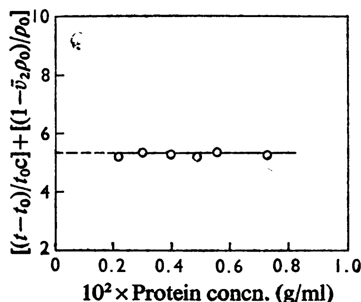


Fig. 3. *Concentration dependence of the reduced viscosity of ovomucoid*

The straight line was obtained by the method of least-squares in the protein concentration range, 0.2–0.8g/100ml.

The elution volume of ovomucoid was determined on the calibrated (Ansari *et al.*, 1975) column (70.5cm × 2cm) of Sephadex G-100 equilibrated and eluted with phosphate buffer, pH7.0, ionic strength 0.15, and was 143.5ml. Analysis of the gel-filtration data by the method of Ackers (1970) gave a Stokes radius, r, of 3.15±0.05nm corresponding to the elution volume of 143.5ml. The diffusion coefficient, D, and the frictional ratio, f/f_0, corresponding to this value of r were calculated by using eqns. (4) and (5).

$$D = kT/6\pi\eta r \quad (4)$$

$$f/f_0 = r/(3\bar{v}_2M/4\pi N)^{\frac{1}{3}} \quad (5)$$

where k is the Boltzmann constant. The values of D and f/f_0 for ovomucoid thus calculated are listed in Table 2.

Discussion

The procedure involving the salt precipitation and subsequent linear salt gradient chromatography on an SE-Sephadex column which was used in the isolation and purification of a sialic acid-deficient ovomucoid is simple and can be used on a preparative scale. The purity of the preparation seems to be better than that achieved previously. The two procedures recently used in the isolation of ovomucoid are batchwise purification of a commercial sample by using cellulose ion-exchangers (Davis *et al.*, 1971) and fractionation on an SE-Sephadex C-50 column without salt gradient (Beeley, 1971). The purity of the former has been checked by starch-gel electrophoresis where the protein band was not sharp (Davis *et al.*, 1971). This preparation has been shown by polyacrylamide-gel electrophoresis to contain about 2.0% ovalbumin (Kay *et al.*, 1974). Beeley (1971) obtained only two fractions, compared with at least three found in this study, by chromatography of ethanol-precipitated ovomucoid on an SE-Sephadex C-50 column. The poor resolution in Beeley's experiment (1971) may be due to (i) the lack of salt gradient and/or (ii) the choice of eluting buffer of pH4.95; optimal conditions for better resolutions would be a pH close to the isoelectric point (4.41) and lower ionic strength.

The ovomucoid preparation was homogeneous with respect to size as is evident from its electrophoretic (in sodium dodecyl sulphate) and gel-filtration behaviour. The presence of two bands in aqueous buffer and one band in 9M-urea, detected on polyacrylamide-gel electrophoresis, suggested minor conformational heterogeneity. The movement of ovomucoid in a single band in 9M-urea indicated the absence of charge heterogeneity. It is hard to attribute this conformational

heterogeneity to difference in sialic acid content, for the latter is barely detectable in our ovomucoid preparation. Such conformational heterogeneity has been observed in concanavalin A and has been ascribed to the difference in carbohydrate composition (Pflumm *et al.*, 1971). A similar explanation may be given for the observed behaviour of ovomucoid in polyacrylamide gel.

The antitryptic activity of our ovomucoid preparation was comparable with or even better than those of earlier preparations (Green, 1953; Beeley, 1971). It was devoid of antichymotryptic activity which is usually associated with ovomucoid (Weil & Timasheff, 1960) and is due to contamination by ovoinhibitor (Feeney *et al.*, 1963).

The *N*-terminal and *C*-terminal residues of ovomucoid were found to be the same, i.e. alanine and phenylalanine respectively, as reported for earlier preparations (Osuga & Feeney, 1968; Penasse *et al.*, 1952). The carbohydrate composition, the total number of basic nitrogen groups, tyrosine and tryptophan contents, and the percentage inhibition of our preparation were the same as those for fraction IIRC of Beeley (1971).

The hydrodynamic results presented in Table 2 provide information about the molecular morphology of ovomucoid. Evidently, intrinsic viscosities obtained for this preparation as well as for others were far greater than the 3–4ml/g expected for globular proteins. Other parameters such as equivalent hydrodynamic radius, diffusion coefficient, axial ratio, frictional ratio and Stokes radius were not consistent with a globular protein of molecular weight 28300. This deviation may be due to marked asymmetry and hydration of the ovomucoid molecule.

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