The Isolation of Ovomucoid Variants Differing in Carbohydrate Composition

By J. G. BEELEY

Department of Biochemistry, University of Glasgow, Glasgow W.2, U.K.

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Three major and two minor species of ovomucoid were separated by chromatography on sulphoethyl-Sephadex. The predominant sialic acid-free species was further resolved into three fractions by DEAE-cellulose chromatography. Although all species of ovomucoid had closely similar trypsin-inhibiting activity, immunochemical properties and amino acid composition, they differ in carbohydrate composition. Wide variation was observed in the content of galactose, *N*-acetylglucosamine and sialic acid. Charge heterogeneity was related, in part, to variation in sialic acid content. The implications of variable carbohydrate composition for the structure and function of ovomucoid are discussed.

Ovomucoid is a glycoprotein trypsin-inhibitor obtained from hen's-egg white. The ready availability and substantial carbohydrate content of ovomucoid have made it the subject of several structural studies (Monsigny, Adam-Chosson & Montreuil, 1968; Montgomery & Wu, 1963; Neuberger & Papkoff, 1963; Bragg & Hough, 1961; Gottschalk & Ada, 1956; Dixon, 1955; Stacey & Woolley, 1940).

Like many other glycoproteins ovomucoid can be resolved into several closely related components by electrophoresis (Fredericq & Deutsch, 1949; Bier, Terminiello, Duke, Gibbs & Nord, 1953; Wise, Ketterer & Hansen, 1964; Beeley & Jevons, 1965; Melamed, 1967) and by isoelectric focusing (Beeley, 1969, 1971). The differences between these species, although residing partly in the sialic acid content (Beeley & Jevons, 1965; Melamed, 1967), have not been fully established. This imposes restrictions on the interpretation of data obtained from experiments made on the whole protein.

A number of chromatographic fractionation procedures have been described for ovomucoid (Feeney, Stevens & Osuga, 1963; Chatterjee & Montgomery, 1962; Beeley & Jevons, 1965; Feeney, Osuga & Maeda, 1967). However, none of these preparations has been shown to consist of a single electrophoretic species. The isolation of two ovomucoid species by starch-gel electrophoresis has been reported by Melamed (1967). This method was applied to small quantities of material and the products were characterized only in terms of sialic acid content.

This paper describes the separation by chromatography on a preparative scale of three major and two minor ovomucoid components differing in isoelectric

point. These species have been characterized by analysis of their protein and carbohydrate composition. It has also been demonstrated that a fraction that appeared to be homogeneous in isoelectric point was heterogeneous in its carbohydrate moiety.

A preliminary account of some of this work has been presented (Beeley, 1969).

EXPERIMENTAL

Preparation of ovomucoid. Eggs were obtained from a flock of White Leghorn hens maintained in an animal house. The eggs were stored under refrigeration and used within 48 h of laying. Ovomucoid was prepared from separated egg white by the method of Fredericq & Deutsch (1949) up to the stage of the first ethanol precipitation. The precipitated protein was taken up in water, adjusted to pH 4.6 with acetic acid (1 M), dialysed, centrifuged and the supernatant freeze-dried.

Sulphoethyl-Sephadex chromatography. Further purification and fractionation of ovomucoid was carried out on sulphoethyl (SE)-Sephadex C-50 (Pharmacia, Uppsala, Sweden). Columns (82 cm × 5 cm) of SE-Sephadex were equilibrated at room temperature with 0.014 M-sodium acetate containing 1 mM-Na₂SO₄ and adjusted either to pH 4.95 or pH 4.85 with acetic acid. These buffers were made up in batches of 10 litres to ensure that several chromatography runs could be carried out with identical buffer. The columns were washed at a flow rate of 40 ml/h, controlled by a peristaltic pump regulating the outflow, until the pH of the eluted buffer was indistinguishable from that of the applied buffer. After reaching a stable pH the columns were washed with a further 1 litre of buffer before the sample was applied. Samples of ovomucoid (1–3 g) were dissolved in water (10 ml) and dialysed overnight against the appropriate buffer. After application of a

sample to the column elution was carried out with the buffer with which sample and column had been equilibrated. Fractions (15 ml) were collected and the E_{280} was read.

The pH4.95 system was employed for preliminary separation of ovomucoid into sialic acid-containing and sialic acid-free fractions. Chromatography at pH 4.85 was used to resolve the sialic acid-containing components.

Columns (38 cm $\times$ 2.5 cm) of Whatman DE32 DEAE-cellulose were equilibrated with 0.02 M-sodium phosphate buffer, adjusted with NaOH to pH 6.7. After application of the sample, equilibrated with starting buffer by dialysis, elution was carried out with a linear gradient generated from two similar reservoirs connected by a siphon. These vessels contained 0.02 M-sodium phosphate, pH 6.7 (500 ml), and 0.02 M-sodium phosphate containing 0.12 M-NaCl, pH 6.7 (500 ml). A flow rate of 21 ml/h was maintained by a peristaltic pump and the fraction size was 9.5 ml. Chromatography on Whatman DE32 was performed at 4°C.

Pooled fractions were adjusted to pH 4.6 with 0.1 M-acetic acid and dialysed against repeated changes of water.

Isoelectric focusing. Details of the procedure for isoelectric focusing were as described by Beeley (1971).

Analytical methods

Carbohydrates. Glucosamine hydrochloride, galactose, mannose and *N*-acetylneuraminic acid were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Arabinol, dulcitol and mannitol were purchased from British Drug Houses Ltd., Poole, Dorset, U.K.

Hexose. This was determined by an orcinol method (Hartley & Jevons, 1962) with mannose as standard. Mannose and galactose gave the same colour yield. The orcinol was recrystallized from benzene before use.

Glucosamine. This was determined by the method of Cessi & Piliego (1960) and additional analyses were performed in some cases by the method of Gatt & Berman (1966). Before analysis samples were hydrolysed for 3 h in 4 M-HCl at 100°C and the acid was removed on a rotary evaporator at 40°C.

Sialic acid. This was measured, after release by hydrolysis in 0.05 M-H₂SO₄ at 80°C for 1 h, by the method of Aminoff (1961).

Mannose/galactose ratio. The proportions of mannose and galactose in glycoprotein samples were determined by g.l.c. of the corresponding alditol acetates. Hydrolysis of samples (2 mg) and preparation of derivatives was carried out by the method of Lenhardt & Winzler (1968). Samples (1–5 μ l) of the alditol acetates were separated by chromatography on 3% ECNSS-M on Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa., U.S.A.) packed into coiled stainless-steel columns (10 ft $\times$ 0.125 in outside diam.) (Sawardeker & Sloneker, 1965). A Perkin-Elmer FII gas chromatograph with flame-ionization detector was used with an oven temperature of 195°C and nitrogen carrier flow rate of 20 ml/min. The peak areas were traced and the tracings cut out and weighed. Mannose/galactose ratios were determined from the ratio of the weights of the mannitol and dulcitol peaks divided by the ratio of weight obtained by chromatographing a standard mixture containing equal amounts of mannitol

and dulcitol. No peaks other than those corresponding to mannitol and dulcitol were observed.

Amino acid analysis. Samples were hydrolysed for 22 h in evacuated sealed tubes at 110°C in 6 M-HCl at a glycoprotein concentration of 0.1% (w/v). An internal standard of norleucine was employed so that correction could be made for transfer losses.

Analyses were performed with a Locarte amino acid analyser on the 50 cm $\times$ 0.9 cm column of Locarte amino acid analyser resin.

With this system a peak corresponding in elution time to mesocystine was observed in addition to the cystine peak. Analytical values for cystine were determined after oxidation to cysteic acid (Hirs, 1967) and a correction was applied for incomplete (90%) conversion of cystine into cysteic acid.

N-Terminal groups. The method used was that of Gray (1967). DNS derivatives were identified by paper electrophoresis.

Trypsin inhibition. This was measured with Hammarsten casein (British Drug Houses Ltd.) as substrate by the method of Northrop, Kunitz & Herriott (1948).

Immunodiffusion. Rabbit antiserum to ovomucoid, purified to the ethanol-precipitation stage, was obtained by an immunization regimen involving subcutaneous, intramuscular and intraperitoneal injection with Freund's adjuvant, followed by intravenous injection without adjuvant. Of three rabbits immunized two gave antisera that formed sharp precipitin arcs with antigen. Immunodiffusion of ovomucoid fractions, at concentrations from 0.1 to 2% (w/v) in 0.85% NaCl, was carried out in borate-saline or phosphate-saline-agar by the method of Ouchterlony (1958). Precipitin arcs obtained by immunodiffusion of ovomucoid fractions (2%, w/v, in saline) were compared with arcs obtained from ovoglycoprotein, prepared by the method of Ketterer (1965) at concentrations from 0.01 to 0.1% (w/v) in saline.

Protein concentration. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) by using standards of ovomucoid fraction IIR of known moisture content. Moisture content was determined by drying to constant weight at 110°C.

RESULTS

Chromatography of ovomucoid on SE-Sephadex with buffers of constant composition was used to separate differently charged species of the glycoprotein. Satisfactory resolution was obtained only in the range pH 4–5 and at low ionic strength. Similar conditions are required for the observation of electrophoretic heterogeneity (Bier *et al.* 1953).

Although ovomucoid could be fractionated in a single chromatography run this led to broadening of later peaks and for this reason two separate chromatographic systems were employed. Fig. 1(a) shows the initial separation of ovomucoid into fractions I and II with the pH4.95 buffer. Rechromatography of fraction II in the same system resulted in a single symmetrical peak (IIR), which emerged at the expected elution volume. Further fractionation of fraction I (Fig. 1b) was carried out

Fig. 1. Chromatography of ovomucoid on SE-Sephadex C-50. Columns (82 cm $\times$ 5 cm) were eluted with buffers of constant composition containing 0.014 M-sodium acetate and 1 mM- NaN_3 , adjusted to pH 4.85 or pH 4.95 with acetic acid. Fractions (15 ml) were collected at a flow rate of 40 ml/h. (a) Preliminary fractionation of ovomucoid at pH 4.95 into sialic acid-enriched (I) and -deficient (II) components. Fraction II was rechromatographed in the same system to give fraction IIR. (b) First rechromatography of fraction I at pH 4.85. The ovomucoid species IA, IB and IC (but not ID) were rechromatographed in this system until symmetrical peaks were obtained.

Fig. 2. Rechromatography of sialic acid-deficient ovomucoid (IIR) on a DEAE-cellulose column (38 cm $\times$ 2.5 cm) in 0.02 M-sodium phosphate buffer, pH 6.7, with a linear gradient of NaCl. Fractions (9.5 ml) were collected at a flow rate of 21 ml/h.

by chromatography at pH 4.85. The central portions of the three peaks (IA, IB, IC) were rechromatographed until they appeared symmetrical.

Further fractionation of fraction IIR was obtained by chromatography on DEAE-cellulose with

a shallow linear gradient of sodium chloride concentration (Fig. 2). Rechromatography of fractions IIRA and IIRB under identical conditions resulted in peaks emerging at the same elution volumes as on initial chromatography.

The result of isoelectric focusing of ovomucoid fractions is shown in Fig. 3. Peaks separated by chromatography on SE-Sephadex correspond to ovomucoid variants with different isoelectric points. Fractions IIR and IC gave single isoelectric bands. In addition to the major component in fraction IB, small amounts of a more acidic and a more basic species were observed. Fraction IA contained two major components and a small amount of a more basic species. Both fraction ID and IIR had indistinguishable isoelectric points. Fractionation of fraction IIR on DEAE-cellulose (see Fig. 2) resulted in resolution of chromatographically distinct components IIRA, IIRB and IIRC, which were all, however, indistinguishable from fraction IIR on isoelectric focusing. Thus several ovomucoid species separated by chromatography (ID, IIRA, IIRB, IIRC) had no apparent difference in net charge.

Immunodiffusion was used to monitor the purity of ovomucoid. Antisera were prepared against ethanol-precipitated ovomucoid before chromatography. Before chromatography the ethanol-precipitated preparation showed a broad precipitin arc characteristic of ovomucoid itself and, in addition, minor arcs: one corresponded to ovalbumin and the other weaker arc was continuous with the major component in ovoglycoprotein.

After fractionation by SE-Sephadex chromatography all ovomucoid variants gave a continuous precipitin arc, indicating their antigenic identity (Fig. 4). No ovalbumin was detected in any of the fractions. When high concentrations (2%, w/v) of chromatographed ovomucoid fractions were employed all were found to contain trace quantities of ovoglycoprotein. However, by visual comparison of the density and distance moved by precipitin arcs at a series of ovoglycoprotein concentrations the extent of contamination was found to be less than 3% for fraction IA and less than 1% for all other fractions.

Analysis of the *N*-terminal groups of ovomucoid fractions I and II showed the presence of a spot corresponding in electrophoretic mobility to alanine in both fractions. No *N*-terminal threonine, the end group of ovoglycoprotein reported by Ketterer (1965), was detected.

Homogeneity was also examined by measurement of trypsin inhibition. This was identical within the limits of experimental error for all fractions (Table 1).

Values obtained from the amino acid analysis of ovomucoid fractions separated on SE-Sephadex are shown in Table 1. The results for fraction IIR

Fig. 3. Gel isoelectric focusing of ovomucoid variants. The conditions are indicated in the text. Samples were: (a) unfractionated ovomucoid, (b) ovomucoid fraction IIR, (c) fraction IC, (d) fraction IB and (e) fraction IA. Fractions IA, IB and IC had been rechromatographed as indicated in Fig. 1(b).

Fig. 4. Immunodiffusion of differently charged ovomucoid variants. Samples of ovomucoid (1%, w/v, in saline) were allowed to diffuse in borate-agar against rabbit antiserum to unfractionated ovomucoid. Samples were: (a) ovomucoid fraction IA, (b) fraction IB, (c) fraction IC, (d) fraction ID, (e) fraction IIR and (f) unfractionated ovomucoid.

were calculated on the basis of a molecular weight of 27 000 (Rhodes, Bennett & Feeney, 1960; Fredericq & Deutsch, 1949). The protein portions of the fractions were very similar if not identical in composition. The agreement between values for half-cystine, determined from the sum of cystine and mesocystine measured in hydrolysates of fractions that had not been oxidized with performic acid, and values for cysteic acid suggest that little destruction of cystine occurred under the conditions of hydrolysis used.

The results of carbohydrate analyses (Table 2) show significant variations in sialic acid content between ovomucoid species. Although fraction II contained less than 0.1 mol of sialic acid per 27 000g the fraction with lowest isoelectric point (IA) contained 1.95 mol. However, the lack of stoichiometry suggests that variation in sialic acid content alone may not entirely account for the charge heterogeneity of ovomucoid. For instance, component IC contains only 0.6 residue of sialic acid and differs from component IB by 0.6 residue of sialic acid. Thus the sialic acid content of these species is insufficient to lead to a difference of one charge per molecule.

The galactose content of ovomucoid fractions was found to decrease in the same order as sialic acid content. This variation in galactose content (1–6 residues/molecule) accounted for most of the variation in total hexose content between the differently charged species (IA–ID), mannose content being relatively constant (10–13 residues/molecule).

The glucosamine contents of fractions IA, IB, IC and ID were all closely similar or identical, falling within the range 22–25 residues. However, fractionation of the sialic acid-free component indicated a considerably wider distribution of values (14–27 residues).

DISCUSSION

The purity of the ovomucoid variants isolated by SE-Sephadex chromatography was demonstrated by the absence of other egg-white proteins on gel isoelectric focusing, the constant trypsin-inhibiting activity of all fractions and the presence of a single *N*-terminal group. Application of the sensitive technique of immunodiffusion confirmed that the protein was essentially homogeneous. The only impurity detected was ovoglycoprotein (Ketterer, 1965), which was present in amounts too small

Table 1. *Amino acid analyses and relative trypsin-inhibiting activity of ovomucoid variants of different charge*

Amino acid results are the mean of two analyses of 22 h hydrolysates and are not corrected for destruction. The composition of fraction IIR is expressed as mol of residues/27 000 g. For the purpose of comparison, analyses of other variants are expressed as ratio of residues to 10 residues of leucine. Half-cystine was determined, after oxidation, as cysteic acid: values given in parentheses were obtained from measurements of both cystine and mesocystine in the acid hydrolysates performed without performic acid oxidation. Trypsin inhibition was measured as described in the text. Values are expressed as percentages relative to the inhibitory activity of fraction IIR.

Component	Composition				
	IA	IB	IC	ID	IIR
Asp	25.0	25.0	27.3	27.3	27.2
Thr	11.7	12.0	12.6	12.9	12.4
Ser	9.4	9.4	9.9	9.1	9.9
Glu	13.2	13.0	13.1	13.3	13.0
Pro	6.5	6.3	6.5	6.2	5.9
Gly	13.2	13.0	13.7	13.8	13.5
Ala	10.3	10.1	10.1	10.5	10.0
Cys (half)	17.3 (16.9)	18.1 (17.4)	18.2 (18.5)	18.4 (18.8)	18.6 (19.0)
Val	12.1	12.7	13.3	13.5	13.0
Met	1.7	1.9	1.6	1.9	1.6
Ile	2.8	2.7	2.7	2.6	2.6
Leu	10.0	10.0	10.0	10.0	10.0
Tyr	4.7	4.9	5.1	4.9	5.2
Phe	4.4	4.4	4.4	4.5	4.6
His	4.0	4.2	4.1	4.1	3.9
Arg	4.6	4.6	4.6	4.2	4.7
Lys	11.1	11.2	11.2	11.2	11.7
Trypsin inhibition (%)	104	106	98	99	100

(<3% in fraction IA, <1% in all other fractions) to influence analytical results.

To facilitate discussion the charged species of ovomucoid separated by isoelectric focusing will be referred to as O₁, O₂, O₃, O₄ and O₅, in order of decreasing isoelectric point. These probably correspond to the five species with isoelectric points of 4.41, 4.28, 4.17, 4.01 and 3.83 separated by zone electrophoresis (Bier *et al.* 1953), although this identity has not been verified experimentally.

Electrophoretic heterogeneity of ovomucoid (Fredericq & Deutsch, 1949; Melamed, 1967) and of other glycoproteins (Schmidt, Kamyama, Pfister, Takahashi & Binette, 1962; Oshiro & Eylar, 1968) is detectable only under conditions of low ionic strength and at pH values close to the isoelectric point. This report demonstrates that chromatography on SE-Sephadex under similar conditions of pH and ionic strength leads to the separation of charged variants of ovomucoid on a preparative scale. This technique may well be applicable to other glycoproteins.

The charged variants of ovomucoid separated by

SE-Sephadex chromatography were O₁ (fractions ID, IIR, IIRA, IIRB and IIRC), O₂ (fraction IC), O₃ (fraction IB) and a mixture of O₄ and O₅ (fraction IA). The quantity of these components decreased in the order O₁ > O₂ > O₃ > O₄ > O₅. This was indicated both by the relative intensity of staining after gel electrofocusing and by the areas under chromatographic peaks.

Amino acid analyses showed that the protein moiety of all species separated must be closely similar, although minor variation in amino acid sequence cannot be completely ruled out as a possible source of heterogeneity. The immunological identity of all the ovomucoid fractions confirms their very close structural similarity. However, the analytical results demonstrated clear differences in carbohydrate composition.

Although the major and most basic ovomucoid species, O₁, was essentially free of sialic acid, the content of sialic acid in the other charged variants was found to increase with decreasing isoelectric point (Table 2). However, the difference in amount of sialic acid between charged species was too low to

Table 2. Carbohydrate composition of ovomucoid variants

Mannose and galactose contents were calculated from the hexose content and the mannose/galactose ratio.

Fraction	Glucosamine content (g/100 g)	Hexose content (g/100 g)	N-Acetyl-neuraminic acid content (g/100 g)	Ratio		Content (mol of residues/27 000 g)				
				GlcN	Man	Hexose	Man	Gal	N-Acetyl-neuraminic acid	
IA	15.9	10.5	2.23	1.51	1.6	15.8	9.7	6.1	1.95	
IB	14.9	10.6	1.32	1.41	2.8	15.9	11.8	4.1	1.15	
IC	14.4	9.3	0.65	1.55	3.4	14.0	10.8	3.2	0.57	
ID	14.5	8.9	0.18	1.62	4.4	13.4	10.9	2.5	0.16	
IIR	14.3	8.3	0.10	1.72	5.1	12.5	10.5	2.0	0.09	
IIRA	17.7	9.6	0.04	1.84	7.9	14.5	12.9	1.6	0.03	
IIRB	14.5	7.9	0.03	1.86	4.3	11.8	9.6	2.2	0.02	
IIRC	9.5	7.8	0.04	1.23	13.5	11.6	10.8	0.8	0.03	

account for a difference of one charge per mol. It has been observed by moving-boundary electrophoresis (Beeley & Jevons, 1965) and starch-gel electrophoresis (Melamed, 1967) that removal of sialic acid from ovomucoid with neuraminidase leads to disappearance of variants O₅, O₄ and O₃ whereas components with the mobilities of variants O₂ and O₁ remain. This observation has been confirmed by using isoelectric focusing (Beeley, 1971). Melamed (1967) has reported that an electrophoretically isolated species corresponding to variant O₂ was made up of two components, one of which contained sialic acid and could be converted by neuraminidase treatment into a component with the mobility of variant O₁. Thus the lack of stoichiometry in the sialic acid content may reflect the existence of two ovomucoid variants differing by a single charge on some residue other than sialic acid.

Although fractionation of ovomucoid variants on SE-Sephadex was related to differences in isoelectric point, fractionation of ovomucoid variant O₁ on DEAE-cellulose was strongly influenced by the neutral carbohydrate composition of ovomucoid species. The content of mannose and glucosamine was similar for the various differently charged species of ovomucoid. However, DEAE-cellulose fractionation of sialic acid-free ovomucoid revealed that variation occurred in the content of glucosamine (14–27 residues/molecule). The homogeneity of the other charged species was not examined by further chromatography but it is possible that they were similarly heterogeneous. The occurrence of neutral carbohydrate heterogeneity in conjunction with heterogeneity of charge explains the difficulties encountered in previous attempts to obtain electrophoretically homogeneous ovomucoid by chromatographic methods.

Monsigny *et al.* (1968) concluded, on the basis of the carbohydrate composition of glycopeptides isolated from digests of ovomucoid prepared as described by Fredericq & Deutsch (1949), that the sialic acid content and variability in carbohydrate composition arise from contamination with ovoglycoprotein. However, a preliminary report (Jakubczak & Montreuil, 1970) from the same laboratory indicates, in agreement with the findings reported here, that the ovoglycoprotein concentration was much too low (1–2%) to account for the observed heterogeneity of glycopeptides. Further, these authors reported the separation of ovomucoid into several electrophoretically distinct fractions, which varied in glucosamine, mannose, galactose and sialic acid content but not in amino acid composition. Although different fractionation methods were employed it appears that their recent results are generally consistent with those reported here.

The origin of glycoprotein heterogeneity may be

in the incomplete specificity of biosynthetic enzymes (Roseman, 1966; Gottschalk, 1969). Other possibilities include degradation by glycosidases or the secretion of some glycoprotein molecules before their carbohydrate groups have been completed. However, direct evidence allowing a distinction to be made between these possibilities is not presently available. It appears that the heterogeneity of charge, at least, exists in ovomucoid before its secretion, as isoelectric focusing of ovomucoid extracted from oviduct by an insoluble trypsin derivative gave a similar pattern to ovomucoid from egg white (Beeley, 1970). At least two other glycoproteins synthesized in the magnum of hen oviduct, ovalbumin (Cunningham, Ford & Rainey, 1965) and ovoinhibitor (Davis, Zahnley & Donovan, 1969), have been shown to be heterogeneous in carbohydrate contents.

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