Turtle-Dove Ovomucoid, a Glycoprotein Proteinase Inhibitor with P₁-Blood-Group Antigen Activity

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Ovomucoid from the egg white of turtle-dove (*Streptopelia risoria*) was purified and shown to be a glycoprotein of mol.wt. 29400, with valine as *N*-terminal residue. It is an inhibitor of both trypsin and chymotrypsin, but has a lower affinity for trypsin than has hen ovomucoid. Turtle-dove ovomucoid contains antigenic activity cross-reacting with the blood-group-P₁ antigen of human erythrocytes. Hen ovomucoid has no detectable blood group-P₁ activity. The carbohydrate composition of turtle-dove ovomucoid differs from hen ovomucoid in having substantially higher galactose content. The possible relationship between carbohydrate composition and antigenic activity is discussed.

The glycoprotein component of hen's-egg white not coagulated by heat was termed 'ovomucoid' by Morner (1894). Lineweaver & Murray (1947) identified ovomucoid as the substance responsible for the anti-tryptic activity of egg white and described a preparative method based on the solubility of ovomucoid in 10% (w/v) trichloroacetic acid. Further purification has been obtained by ion-exchange chromatography on CM-cellulose (Rhodes *et al.*, 1960; Chatterjee & Montgomery, 1962), DEAE-cellulose (Feeney *et al.*, 1967) and SE (sulphoethyl)-Sephadex (Beeley, 1971a).

Although purified ovomucoid was found to be homogeneous in size, it was shown to be electrophoretically heterogeneous, containing components with isoelectric points in the range 3.83–4.41 (Bier *et al.*, 1953). The heterogeneity is partly due to variation in the sialic acid content of the protein (Beeley, 1971b). After neuraminidase treatment the number of ovomucoid components observed on gel isoelectric focusing was decreased from five to two, suggesting further heterogeneity at the level of the polypeptide chain (Beeley, 1976a).

Ovomucoids isolated from several avian species (Rhodes *et al.*, 1960) were shown to be similar in chemical behaviour, all having acidic isoelectric points and containing no tryptophan residues. However, major differences were observed in proteinase-inhibition activity, and it was proposed that ovomucoids might be classified according to whether they inhibited trypsin, chymotrypsin or both proteinases. Sequence studies on ovomucoids have revealed internal homologies (Beeley, 1974; Kato *et al.*, 1975), indicating the presence of three domains within the molecule. Inhibition sites for

different proteinases may be located within separate domains (Kato *et al.*, 1975). Certain of the carbohydrate side chains of ovomucoid are located on homologous internal sequences in different domains (Beeley, 1976b).

Immunological investigations at the University of Liège of antigens in the blood of several avian species indicated that a substance cross-reacting with the human blood-group glycolipid P₁ antigen was present in the blood of pigeon (Radermecker *et al.*, 1975) and budgerigar, but absent from the erythrocytes of chicken, duck, goose and turkey (Brocteur *et al.*, 1975). This substance was shown to occur not only in the erythrocyte membrane but also, in soluble form, in plasma and in extracts of pigeon droppings (Brocteur *et al.*, 1975).

The present paper reports the isolation and physicochemical characterization, of turtle-dove ovomucoid, which is shown to have marked P₁-antigen-like activity and differs in both protein and carbohydrate moieties from hen ovomucoid.

Experimental

Materials

Chymotrypsin and carboxypeptidase B were obtained from Worthington (Freehold, NJ, U.S.A.); trypsin was from Boehringer (Mannheim, W. Germany); carboxypeptidase A was from Miles Laboratories (Lausanne, Switzerland) and neuraminidase (*Vibrio cholerae*) from Boehringerwerke (Marburg/Lahn, W. Germany). Ampholines were purchased from LKB (Stockholm, Sweden).

Purification of ovomucoid

Turtle-dove (*Streptopelia risoria*) eggs, obtained from the issue of a single pair of birds, were collected

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

daily and kept in a cold-room for a maximum of 8 days. The egg whites were pooled and frozen at -32°C until they were fractionated.

Ovomucoid was prepared by the method of Lineweaver & Murray (1947). To 1 vol. of turtle-dove egg white was added 1 vol. of 0.5M-trichloroacetic acid/acetone (1:2, v/v) at 4°C . The precipitate was discarded after centrifugation at 5000 g for 20 min. Ovomucoid was then precipitated by adding 2.5 vol. of acetone. The precipitate was suspended in water, dialysed against distilled water and then freeze-dried. Further purification was carried out by chromatography on DEAE-cellulose (Feeney *et al.*, 1967). Crude ovomucoid (200mg) was dialysed overnight against phosphate buffer (0.02M- NaH_2PO_4 adjusted to pH 6.5 with NaOH). An ion-exchange column was washed and equilibrated with the same buffer. Elution was performed initially with a gradient made by mixing 250ml of 0.02M-phosphate buffer and 250ml of 0.02M-phosphate buffer containing 0.1M-NaCl. A second gradient prepared from 100ml of 0.02M-phosphate buffer containing 0.1M-NaCl and 100ml of 0.02M-phosphate buffer containing 0.2M-NaCl was then applied. The column was finally washed with 300ml of 0.02M-phosphate buffer, pH 6.5, containing 0.2M-NaCl.

Isoelectric focusing

The technique used was based on the method of Huriaux & Focant (1978). Gels were prepared by mixing 3.75g of acrylamide, 0.125g of bisacrylamide and 24g of urea in 50ml of water. Ampholine pH 3.5–10 (1.25ml of 40%, w/v, solution) was added with 0.29ml of a 10% (w/v) ammonium persulphate solution. The gel was poured into a vertical Plexiglas box (18cm $\times$ 8cm $\times$ 0.2cm). The electrode compartments contain 0.2% (v/v) H_2SO_4 (anode, lower reservoir) and 0.4% (v/v) ethanalamine (cathode, upper reservoir) respectively. After a pre-run of 45 min at 100 V, protein samples (1–3mg) dissolved in 8M-urea (25–100 μl) containing 1% Ampholine were applied on the upper surface of the gel with a Hamilton syringe. Samples were covered with a solution of 4M-urea containing 1% (w/v) Ampholine. Electrophoresis was conducted for 16h at room temperature (22°C). The initial current of 10mA fell to 2–3mA at the end of the run. The gel was dipped for 2h in 0.2% Coomassie Brilliant Blue G-250, dissolved in water/ethanol/acetic acid (10:9:1, by vol.) and destained with the same solvent. As the bands disappeared on prolonged destaining, photographs were taken after no more than 6h.

Measurement of proteinase inhibition

Tryptic-activity measurements were made in the presence of different amounts of inhibitor by the method of Waheed & Salahuddin (1975).

Typical assays were as follows: 0.4nmol of trypsin dissolved in 50 μl of 1M-HCl was incubated for 5min with various amounts of ovomucoid dissolved in 0.05M-Tris buffer adjusted to pH 8 with HCl and containing 0.01M- CaCl_2 . The inhibitor/enzyme (I/E) ratio (w/w) was varied from 0.2 to 2. The substrate, *N*-benzoyl-L-arginine ethyl ester (1.5mmol in 3ml of 0.05M-Tris/HCl buffer, pH 8, containing 0.01M- CaCl_2), was then added and the A_{253} measured at 60s intervals. The total volume in the cell was always 3.25ml.

Parallel experiments were carried out with hen and turtle-dove ovomucoid.

Anti-chymotryptic activity of turtle-dove ovomucoid was measured by the same experimental procedure. Chymotrypsin (0.2nmol in 50 μl of 1M-HCl) was incubated for 5min with increasing amounts of turtle-dove ovomucoid (I/E was varied from 0.2 to 4). *N*-Acetyl-L-tyrosine ethyl ester (2.4nmol in 3ml of 0.05M-phosphate buffer, pH 7) was added and the change in A_{237} followed as a function of time.

Neuraminidase treatment

Ovomucoid (16mg) was dissolved in 500 μl of 0.05M-sodium acetate buffer adjusted to pH 5.5 with NaOH containing 0.15M-NaCl and 0.007M- CaCl_2 . Neuraminidase (10 units/mg of ovomucoid) was added and the mixture was incubated at 37°C for 24h. The liberation of sialic acid was followed as a function of time by analysis of samples by the method of Aminoff (1961).

U.v. spectra

The absorption spectra of the purified ovomucoid dissolved at a concentration of 0.12% (w/v) in water and at a concentration of 0.06% (w/v) in 0.1M-NaOH were recorded with a double-beam spectrophotometer (Hitachi Perkin Elmer, model 124). The protein concentration was determined from the dry weight.

The absorption coefficient $A_{278}^{1\%, 1\text{cm}}$ was deduced from the spectrum. The tyrosine content of turtle-dove ovomucoid was calculated from the spectrum obtained at alkaline pH by assuming a molar absorption coefficient of $2340\text{M}^{-1}\cdot\text{cm}^{-1}$ (Fromageot & Schnek, 1950).

Ultracentrifugal analysis

Sedimentation-equilibrium analysis was carried out in a Spinco model E ultracentrifuge at 20°C under the conditions described previously (Gerday & Teuwis, 1972). Turtle-dove ovomucoid solution (0.5%, w/v) was dialysed against 0.1M-NaCl/0.05M-Tris/HCl, pH 8.5.

Analytical methods

Hexose was determined by a phenol/H₂SO₄ method (Dubois *et al.*, 1956) by using an equimolar mixture of mannose and galactose as standard and individual monosaccharides were assayed as described by Chambers & Clamp (1971).

Sialic acid was measured (Aminoff, 1961) after hydrolysis with 0.05M-H₂SO₄ at 80°C for 1h. *N*-Acetylneuraminic acid was used as standard.

Glucosamine was determined by amino acid analysis after hydrolysis of the glycoprotein with 6M-HCl at 110°C for 24, 48 and 72h. The results were extrapolated to zero time.

The *N*-terminal residue of ovomucoid was determined by the method of Gray (1972), and dansyl derivatives of the amino acids were identified as described by Woods & Wang (1967).

Identification of the C-terminal residue was attempted by hydrazinolysis (Fraenkel-Conrat & Tsung, 1967) and by carboxypeptidase digestion.

Amino acid analysis was carried out after hydrolysis with 6M-HCl for 24, 48 and 72h at 110°C in evacuated sealed tubes. Protein concentration in samples was about 0.1% (w/v). Analyses were made by the method of Benson & Patterson (1965) with a Beckman 120B amino acid analyser.

Haemagglutination-inhibition assay of blood-group-P₁ antigenic activity

Human erythrocytes of group OP₁ were obtained from normal blood donors. Goat anti-(blood-group-P₁) serum was a gift from Professor O. Prokop (Berlin, E. Germany).

Washed erythrocytes suspended in iso-osmotic buffer (Isoton II; Coulter Electronics) were incubated with antiserum in the presence of a series of dilutions of hen or turtle-dove ovomucoid. Haemagglutination was measured by using a Coulter model B automatic cell counter. Percentage haemagglutination was plotted as a function of the ovomucoid dilution on a semilogarithmic scale, and the concentration producing 50% inhibition was obtained by interpolation.

Results

Extraction and purification

The acetone/trichloroacetic acid precipitation makes it possible to obtain a crude extract containing about 600mg of ovomucoid from 100ml of egg white.

The chromatography on DEAE-cellulose shown in Fig. 1 gave a partial resolution of acidic and less-acidic ovomucoid components. The four fractions pooled, I, II, III and C, were examined by isoelectric focusing on polyacrylamide gels (Fig. 2): pool I,

Fig. 1. DEAE-cellulose chromatography of turtle-dove ovomucoid

Turtle-dove ovomucoid (180mg) was applied to a DEAE-cellulose column (2.5cm x 30cm) equilibrated with 0.02M-NaH₂PO₄ buffer adjusted to pH 6.5 with NaOH. Fractions (5ml) were collected at a flow rate of 25ml/h and were monitored for their *A*₂₁₅ (—) and conductivity (---). Elution was carried out with a gradient of ionic strength as described in the text. Fractions (I, II, III and C) were pooled as indicated by the bars.

Fig. 2. Polyacrylamide-gel isoelectric focusing of turtle-dove ovomucoid

The samples were: (1) the crude extract obtained after trichloroacetic acid/acetone precipitation; (2) ovomucoid fraction I; (3) fraction II; (4) fraction III; (5) a fraction containing a more basic contaminant (all isolated from DEAE-cellulose); (6) the products of digestion of the pooled fractions I, II and III with neuraminidase.

for example, is enriched in the three more basic components, whereas pools II and III contain more acidic ones. The crude extract contains contaminants with basic isoelectric points. Impurities present in the crude extract probably include lysozyme, ovoinhibitor and ovalbumin, as in hen ovomucoid preparations (Feeney *et al.*, 1967).

U.v. spectra

The u.v.-absorption spectra of the three main fractions obtained from the DEAE-cellulose chromatography were recorded at protein concentrations

close to 0.1% (w/v). The values of the absorption coefficient at 280nm of 1% solutions were close to each other and gave the following results for three different samples of fraction I: $A_{280\text{nm}}^{1\%} = 7.55, 7.97$ and 7.95cm^{-1} .

Neuraminidase treatment

The amount of sialic acid liberated after a 10h digestion was $13\mu\text{g}/\text{mg}$ of turtle-dove ovomucoid. This represented 98% of the sialic acid released by acid hydrolysis.

The product of digestion was subjected to isoelectric focusing (Fig. 2). Release of sialic acid decreases the number of turtle-dove ovomucoid components to two.

Proteinase inhibition

The differences between the inhibitory power of hen and turtle-dove ovomucoid towards trypsin and chymotrypsin are illustrated in Fig. 3.

Turtle-dove ovomucoid inhibits both trypsin and chymotrypsin to some extent; these inhibitory effects differ from what is observed for hen ovomucoid, which has no effect on chymotrypsin, but which completely inhibits trypsin.

During the progress of assays for the free trypsin remaining after addition of increasing concentrations of inhibitors the amount of substrate hydrolysed increased with time, giving, with hen ovomucoid, a

series of straight lines having decreasing slopes. With turtle-dove ovomucoid the progress curves were sigmoid in shape, initial inhibition being followed by a progressive increase in the rate of hydrolysis. This suggests that inhibitor is displaced from the enzyme-inhibitor complex by substrate, as the reaction proceeds. With chymotrypsin as inhibitor, sigmoid reaction-progress curves were also obtained.

Ultracentrifugal analysis

Sedimentation-equilibrium analysis was performed on fraction II from DEAE-cellulose (Fig. 1), illustrating the degree of homogeneity of the protein. The apparent molecular weight deduced is 29400.

Terminal amino acids

The identification by polyamide t.l.c. of the dansyl-amino acid liberated by hydrolysis of the dansylated protein gave only one spot, corresponding to valine, regardless of the ovomucoid fraction treated.

It was not possible to identify the C-terminal amino acid residue of native ovomucoid by an enzymic method or by hydrazinolysis of the performic acid-oxidized protein.

Amino acid analysis

Table 1 summarizes the amino acid, glucosamine, neutral hexose and sialic acid content of turtle-dove ovomucoid.

Blood-group- P_1 antigenic activity

All fractions of turtle-dove ovomucoid showed strong blood-group- P_1 antigenic activity in the haemagglutination-inhibition assay (Table 2), with the highest activity occurring in fraction I. In the same assay system hen ovomucoid showed no inhibition at a concentration 500–1000 times that required for 50% inhibition of agglutination by turtle-dove ovomucoid fractions.

Discussion

Precipitation of other egg-white proteins with a trichloroacetic acid/acetone mixture was useful as a first step in the isolation of turtle-dove ovomucoid. Subsequent ion-exchange chromatography on DEAE-cellulose (Fig. 1) removed other contaminating proteins. The purity of turtle-dove ovomucoid obtained from DEAE-cellulose was demonstrated by the absence of any visible contaminating material

Fig. 3. Proteinase-inhibition activity of hen and turtle-dove ovomucoids

The percentage of residual proteinase activity is expressed as a function of the inhibitor/enzyme ratio for hen ovomucoid with trypsin (■) or chymotrypsin (□) and turtle-dove ovomucoid with trypsin (●) or chymotrypsin (○).

Table 1. *Amino acid compositions of the three ovomucoid fractions obtained after chromatography on DEAE-cellulose (Fig. 1)*
The results are expressed as residues per 29400 mol.wt. Numbers in parentheses indicate the probable integral number of residues per molecule.

Amino acid	Content (residues/molecule)			
	Turtle-dove ovomucoid			Hen ovomucoid†
	Fraction I	Fraction II	Fraction III	
Lys	6.1 (6)	6.3 (6)	5.4 (5)	13
His	3.0 (3)	3.2 (3)	3.4 (3)	4
Arg	13.9 (14)	14.2 (14)	13.8 (14)	6
Asp	30.8 (31)	31.8 (32)	32.2 (32)	29
Thr	15.4 (15)	16.9 (17)	16.3 (16)	13
Ser	14.1 (14)	14.3 (14)	13.8 (14)	11
Glu	18.1 (18)	17.6 (18)	17.8 (18)	14
Pro	6.3 (6)	7.1 (7)	6.7 (7)	7
Gly	14.9 (15)	15.2 (15)	15.6 (16)	15
Ala	7.7 (8)	6.6 (7)	6.7 (7)	11
‡Cys*	17.2 (17)	17.9 (18)	19.4 (19)	16
Val	13.6 (14)	12.8 (13)	13.4 (13)	16
Met	1.4 (1)	1.4 (1)	1.5 (2)	2
Ile	4.4 (4)	3.8 (4)	3.6 (4)	3
Leu	14.6 (15)	14.8 (15)	14.6 (15)	11
Tyr	10.6 (11)	11.0 (11)	10.3 (10)	6
Phe	3.0 (3)	3.0 (3)	2.6 (3)	5
GlcNH ₂	23.2 (23)	20.5 (21)	20.4 (20)	24
Glc‡	0.9	0.9	0.9	
Man	8.2 (8)	8.1 (8)	7.3 (7)	11
Gal	21.2 (21)	17.0 (17)	12.3 (12)	2
Sialic acid	1.1 (1)	1.6 (2)	2.0 (2)	0§

* Determined as cysteic acid.

† Beeley (1976a).

‡ The glucose present in these samples may have arisen from the chromatographic media used in purification.

§ Sialic acid varies between 0 and 2 residues/molecule depending on the fraction (Beeley, 1971a).

Table 2. *Blood-group-P₁ antigenic activity of turtle-dove and hen ovomucoids*

Haemagglutination inhibition assays were performed as described in the text. No inhibition was detected with hen ovomucoid at a concentration of 0.4mg/ml.

Antigen	Antigen concentration (ng/ml) producing 50% agglutination inhibition
Turtle-dove ovomucoid	
Fraction I	320
Fraction II	420
Fraction III	800
Hen ovomucoid	No inhibition

on isoelectric focusing in polyacrylamide gels (Fig. 2).

As with hen ovomucoid, turtle-dove ovomucoid contains five main components, having different isoelectric points which were partially separated on

the DEAE-cellulose column. On isoelectric focusing turtle-dove ovomucoid has a lower range of pI values than hen's.

As has been described (Beeley, 1971b) for hen ovomucoid, neuraminidase treatment removes around 100% of the original sialic acid present in turtle-dove ovomucoid. This treatment decreases the number of bands visible in the isoelectric-focusing pattern to two. These bands have the same mobility as the two more basic ones present in native ovomucoid. This result confirms that the ovomucoid species differ not only in their sialic acid content but also at the level of their peptide chain and/or their carbohydrate moieties.

The u.v.-absorption spectra of the different fractions of turtle-dove ovomucoid have the same shape as chicken ovomucoid spectra (Krysteva & Dobrev, 1977). However, the higher tyrosine content of turtle-dove ovomucoid (Table 1) was reflected in a significantly higher absorption coefficient.

Equilibrium ultracentrifugal analysis indicated that the turtle-dove ovomucoid fractions (I, II and III from DEAE-cellulose) were homogeneous in molecular weight. The apparent mol.wt. of 29400 was within the range of values usually found (28000–32000) for ovomucoids extracted from different avian species.

All of the turtle-dove ovomucoid fractions (I–III) contained only valine as *N*-terminal residue. Several ovomucoids have this amino acid as *N*-terminal residue (Feeney & Allison, 1969), although hen ovomucoid is an exception.

Turtle-dove and hen ovomucoids differ in their proteinase-inhibition properties. Whereas hen ovomucoid almost completely inactivates trypsin at an inhibitor/enzyme ratio of 1:1, turtle-dove ovomucoid produces only 30% inhibition under similar conditions (Fig. 3). This indicates that the affinity of turtle-dove ovomucoid for trypsin is lower than that of hen ovomucoid, for which the K_{ass} for the enzyme-inhibitor complex has been reported to be 5×10^9 M (Green, 1953). The displacement of turtle-dove ovomucoid from its complex with trypsin by substrate under conditions in which the hen ovomucoid–trypsin complex was stable is also consistent with the turtle-dove inhibitor binding less strongly to trypsin.

Turtle-dove ovomucoid inhibits chymotrypsin, in contrast with hen ovomucoid. However, the ovomucoids from several other avian species are also known to inhibit both trypsin and chymotrypsin (Feeney & Allison, 1969). Displacement of inhibitor by substrate was also observed in the assay of chymotryptic activity of turtle-dove ovomucoid.

Although the overall content of sialic acid and glucosamine was quite similar to that of hen ovomucoid (Table 1), the nature of the neutral hexose in turtle-dove ovomucoid was markedly different. Galactose was the major neutral hexose component in turtle-dove ovomucoid, whereas hen ovomucoid contains substantially more mannose than galactose (Beeley, 1971a).

These differences in carbohydrate composition may be related to the presence of blood-group-P₁-antigen-like activity in turtle-dove, but not in hen, ovomucoid. The erythrocyte P₁-antigen contains five carbohydrate residues bound to ceramide (Naiki & Marcus, 1975; Naiki *et al.*, 1975), Gal α 1 $\rightarrow$ 4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer, in which the terminal α -galactose residue appears to be essential for blood-group-P₁ activity. It may be that the carbohydrate groups of turtle-dove ovomucoid contain similar sequences terminating in α -galactose.

Blood-group-P₁-like antigens have also been reported in some other biological materials, such as

hydatid cyst fluid (Cameron & Staveley, 1957; Corey *et al.*, 1974), worm extracts (Prokop & Schlesinger, 1965) and avian enterobacteria (Roland *et al.*, 1977), as well as in the blood cell and serum components of certain avian species (Brocteur *et al.*, 1975). The discovery of blood-group-P₁-like activities in different biological media from various branches of the animal kingdom raises the question of their exact structural relationship and biological significance. The fact that a blood-group-P₁ activity has been found to be associated with avian Gram-negative bacteria may explain the presence of this activity in bird droppings, but this does not help us to understand why the P₁-like antigen is present in the ovomucoid of certain birds only.

Further systematic investigation of the distribution of P₁ antigens and the elucidation of their structural characteristics will probably bring some light to this question.

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