ISOLATION OF AN ACIDIC GLYCOPROTEIN FROM RABBIT OVIDUCAL FLUID AND ITS ASSOCIATION WITH THE EGG COATING

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Summary. A high molecular weight acidic glycoprotein has been isolated from rabbit oviducal fluid. Electrophoretic analysis of this purified glycoprotein showed one major component. Antibodies to the glycoprotein were raised in the goat, concentrated by ammonium sulphate precipitation and conjugated with fluorescein isothiocyanate. The conjugated antibodies reacted with 3-day-old rabbit eggs causing them to fluoresce green in u.v. light, but 4-day-old eggs, which have largely lost their coat, did not react significantly with the antibody. These observations suggest that the high molecular weight acidic glycoprotein in oviducal fluid participates in the egg-coating process.

INTRODUCTION

In order to understand the functions of the mammalian oviduct, it is necessary to identify the components of oviducal fluid. Hamner & Williams (1965) have reported the total protein, carbohydrate and electrolyte composition of rabbit oviducal fluid. The protein composition was further detailed by Shapiro, Jentsch & Yard (1971), who concluded that proteins in oviducal fluid resulted from secretion as well as transudation from plasma.

In many species (Bacsich & Hamilton, 1954), and particularly in the rabbit, a 'mucin' coat is deposited around the ovum during passage along the oviduct to the uterus. Braden (1952) concluded this material to be acidic mucoprotein after subjecting it to various dyes, proteases, hydrogen ion concentrations and oxidizing and reducing agents.

Hamner & Fox (1969) reported that the rabbit oviduct is rich in secretory granules that are periodic acid–Schiff (PAS) positive and diastase resistant.

We have investigated the nature of the 'mucin'-like material in oviducal fluid as part of a continuing study on the protein composition of rabbit oviducal fluid. We now report on the isolation of an acidic glycoprotein from oviducal fluid which we believe to be involved in coating of the egg.

MATERIALS AND METHODS

Collection of oviducal fluid

Oviducal fluid was collected by the method of Hamner & Williams (1965) and contained 1.5 to 2.5 mg protein/ml.

Purification of acidic glycoprotein

Pooled oviducal fluid (100 ml) was freeze-dried, redissolved in 2.5 ml distilled water and then dialysed against 50 mM-tris-HCl, pH 7.8. The non-dialysable fraction was applied to a 50×2.5 cm Sepharose 4B column and eluted with 50 mM-tris-HCl, pH 7.8 (Text-fig. 1a). The void volume peak (Text-fig. 1a, I, shaded area) was fractionated again on the same column. The tubes from the second fractionation that contained Alcian blue (acidic carbohydrate) and Ponceau S (protein)-staining material were combined (Text-fig. 1b, shaded area) and referred to as the acidic glycoprotein.

Immunization to oviducal fluid acidic glycoprotein

The acidic glycoprotein isolated by Sepharose gel fractionation was concentrated to 2 mg protein/ml (Sepharose 4B has an exclusion limit of 3×10^6 daltons). A 2-ml volume of the glycoprotein was mixed thoroughly with an equal volume of Freund's adjuvant (Baltimore Biological Laboratories) containing *Mycobacterium butyricum* and 1.0-ml portions of the mixture were injected intramuscularly into each thigh of a male goat. After 10 weeks, a 2-ml booster dose of similarly treated antigen was injected subcutaneously and, 3 weeks later, the goat was bled from the jugular vein and antibodies to oviducal fluid were detected by the technique of Ouchterlony (1953).

Preparation of fluorescent goat anti-rabbit oviducal acidic glycoprotein

Goat serum was brought to 50% saturation with respect to $(\text{NH}_4)_2\text{SO}_4$ by slowly adding an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ at 0°C. The precipitate was centrifuged and redissolved in distilled H_2O to give a final volume equal to the original serum. An equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ was again added. The precipitated globular fraction was centrifuged, redissolved in saline, and dialysed against saline overnight at 4°C.

Labelling of the globular proteins with fluorescein isothiocyanate (FITC) was carried out according to the method of Clark & Shepard (1963). The removal of excess dye from the fluorescein conjugate was accomplished with a Sephadex G-25 column (0.5×13 cm). The degree of conjugation was 4.5 mol FITC/mol protein as determined by the absorbance of the protein at 493 nm and 276 nm according to the method of Wells, Miller & Nadel (1966). There was no apparent physicochemical change in the FITC-conjugated antiglycoprotein compared to unconjugated antiglycoprotein as judged by immunoelectrophoresis and double diffusion studies.

Double diffusion studies

Double diffusion studies were performed at room temperature for 48 to 72 hr in agarose gels (Kallestad Labs). A sample of 30 μl antiserum containing 100 mg protein/ml was applied to the centre well. The antigen well contained 30 μl glycoprotein at a concentration of 2 mg protein/ml.

Cellulose acetate electrophoresis

Electrophoresis was performed on cellulose acetate (Gelman) strips in 0.05 M-sodium barbital buffer, pH 8.6, for 120 min at 1.5 mA/strip.

Incubation of fluorescent antibody with egg

The FITC-labelled antibody (0.1 ml containing 100 mg of protein/ml) was incubated with 0.9 ml saline containing two 3-day-old rabbit eggs for 4 hr. After incubation, the eggs were washed twice in 1.5-ml portions of saline for 1 hr each and allowed to stand overnight in a fresh 1.5 ml aliquot of saline. The last wash was clear. When the eggs were examined microscopically with a u.v. light source, they fluoresced green indicating common antigenic determinants on both the isolated glycoprotein and the 3-day-old eggs taken from a rabbit mated with a fertile buck.

Lysis of 3-day-old eggs

Forty-three 3-day-old eggs were lysed in 0.1 ml solution of 2% sodium lauryl sulphate (SLS) and 2% mercaptoethanol in water. After lysis, the SLS and mercaptoethanol were removed by exhaustive dialyses against 0.05 M-tris, pH 8.6, containing 0.25% bovine serum albumin over 4 days with frequent changes of buffer. The resulting dialysate was concentrated to 30 μ l by ultrafiltration (Schleicher & Schuell, Inc.) at room temperature. This concentrated lysate was used for reaction against anti-acidic glycoprotein.

Amino acid analysis

Samples of the glycoprotein were dialysed exhaustively against distilled water and then evaporated to dryness. The dried material was dissolved in 3.0 ml of 6.0 N-HCl and hydrolysed *in vacuo* for 24 hr at 100°C. The hydrolysate was evaporated to dryness, dissolved in 3.0 ml distilled water, and again taken to dryness. This operation was performed three times before finally drying the sample *in vacuo* over NaOH. The dried sample was dissolved in 0.6 ml of 0.01 N-HCl and an aliquot of 0.5 ml was applied to a Technicon amino acid analyser.

Neutral and amino sugars

The neutral and amino sugars were analysed by a procedure modified from Albersheim, Nevins, English & Karr (1967). The glycoprotein was hydrolysed in 2 N-HCl for 3 hr at 100°C. The HCl was removed *in vacuo* over NaOH pellets. The sample was dissolved in 0.005 M-NH₄OH, 10 mg NaBH₄ was added and the mixture was allowed to stand for 18 to 20 hr at room temperature. This solution was brought to pH 5 with glacial acetic acid and applied to a Dowex-50 (H⁺) column to remove the sodium. The column was washed with 10 bed volumes of distilled water and these washings were taken to dryness. The dried residue was washed with methanol to remove borate (as the trimethylester). Pyridine (30 μ l) and acetic anhydride (30 μ l) were added to the dried material and heated to 100°C for 20 min to form the sugar acetates. The reactants were removed under reduced pressure.

The dry sample was dissolved in 20 to 50 μ l CHCl₃ and then analysed on a Hewlett-Packard model 402 gas chromatograph equipped with a flame ionization detector and a model 3378 digital integrator. The column was packed with Gas-Chrom Q, 100 to 120 mesh, precoated with a mixture of 0.2% stabilized ethylene glycol succinate, 0.2% stabilized ethylene glycol

adipate, and 0.4% silicone XF-1150. The packing was obtained from Applied Science Labs.

The acetates of the following sugars were prepared and used as standards: 2-deoxyribose, rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, glucose, inositol and 2-deoxyhexose. The detector gave a linear response to all the standards. The variation in response to each sugar was determined. Samples were usually injected three times and the values for the areas under the peaks were averaged.

Sialic acid

Free sialic acid was determined by the thiobarbituric acid method of Warren as described by Spiro (1966). Free sialic acid was generated by hydrolysis of the glycoprotein with 0.5 N-H₂SO₄ at 80°C for 1 hr or by incubation overnight with neuraminidase in 0.01 M-acetate buffer, pH 5.8. The sample was dialysed against 50 mM-tris-HCl, pH 7.8. The dialysate was concentrated before assaying for sialic acid.

Uronic acids were determined by the carbazole method of Dische (1947).

Sulphate determination

Covalent sulphate was determined by the method of Antonopoulos (1962).

Protein determination

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Molecular weight determination

The molecular weight of the acidic glycoprotein subunit was determined by the method of Shapiro, Viñuela & Maizel (1967). Ovalbumin, catalase and phosphorylase (Worthington), and γ -globulin (Miles) were used as standards.

RESULTS

Purification of acidic glycoprotein

The fractionation of oviducal fluid on Sepharose 4B column resulted in four major protein peaks (Text-fig. 1a). Acidic glycoprotein(s) were detectable only in the excluded fraction (I), which stained with both Alcian blue and Ponceau S. Fractions II, III and IV were stained by Ponceau S but not Alcian blue. The void volume (Peak I) was opaque and appeared to represent a major constituent of the fluid, but it contained only 2 to 3% of the oviducal fluid protein. This void volume peak was fractionated a second time on a Sepharose 4B column and eluted as a single peak with a small but consistent amount of tailing (Text-fig. 1b). Disc gel electrophoresis was not possible due to the large molecular weight of glycoprotein.

After purification, the acidic glycoprotein had a tendency to precipitate and exist as a viscous gelatinous material. This transformation was accelerated by freezing and thawing.

Electrophoresis of the eluate from the Sepharose 4B column was performed

TEXT-FIG. 1. The Sepharose 4B fractionation of pooled rabbit oviducal fluid. Column conditions are described under 'Materials and Methods'. (a) Fractionation of rabbit oviducal fluid, flow rate = 12 ml/hr, 4 ml/tube collected. The shaded area (void volume) was pooled, concentrated and fractionated. (b) Refractionation of void volume peak from 'a'. The conditions were similar to those described for 'a'. The shaded area represents pooled tubes. V_0 = void volume.

TEXT-FIG. 2. Cellulose acetate electrophoresis of acidic glycoprotein. The conditions of electrophoresis are described under 'Materials and Methods'. (a) Oviduct fluid, stained with Ponceau S; (b) Peak I, stained with Ponceau S or Alcian blue; (c) Peaks II, III and IV, stained with Ponceau S.

on cellulose acetate strips in sodium barbital buffer at pH 8.6. Peak I was compared with the combined Peaks II, III and IV. The strips were stained for protein with Ponceau S and for glycosaminoglycans with Alcian blue. Peak I from the Sepharose 4B column showed only one protein band which also stained with Alcian blue (Text-fig. 2b). Pooled Peaks II, III and IV from the Sepharose 4B column showed three major Ponceau S-positive bands and no Alcian blue-positive bands. None of the three protein bands of pooled Peaks II, III and IV corresponded to the protein band in Peak I (Text-fig. 2c).

TEXT-FIG. 3. Densitometric tracing of acidic glycoprotein subjected to electrophoresis on cellulose acetate strips. The conditions of electrophoresis are described under 'Materials and Methods'. The tracing was obtained with a photovolt densitometer after staining the strips with Ponceau S.

When the glycoprotein sample was subjected to electrophoresis in barbital-acetate buffer at pH 8.6 and stained for either protein (Ponceau S) or carbohydrate (PAS or Alcian blue), only one major band was observed with a densitometer (Text-fig. 3), but there was usually some glycoprotein material at the origin. The relative ratio of the mobile band to the origin band varied from preparation to preparation and with the age of the sample. For some preparations, the major portion of the material subjected to electrophoresis remained at the origin. The mobile band was always converted to the immobile insoluble band by repeated freezing and thawing or by ageing.

Freshly prepared acidic glycoprotein in combination with antibody gave a single precipitant band (Pl. 1, Fig. 1). As the sample became more gelatinous after ageing or freezing and thawing, multiple bands appeared in the Ouchterlony plate and remained very close to the antigen well.

EXPLANATION OF PLATE 1

FIG. 1. Double-diffusion study of glycoprotein and anti-glycoprotein. The conditions for the double-diffusion studies are described under 'Materials and Methods'. The centre well (A) contained 30 μ l immunized goat serum containing 100 mg protein/ml. Antigen wells (G) contained 30 μ l glycoprotein containing approximately 2 mg protein/ml.

FIG. 2. Disc gel electrophoresis of sodium lauryl sulphate-treated glycoprotein. The conditions for dissociation and electrophoresis are described under 'Materials and Methods' (50 μ g protein was subjected to electrophoresis).

FIG. 3. Reaction of lysed eggs with anti-glycoprotein. The conditions for lysis are described under 'Materials and Methods'. The centre well contained 30 μ l immunized goat serum containing 100 mg protein/ml. Well E contained the lysate from seventeen 3-day-old eggs in 30 μ l. Well G contained 30 μ l glycoprotein containing 1 mg protein/ml.

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FIG. 4. Reaction of 3-day-old rabbit eggs with FITC-labelled antiglycoprotein. The conditions for the reaction are described under 'Materials and Methods'.

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Dissociation of acidic glycoprotein

The glycoprotein was dissociated into smaller sub-units by incubation at 37°C for 2 hr in the presence of 0.1% SLS and 0.1% mercaptoethanol. The dissociated glycoprotein (50 µg) was subjected to disc gel electrophoresis with the standard alkaline gel and buffer containing 0.1% SLS and 0.1% mercaptoethanol (Davis, 1964). One major component was observed when the gels were stained with amido black or PAS, but minor protein components were also present (Pl. 1, Fig. 2).

The molecular weight of the dissociated sub-unit was calculated using the technique developed by Shapiro *et al.* (1967). The polydisperse glycoprotein showed predominately one major band after treatment with SLS. This band migrated as a sub-unit or monomer of 73,000 mol. wt (Text-fig. 4).

TEXT-FIG. 4. Molecular weight determination of dissociated glycoprotein. The conditions for electrophoresis are described under 'Materials and Methods'. The determined molecular weight was 73,000 daltons.

Amino acid and carbohydrate analyses

The isolated macromolecule contained almost equal (approximately 50 mol/73,000 g of protein) amounts of aspartic acid, threonine, serine, glutamic acid and leucine (Table 1). The molecule was composed of approximately 30% by weight of carbohydrate, galactose being the most prominent sugar (Table 2). In addition to hexuronic acid, the glycoprotein also contained covalent sulphate (Table 2) indicating the presence of sulphated sugars. The identification of these sulphate esters has not been accomplished.

Reaction of fluorescent antibody with 3-day-old eggs

The FITC-antibody was incubated with 3-day-old eggs for 4 hr. Excess

antibody was removed by washing with saline for 18 hr. A green fluorescence in ultraviolet light (Pl. 2, Fig. 4) was seen after reaction of the egg with labelled antibody. The FITC-control (non-immunized) goat serum did not react with the 3-day-old eggs. Fluorescent antibody was also incubated with 4-day-old eggs that had lost most of their 'mucin' coat. When these eggs were examined microscopically with a u.v. light source, only a very low degree of fluorescence relative to the 3-day-old eggs was observed.

Table 1. Amino acid composition of glycoprotein in rabbit oviducal fluid

<i>Amino acid</i>	<i>μmol Amino acid/0.190 mg glycoprotein</i>	<i>mol Amino acid/73,000 g glycoprotein</i>	<i>Nearest integer/73,000 g glycoprotein</i>
Aspartic acid	0.1299	49.96	50
Threonine	0.1329	51.11	51
Serine	0.1264	47.92	48
Glutamic acid	0.1227	47.19	47
Proline	0.0992	38.15	38
Cysteine	0.0085	3.26	3
Glycine	0.1057	40.65	41
Alanine	0.1100	42.31	42
Valine	0.0920	35.38	35
Methionine	0.01437	5.53	6
Isoleucine	0.0568	21.84	22
Leucine	0.1366	52.54	53
Tyrosine	0.0364	14.00	14
Phenylalanine	0.0704	27.08	27
Lysine	0.09123	35.09	35
Histidine	0.0426	16.38	16
Arginine	0.0658	25.31	25

Table 2. Carbohydrate composition of glycoprotein in rabbit oviducal fluid

<i>Carbohydrate</i>	<i>μmol Carbo-hydrate/0.190 mg glycoprotein</i>	<i>mol Carbo-hydrate/73,000 g glycoprotein</i>	<i>Nearest integer/73,000 g glycoprotein</i>
Sialic acid	0.0494	19.00	19
Galactose	0.2245	86.35	86
Uronic acid	0.0696	26.54	27
Glucosamine	0.0893	34.35	34
Galactosamine	0.05691	21.88	22
Fucose	0.00810	3.11	3
SO ₄ ⁼	0.07588	29.18	29

Reaction of lysed eggs with antibody

Forty-three 3-day-old rabbit eggs were lysed in 0.1 ml of a 2% SLS and 2% mercaptoethanol solution. After dissolution, the SLS and mercaptoethanol were removed by exhaustive dialyses (4 days). The dialysate was concentrated and allowed to react against anti-glycoprotein (Pl. 1, Fig. 3). After SLS dissociation and dialysis, both the coated egg and the purified glycoprotein

antigens formed multiple precipitant bands when allowed to react against anti-glycoprotein.

DISCUSSION

We have isolated a large molecular weight acidic glycoprotein from rabbit oviducal fluid. The fractionation pattern indicates that the glycoprotein exists as a very high molecular weight polydisperse aggregate. Ultracentrifugation of the glycoprotein also indicated polydispersion. The purified material aggregated with ageing or freezing and thawing. The glycoprotein became immobile in electrophoresis and much less mobile in agarose gel.

The change in the physical properties of this glycoprotein was assumed to be the result of aggregation. However, we cannot explain why similarly prepared samples, which had not aged, varied in the degree of aggregation.

The large molecular weight of the glycoprotein, indicated by the fractionation pattern on Sepharose 4B, facilitated its purification from the rest of the proteins in the fluid. Except for DNA or RNA, the presence of such high molecular weight physiological polymers would not be expected in a biological fluid. Since no ribose, deoxyribose or phosphate was detected with standard methods in the isolated material, the presence of DNA and RNA can be excluded.

Fractionation of the oviducal fluid resulted in the isolation of an acidic glycoprotein which gave one major band which stained for both protein and carbohydrate when subjected to electrophoresis on cellulose acetate strips. Surprisingly, this sample gave a single precipitant band when allowed to react against antibody in agarose gel. Treatment of the acidic glycoprotein with SLS resulted in dissociation of the aggregate to one major sub-unit with a molecular weight of 73,000 daltons. The minor bands may be additional sub-units of the same high molecular oligomer or represent impurities. Further purification of the high molecular aggregate may help resolve this point.

Fluorescent antibody (raised in the goat) to this acidic glycoprotein fraction reacted with 3-day-old rabbit eggs giving a green fluorescence which indicated common antigenic determinants for both the glycoprotein and the egg coat. Furthermore, disrupted eggs gave a positive precipitant reaction with the antibody which suggested that the acidic glycoprotein is associated with the tertiary or 'mucin' coat of the egg.

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