Bufo arenarum egg jelly coat: purification and characterization of two highly glycosylated proteins

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Egg jelly coats from *Bufo arenarum* are formed by components secreted along the oviduct. These secretion products overlay the oocytes as they transit along the different oviductal portions. In this study, we have isolated two highly glycosylated proteins of the jelly coat, which are secreted almost all the way along the oviduct. Both glycoproteins [designated as highly glycosylated protein (HGP) and low-molecular-mass highly glycosylated protein (L-HGP)] were purified to homogeneity, from the secretion of the caudal oviduct portion, by CsCl density gradient ultracentrifugation. HGP is a high-molecular-mass protein with mucin-like characteristics: high viscosity, a high content of serine and threonine, about 70% carbohydrate by weight, and a protease-resistant domain. Cleavage of disulphide bridges with reducing agents resulted in the release of a single subunit (300 000 Da). L-HGP is also a disulphide-cross-linked protein with lower apparent monomeric molecular mass, in the range

100–120 kDa and containing 50% carbohydrate by weight. HGP contains galactose, fucose, *N*-acetylgalactosamine and sialic acid, but no mannose, suggesting the presence of O-linked oligosaccharides exclusively. The secretion ratio of HGP increases from cephalic (16% of total protein in *pars preconvoluta*) to caudal (40% of total protein in *pars convoluta*) oviductal portions. It appears to be the major structural component of the jelly coat. Our purification data suggest that HGP is non-covalently linked to the other egg jelly proteins. Polyclonal antiserum to each purified glycoprotein from secretion was raised in rabbits and used to localize both glycoproteins in the different oviductal portions, total egg jelly and the aqueous medium where oocyte strings were incubated. HGP forms a stable fibre matrix around the oocyte. L-HGP is present in the jelly coat and is released into the incubation medium.

INTRODUCTION

Amphibian oviductal secretions overlay the oocytes with transparent gelatinous layers (jelly coats), as they transit along the oviduct after their release from the ovary [1–3]. These coats are mainly composed of glycoproteins and, together with the vitelline envelope, are the first barriers through which fertilizing sperm has to penetrate before reaching the oocyte plasma membrane [4–8].

Many functions have been attributed to the egg jelly components, such as the provision of specific binding sites for sperm recognition, sperm capacitation, induction of the acrosome reaction and the provision of a protective environment for the developing embryo [9,10]. However, the molecular mechanisms underlying such functions are not yet well characterized. Incomplete understanding of the functions was due to deficient purification of the glycoproteins and peptides that constitute the jelly coats. Recently, a major glycoprotein from egg jelly coat of *Bufo japonicus* has been isolated and was shown to display calcium-binding properties [11].

The requirement of the egg jelly coat for fertilization is well established [12,13]. Oocytes whose jelly coat has been removed (dejellied oocytes), or that have not travelled down the oviduct (body cavity oocytes) are not fertilized when inseminated [14–16]. On the other hand, jelly layers become a blocking site to sperm penetration upon hydration [17–19]. As reported for *Bufo arenarum*, dejellied oocytes are successfully fertilized by treating the gametes with the diffusible factors released from the jelly coat when egg strings are incubated in distilled water [20,21]. This

material, called egg water (EW), contains a variety of compounds, including glycoproteins, lipids and salts [18,21,22], but the precise composition has not been fully characterized.

The extracellular matrix of amphibian oocytes is arranged in several layers, suggesting that the sperm could be progressively modified through a sequence of interactions with the glycoproteins in the layers [8]. In *Xenopus laevis*, a model of a carbohydrate-rich fibre matrix to which low-molecular-mass proteins are bound has been reported [23]. The isolation and comprehensive analysis of such components from each jelly layer should be useful to study with more detail their interactions with sperm.

In the present paper we report the isolation of two highly glycosylated proteins of *B. arenarum* egg jelly coat: a high-molecular-mass, highly glycosylated protein (HGP) with mucin-like characteristics, and a lower-molecular-mass heavily glycosylated one (L-HGP) that is released from the jelly coat to the surrounding medium. Using polyclonal antibodies, we studied their site of biosynthesis in the different portions of the oviduct: *pars recta* (PR), *pars preconvoluta* (PPC) and *pars convoluta* (PC). Our results strongly support the idea that both highly glycosylated proteins are secreted along the oviduct and contribute towards forming the jelly layers.

MATERIALS AND METHODS

Reagents

The following reagents were purchased from Sigma Chemical Co.: pepstatin, leupeptin, aprotinin, phenylmethanesulphonyl

Abbreviations used: HGP, highly glycosylated protein; L-HGP, low-molecular-mass highly glycosylated protein; EW, egg water; PR, *pars recta*; PPC, *pars preconvoluta*; PC, *pars convoluta*; PAS, periodic acid–Schiff reaction; DTT, dithiothreitol.

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fluoride, *N*-ethylmaleimide, EDTA, anti-rabbit IgG (whole molecule)–peroxidase conjugate, complete Freund's adjuvant, 3,3-diaminobenzidine, SDS molecular-mass markers, Schiff reagent and silver stain kit. Pure nitrocellulose membranes were from Trans-Blot, Bio-Rad, and molecular porous membrane tubing was from Spectra/Por, Spectrum Medical Industries.

Animals

B. arenarum specimens were collected in the neighbourhood of Rosario city and maintained in the dark, in a moist chamber, between 15 and 17 °C until use.

Oviduct secretions

Oviducts of *B. arenarum* females were ligated under anaesthesia to obtain secretions from three different portions: PR, PPC and PC. Animals were allowed to recover from the surgery for 48 h. Ovulation was induced by intracoelomic injection of one homologous hypophysis [24,25]. Twelve to fourteen hours after keeping the animals at 18–20 °C, they were pithed and the oviducts were removed. Secretions from the different portions were obtained by flushing with a syringe containing Ringer solution. Protease inhibitors, pepstatin, leupeptin and aprotinin, were added to the resulting solution at the final concentration of 1 µg/ml followed by 1 mM phenylmethanesulphonyl fluoride/5 mM EDTA/5 mM *N*-ethylmaleimide. Samples were stored at 20 °C until use.

Dissolved jelly coat

The strings of oocytes, obtained from ovisacs, were treated with thioglycolic acid, pH 8.0 [26], and the remaining solubilized jelly was stored at 20 °C until used.

Egg water

EW was obtained as described [20,21]. Briefly, strings of *B. arenarum* oocytes were removed from the ovisacs and extracted for 20 min with distilled water, Ringer solution or 10% (w/w) Ringer solution, with occasional stirring at 20–22 °C. Equal volumes of medium from each extraction were assayed for proteins.

Oviductal tissue

PR, PPC and PC portions of the oviduct were removed 12 h after ovulation induction, and opened longitudinally. The internal surface was washed and gently scraped in modified Barth solution (88 mM NaCl/0.1 mM KCl/0.24 mM NaHCO₃/0.082 mM MgSO₄/0.033 mM CaCl₂/0.75 mM Tris/HCl, pH 7.6) to obtain the secretory cells. The homogenization was carried out in modified Barth solution with the same protease inhibitory cocktail as described for secretion extraction. Ca²⁺ was added to a final concentration of 10 mM to allow the secretory granules to break down. The homogenate was centrifuged for 15 min at 20000 *g*, and the supernatant precipitated with 80% ethanol. The pellet was washed with ethanol and stored at 20 °C.

Gel electrophoresis

SDS/PAGE was performed in 4–15% (w/v) polyacrylamide gradient gels [27], with a 3% (w/v) stacking gel. Samples were dissolved in 60 mM Tris/HCl (pH 6.8) containing 2% SDS and 10% glycerol, and denatured by heating in a boiling-water bath for 5 min. For electrophoresis under reducing conditions the sample buffer also contained 10% β-mercaptoethanol. The gels were stained with either Coomassie Brilliant Blue or by using the

periodic acid–Schiff reaction (PAS) for carbohydrates [28]. In other cases, silver kit or PAS–silver [29] stainings were used.

Purification of oviductal secretion glycoproteins

Secretion glycoproteins were isolated basically as described by Dekker et al. [30] for purification of rat gastric mucin. Oviductal secretion (approx. 4 mg of total protein), extracted from PC in homogenization buffer, was solubilized in 4 M guanidine/HCl. The insoluble material was clarified by centrifugation. CsCl was added to the soluble fraction up to a density of 1.4 g/ml. Isopycnic density-gradient centrifugation was performed in a Beckman ultracentrifuge at 150000 *g* for 48 h at 4 °C. Aliquots of 1 ml were removed from the gradient and the density of each fraction was measured. Samples were dialysed against water for 24 h at 4 °C using a 12000-Da cut-off dialysis membrane. Aliquots from the fractions were assayed for proteins by the method of Lowry et al. [31] and for hexoses by the phenol/sulphuric acid method (490 nm) [32]. The position of each glycoprotein (HGP or L-HGP) in the gradient was determined by analysis of small aliquots by using SDS/PAGE.

In order to separate possible glycoprotein-linked glycopeptides, HGP-containing fractions were pooled and run again on a similar CsCl gradient (gradient density 1.40 g/ml; centrifugation conditions as above) after reduction and alkylation (see below). The resulting fractions were analysed as described above, and the HGP-containing fractions were pooled, dialysed exhaustively against water and freeze-dried.

In other cases, HGP purification was performed by the method described to purify mucin-like proteins using electrophoresis and electroelution after reversing electrode polarity [33].

Production of antiserum

HGP and L-HGP were used to produce antiserum in rabbits. The antigens used for immunizations were purified by preparative electrophoresis under reducing and denaturing conditions on 4–15% polyacrylamide gradient gels, and electroeluted using the method described by Leppard et al. [34]. Electroeluted proteins were dialysed against distilled water, precipitated in 80% ethanol and resuspended in 200 µl of water. Approx. 100 µg of purified glycoprotein was injected into the rabbit at 0, 4, 8 and 12 weeks. Blood was collected from each rabbit 15 days after injections, allowed to clot at 37 °C and centrifuged to separate the serum [35]. Antisera were stored at –20 °C. Preimmune serum was obtained from the same animals before the first injection.

Immunoblots

Electrophoretically separated proteins were transferred to 0.2 µm nitrocellulose membrane, at 95 mA (17 h at 4 °C), in 20% (v/v) methanol containing 0.15 M glycine, 0.03% SDS, 25 mM Tris/HCl, pH 8.3. Blotted nitrocellulose strips were equilibrated in 10 mM PBS, pH 7.2, and blocked for 2 h in Blotto (5% non-fat dry milk, 0.02% sodium azide in PBS) at room temperature. Strips were incubated in a 1:500 dilution of the antiserum for 2 h in Blotto, fully washed and allowed to react for 1 h with horseradish peroxidase-labelled goat anti-rabbit IgG (1:1500 dilution in Blotto). After washing in blotto (3 × 15 min) the strips were rinsed briefly in PBS and finally developed by incubation for 15–20 min in a freshly prepared substrate solution of 20 ml of 100 mM Tris/HCl (pH 7.2) containing 2 mg of 3,3-diaminobenzidine and 7.5 µl of ice-cold 30% hydrogen peroxide. Colour development was stopped by washing the strips in water [35].

Treatments of HGP and L-HGP

For protease digestion, incubation of HGP and L-HGP with proteinase K was carried out during 24 h at 37 °C, at 1 µg of enzyme/µg of glycoprotein, in 10 mM sodium phosphate (pH 7.0) containing 250 µg/ml BSA.

Disulphide reduction and S-alkylation

Fractions containing purified HGP (lanes 3–5, Figure 4) were pooled and dialysed for 24 h against water and freeze-dried. The sample (600 µg of protein) was resuspended in 0.1 M Tris/HCl (pH 8.5) containing 6 M guanidine/HCl, reduced with 10 mM dithiothreitol for 5 min in a boiling-water bath, cooled to room temperature and treated with 20 mM iodoacetamide for 4 h at 4 °C in the dark. The solution was dialysed against water for 24 h and freeze-dried.

Compositional analysis

Amino acid analysis was performed on an ABI Model 130A HPLC after hydrolysis in 6 M HCl in the ABI 420H derivatizer/hydrolyser, and the data were analysed in an ABI Model 610 data acquisition system [36,37]. Carbohydrate analysis was performed on a Dionex BioLC instrument especially configured for carbohydrates with an anion-exchange column, pulsed amperometric detection, and a gold working electrode. Neutral and amino sugar were analysed after release by hydrolysis in 400 µl of 2 M trifluoroacetic acid for 4 h at 100 °C, using 2-deoxyglucose as internal standard. Sialic acid was determined by the thiobarbituric acid assay following hydrolysis in 0.1 M HCl at 80 °C for 4 h [38], using *N*-acetylneuraminic acid as a standard.

RESULTS

Analysis of *B. arenarum* jelly coat and oviductal secretion

On the basis of electrophoretic analysis under reducing and denaturing conditions, secretion from PC and dissolved jelly coat (Figure 1, lanes 2 and 3 respectively) were shown to contain a component of high apparent molecular mass that stained for

Figure 1 Comparison of oviductal secretion with jelly coat and EW homogenates

Unfractionated oviductal secretion, jelly coat and EW were analysed on 4–15% SDS/PAGE under reducing conditions with 3% stacking gel. After electrophoresis the gel was stained with either Coomassie Blue (a), PAS (b) or PAS–silver (c). Lane 1, molecular-mass markers; lane 2, unfractionated oviductal secretion (5 µg/lane); lane 3, unfractionated jelly coat (5 µg/lane); lane 4, unfractionated EW (5 µg/lane).

Figure 2 SDS/PAGE of PC secretion proteins

Unfractionated PC secretion (3 µg/lane) was electrophoresed under reducing (+) and non-reducing conditions (–) on 4–15% gradient polyacrylamide gels. The gels were stained with either silver or PAS–silver.

both protein and carbohydrate. These samples also contained another glycosylated component with lower apparent molecular mass (in the range 100000–120000 Da) that stained weakly with PAS but not with either Coomassie Blue or silver. PAS staining was intensified with silver to reveal this band (Figure 1, compare 1b and 1c). Protein staining also revealed a major secretion protein band of 70 kDa and several minor bands that did not stain with the Schiff reagent. The PAS-staining band of lower molecular mass was the only highly glycosylated component in EW (Figure 1c, lane 4).

Under non-reducing conditions, the carbohydrate-containing band that did not stain for protein on SDS/PAGE showed a decreased mobility, suggesting the presence of interchain disulphide bonds in the native form of this protein (Figure 2, compare lanes 3 and 4). The band of high apparent molecular mass that just entered the separation gel, failed to enter even in the stacking gel under non-reducing conditions (not shown), and the band of 70 kDa increased its mobility under reducing conditions, suggesting intrachain disulphide bridges.

In order to determine if the band that just entered the gel was a molecular aggregation, the secretion homogenate from PC was treated with different concentrations of reducing agents (β -mercaptoethanol or dithiothreitol, DTT), or was reduced and subsequently alkylated. These treatments did not produce a lower molecular mass band on SDS/PAGE (results not shown).

Oviductal fluid is a mixture of proteins and glycoproteins secreted all along the oviduct. The material from PC is more viscous than PR and PPC secretions. The different oviductal secretions are partially solubilized in 4 M guanidine/HCl under non-reducing conditions, while jelly coat is soluble under reducing conditions but not by treatment with denaturing agents only. These data suggest that the components of oviductal secretions interact via interchain disulphide bonds to form the jelly layers. Therefore purification of jelly coat proteins was carried out from oviductal secretions.

Purification of HGP and L-HGP

To purify the two highly glycosylated proteins present in the jelly coat, PC oviductal secretion was subjected to CsCl gradient ultracentrifugation. The homogenization of the secretion in a buffer containing *N*-ethylmaleimide (to block free thiol groups

Figure 3 Purification of glycoproteins from PC secretion by CsCl density gradient centrifugation

(a) PC secretion (9 ml, 0.44 mg/ml of total protein), solubilized in 4 M guanidine/HCl, was centrifuged in a Beckman 80 Ti rotor at 150 000 *g* for 48 h at 4 °C. Twelve aliquots (1 ml each) were aspirated from the top of the tube, and 100 μ l of each dialysed fraction were assayed for protein ($\square$, Abs. 750 nm); for hexoses by the phenol/sulphuric acid method ($\blacksquare$, Abs. 450 nm) and density ($\bullet$). (b) Aliquots of dialysed fractions were analysed on 4–15% gradient polyacrylamide gels under reducing conditions with a 3% stacking gel. After electrophoresis the gel was stained with the PAS–silver method. Lanes 1–7, fractions 5–11 from the CsCl gradient respectively (15 μ l/lane).

and inhibit possible disulphide exchange) and under denaturing but non-reducing conditions, produced a partially solubilized fraction. The CsCl gradient ultracentrifugation analysis of the soluble fraction showed a single peak of carbohydrate-containing material (Figure 3a, fractions 5–9), with the two highly glycosylated components focusing at different buoyant densities, as shown by SDS/PAGE under reducing conditions (Figure 3b). The glycoprotein that focused at higher densities (Figure 3a, fractions 6–9, 1.34–1.40 g/ml; Figure 3b, lanes 2–5) was named HGP and the name L-HGP was given to the other highly glycosylated protein (Figure 3a, fractions 5–6, 1.28–1.34 g/ml; Figure 3b, lanes 1 and 2), because it migrated at a lower apparent molecular-mass band on SDS/PAGE under reducing conditions. L-HGP appeared to be disulphide cross-linked with subunits migrating as a broad band of 100–120 kDa. It was not possible to estimate the molecular mass of HGP subunits by this method, but the apparent monomeric molecular mass must be above 300 kDa. Both glycoproteins do not appear to be covalently linked with other secretion proteins, since no other bands were

visualized in the respective lanes of the gel by silver and PAS–silver staining.

Dialysis against water of fraction 5 (Figure 3a) gave a low-viscosity solution, whereas fractions 7–9 formed highly viscous solutions after the same treatment.

To ensure purity of HGP in the collected fractions, a second CsCl density gradient centrifugation was carried out on HGP reduced and alkylated. This procedure yielded identical results when compared with the first gradient. HGP was located in the second gradient at a buoyant density of 1.34–1.40 g/ml. The purity was assessed by SDS/PAGE of the fractions, followed by silver or PAS–silver stain. Under these conditions no other proteins were detected (results not shown).

Highly purified HGP from PPC and PC was also obtained by a novel technique reported for purification of glycoproteins from porcine gastric mucus [33]. HGP was purified by this method in both oviductal secretions. The ratio of HGP/total protein is higher in PC (40% HGP/total protein) than in PPC (16% HGP/total protein).

Jelly coat hydration

Analysis of the EW fractions obtained from strings of deposited oocytes incubated at different salt concentrations in aqueous solution yielded similar patterns of bands on SDS/PAGE. Two main proteins were identified in the extracts. One was L-HGP and the other was the 70 kDa protein secreted in PC (Figures 1a and 1c, lane 4). Protein staining of the gel also showed several minor peptides. The pattern of bands was very similar to the jelly coat, except for the absence of HGP (Figures 1a and 1c, lanes 3 and 4). The amount of total protein released from the jelly envelopes was higher (0.11 mg/ml) when saline solutions were used instead of distilled water (0.03 mg/ml) for the extraction. The analysis of the resulting hydrated jelly coat by SDS/PAGE showed that HGP appeared as the major protein-staining component (Figure 1a, lane 3), whereas it was not detected in EW under the different conditions of extraction (Figure 1c, lane 4).

Comparison of HGP and L-HGP from different portions of the oviduct

Polyclonal antibodies were prepared separately against HGP and L-HGP purified from PC secretion. Each glycoprotein reacted with both the specific antiserum and the antiserum against the other glycoprotein. This may be due to the high content of similar carbohydrates and not because of a common protein core. No other proteins from unfractionated tissue homogenates and secretions of the different oviductal portions investigated reacted with this antiserum.

Analysis by immunoblot was carried out to determine the site of biosynthesis of both glycoproteins along the oviduct and their presence in the gelatinous coat. We used antiserum prepared against HGP and L-HGP purified from PC secretion separately, as described previously. Both HGP and L-HGP were present in PPC and PC tissue homogenate and secretion. L-HGP was also present in PR tissue homogenate and secretion, but showed a decreased mobility in gels if compared with L-HGP from other oviductal portions (Figure 4). Both are present in the gelatinous layer that surrounds the oocytes, but only L-HGP diffuses to the medium (Figure 4, lanes 7 and 8).

Characterization of HGP and L-HGP

Proteinase K treatment of both isolated glycoproteins showed a different sensitivity of each macromolecule to protease. L-HGP was completely hydrolysed, while HGP mobility remained un-

Figure 4 L-HGP and HGP from different sources

Unfractionated oviductal tissue homogenate from PR, PPC and PC, oviductal secretion from PR, PPC and PC, total jelly coat and EW (7 µg/lane) were subjected to 4–15% gradient SDS/PAGE and blotted on to nitrocellulose. Immunodetection was performed using anti-HGP polyclonal antibodies as described under the Materials and methods section. Lanes 1–3, PR, PPC and PC tissue homogenate respectively; lanes 4–6, PR, PPC and PC secretions respectively; lane 7, total jelly coat; lane 8, EW.

Table 1 Amino acid and carbohydrate composition of HGP

Amino acid analysis was performed on an ABI model 130A HPLC following hydrolysis with 6 M HCl. Fucose, galactose, mannose, *N*-acetylgalactosamine and *N*-acetylglucosamine were analysed on a Dionex BioLC instrument especially configured for carbohydrates. *N*-Acetylneuraminic acid was determined by thiobarbituric acid assay [31].

Constituent	Amino acids (mol/100 mol)
Amino acids	
Asx	2.6
Ser	20.9
His	0.7
Thr	34.0
Pro	9.3
Val	6.1
Cys	0
Leu	3.4
Lys	2.4
Glx	3.6
Gly	2.4
Arg	1.8
Ala	5.3
Tyr	0.7
Met	0.3
Ile	2.3
Phe	1.7
Trp	0
Carbohydrates	
Fucose	34
Galactose	60
Mannose	0
<i>N</i> -Acetylgalactosamine	35
<i>N</i> -Acetylglucosamine	13
<i>N</i> -Acetylneuraminic acid	11

changed in SDS/PAGE, although BSA present in the incubation buffer was completely digested (results not shown).

Both HGP and L-HGP are highly glycosylated proteins. HGP purified by CsCl density gradient centrifugation contains approx. 70 % carbohydrate (hexose and hexosamines) and 30 % protein, by weight. L-HGP purified by the same procedure consists of carbohydrate and protein in approximately equal proportions by weight.

The amino acid composition of HGP is shown in Table 1. The

relative amino acid content is typical of mucins and mucin-like glycoproteins: a large proportion of Thr and Ser. These two residues and the next predominant amino acids (Pro, Val, Ala and Lys) account for 80 % of the protein core.

The carbohydrate analysis (Table 1) indicates that PC HGP contains galactose, fucose, *N*-acetylgalactosamine and *N*-acetylglucosamine. HGP appeared also as a sialic acid-rich mucin (15 mol/100 mol of amino acids). Release of sialic acid from PC HGP was 100 % complete after 1 h of treatment with 0.1 M HCl (results not shown). HGP purified from PPC showed similar sialic acid release values. This result suggests similar HGP glycosylation with sialic acid along the oviduct.

DISCUSSION

In the present paper, we report the purification of two macromolecular components of jelly coat from *B. arenarum* oocyte. We have isolated a high-molecular-mass HGP from PC and PPC secretions and a second HGP with lower molecular mass (L-HGP), present all along the oviduct, from PC secretions.

HGP is a typical mucin-type glycoprotein [39–42]. When HGP was reduced with dithiothreitol and alkylated with iodoacetamide, it released mucin-like subunits with a molecular mass of about 300000 Da. This apparently high molecular mass may be due to the extensive O-glycosylation, which affects the electrophoretic mobility (for discussion see [43]). About 70 % of the molecule is carbohydrate. The protein moiety contains six predominant amino acids (Thr, Ser, Pro, Val, Ala and Lys) that comprise 80 % of the total residues.

Our results indicate that HGP presents five different carbohydrate components (GalNAc, Fuc, Gal, GlcNAc and NeuAc). The absence of Man and the high content of Ser and Thr (more than 50 %) suggest that carbohydrate chains are probably the O-linked type only. However, the methods employed may lack the sensitivity to detect a few N-linked glycans among many O-linked glycans. Previous studies on toad-egg jelly coat indicate that the linkage between peptide and carbohydrates is exclusively between Ser and Thr and GalNAc [44].

L-HGP is present in jelly coat and EW. L-HGP is a minor component of the total oviductal secretion proteins, since its presence was detected by PAS-silver, a more sensitive glycoprotein-staining method on SDS/PAGE. Staining for protein failed to reveal this secretion component. It has an apparent molecular mass of 100–120 kDa, and, although 50 % of the molecule corresponds to carbohydrates, L-HGP was shown to be degraded by proteinase K.

Purified HGP appears as a highly viscous, aqueous solution. This property results from the long, expanded, hydrated structure of mucin aggregates, which is caused in part by the mutual repulsion of the anionic charges, represented by the high content of sialic acid residues [45]. Most of the polypeptide is resistant to protease activity, suggesting a domain with an extensive substitution with oligosaccharide units that cause steric hindrance. This resistance may explain the protective role against the attack of proteases from micro-organisms [46–48].

Individual macromolecules of the jelly coat are assembled and organized in a way that is poorly defined [49]. The oocytes from *Bufo* ovisac are covered by four jelly layers, named J1 to J4 from the innermost to the outermost [50]. It has been established that each jelly layer is composed of the material secreted from a different part of the oviduct [1].

Our data indicate that HGP is the major structural component of the gelatinous envelopes and that non-covalent bonds between HGP and the other glycoproteins and peptides are present. Although higher amounts of factors diffused from the jelly when

saline solutions were used, instead of distilled water, HGP was not detected in EW. It is therefore likely that the interactions between HGP and the jelly coat components are of the ionic type, since all of them, except HGP, are released into the surrounding medium. In order to isolate soluble HGP from jelly coat, oviposited eggs must be treated with mercaptan solutions. After the EW was obtained, HGP appeared as the major component of the remaining jelly coat. The combined data suggest that once HGP was secreted, it overlays the oocytes and forms a mesh where HGP subunits interact via interchain disulphide bonds; this interaction prevents HGP from diffusing to the medium. HGP purified from secretion released no other proteins when it was treated with DTT or β -mercaptoethanol, indicating the lack of covalent interactions with other secretion components.

The structural arrangement of HGP in the different jelly layers around the oocyte still remains unknown. It has been suggested that the major glycoprotein present in *Bufo japonicus* egg jelly is present only in J3 and J4 layers [9]. Our results suggest that at least the two highly glycosylated proteins characterized from *Bufo* oviductal secretion are secreted along the oviduct and are probably present in the four jelly layers. Further experimental work will be necessary to understand how HGP influences the structural organization of the different jelly envelopes. The variability in HGP secretion ratio through the oviduct may influence the organization of the jelly layers surrounding the eggs.

The results obtained from immunoblots using polyclonal antibodies suggest a high similarity between the antigenic sites of HGP and L-HGP. In order to investigate whether this cross-reactivity is due to a complex formed by L-HGP subunit interactions, which might lead to the formation of the high-molecular-mass glycoprotein (HGP), the secretion homogenate from PC was treated with different concentrations of reducing agents (β -mercaptoethanol or DTT), or was reduced and subsequently alkylated. These treatments did not give a lower-molecular-mass HGP subunit band on SDS/PAGE. Conversely, HGP purified under non-reducing conditions only showed the band of 300 kDa on SDS/PAGE when treated with β -mercaptoethanol. These results suggest that the band of 100–120 kDa is neither the monomer of HGP nor a covalently linked protein. The cross-reactivity of the respective antibodies with both glycoproteins may be due to the presence of common carbohydrate epitopes, but more work will be necessary to elucidate this.

In conclusion, we purified to homogeneity, in a single step, the main structural component of the jelly coat from *B. arenarum* oviductal secretions and we characterized HGP as a mucin-type glycoprotein. We also purified another heavily glycosylated protein that diffuses to the medium when the strings of oocytes reach the aqueous solution. More experimental work is required to elucidate the role of diffusible factors in the mechanism of oocyte–sperm recognition.

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