Diffusible Highly Glycosylated Protein From *Bufo arenarum* Egg-Jelly Coat: Biological Activity

SILVIA E. ARRANZ AND MARCELO O. CABADA*

IBR (CONICET), Area Biología, Departatmento de Ciencias Biológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina

ABSTRACT L-HGP is a highly glycosylated protein from *Bufo arenarum* egg-jelly coat that diffuses into the surrounding medium when the strings of oocytes are incubated in saline solutions. L-HGP was purified from egg water and the estimated percentage of L-HGP/total protein in egg water was estimated in 30%. In the present study we examine, by indirect immunofluorescence, the effect of L-HGP on acrosome status of homologous spermatozoa. A high percentage (77%) of sperm lost the acrosome when incubated in 10% Ringer solution buffered with 10 mM Tris-HCl, pH 7.6, during 60 min, a condition that resembles egg-jelly osmolarity. The addition of purified L-HGP to the incubation medium prevents acrosome breakdown. The acrosome integrity is maintained for at least 1 hr. This effect is specific for L-HGP at concentration ranging from 0.01 to 0.1 mg/ml since neither BSA nor fetuin seems to have similar activity at similar concentrations. The same effect was observed when spermatozoa were incubated in egg water. Preliminary results suggest that L-HGP binds to *B. arenarum* spermatozoan membranes. *Mol. Reprod. Dev. 56:392–400, 2000.*

Key Words: egg jelly; diffusible factor; acrosome protection; acrosome reaction

INTRODUCTION

Amphibian oocytes are surrounded by several layers of jelly material, which are deposited around the oocytes while they traverse the oviduct. This jelly coat is associated to the fertilization process, since oocytes deprived of their jelly layers exhibit a very low degree of fertilizability when inseminated (Kambara 1953; Katagiri, 1966). *Bufo arenarum* egg-jelly coat is composed of a mucin called HGP, which constitutes the structurally stable matrix of the jelly, and some diffusible molecules, including proteins (Arranz et al., 1997). Under natural conditions, these molecules diffuse into the surrounding medium once the strings of oocytes are shed. It has previously been reported that diffusible substances seem to be essential for fertilization (Barbieri and Villeco, 1966). Since this diffusible factor is rapidly released from the jelly coat, it was assumed that this factor could activate free spermatozoa before they penetrate the jelly coat (Barbieri and Raisman, 1969). The solution containing the substances that diffuse from the oocytes strings is called egg water (EW).

A necessary condition for spermatozoa to fertilize oocytes is to reach the vitelline envelope with the acrosome intact, or at least not completely reacted. It has been shown in *Leptodactylus chaquensis* that spermatozoa with intact acrosomes can reach the vitelline envelope of homologous oocyte (Raisman et al., 1980). A study of the relative roles of jelly layers in fertilization of *Bufo japonicus* (Omata, 1993) also confirms the previous report by Yoshizaki and Katagiri (1982) that acrosome reaction occurs not in the jelly but at or near the surface of the vitelline coat of fully jellied oocytes. It has also been suggested that some molecules of *B. japonicus* jelly coat could act as cofactors in inducing acrosome reaction (Omata, 1993).

The jelly coat of *B. japonicus* contains cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) in a total concentration of about 130 mM (Ishihara et al., 1984). Most ions diffuse from intact jelly when the strings of oocytes are immersed in water for 2–3 min. This is accompanied by the acquisition of fertilizing capacity by sperm. Hypotonicity, which ensures sperm motility, and a relatively high concentration of Ca^{2+} and Mg^{2+} are conditions found in the natural ionic environment surrounding fertilizing sperm in the jelly coat. However, it was reported that *B. arenarum* spermatozoa acrosome reaction occurs after a short period of incubation in hypotonic solutions (Martínez and Cabada, 1996).

In a previous paper we reported the purification of the glycoprotein L-HGP from *B. arenarum* oviductal secretion (Arranz et al., 1997). This protein is present in the egg-jelly coat and diffuses from it when the oocytes strings are incubated in saline solutions (100–120 kDa, 50% glycosylated). The present study was carried out to examine the morphological and biological effect of L-HGP on homologous spermatozoa. Purified L-HGP has no acrosome reaction-inducing activity. Instead, L-HGP protect the acrosome from breakdown

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*Correspondence to: M.O. Cabada, IBR (CONICET), Area Biología, Departatmento de Ciencias Biológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR—Suipacha 531 (2000) Rosario, República Argentina. E-mail: cabada@elsitio.net

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when spermatozoa are incubated in 10% Ringer, a hypotonic solution that resembles egg-jelly osmolarity at fertilization time. This effect is specific for L-HGP. Preliminary results suggest that L-HGP binds to *B. arenarum* spermatozoa membranes.

MATERIALS AND METHODS

Chemicals and Enzymes

Bovine serum albumin (BSA), fetuin, D-biotinyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester, HRP-labeled goat anti rabbit IgG, and streptavidin/peroxidase were from Sigma Chemical Co. (St. Louis, MO). Anti-L-HGP antibodies were obtained previously (Arranz et al., 1997).

Animals

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

Egg Water

EW was obtained as described (Barbieri and del Pino, 1975). Briefly, ovulation was obtained in hypophysis-stimulated females of *B. arenarum*, and strings of oocytes were removed from the ovisacs and extracted 20 min with distilled water three times with occasional stirring at 20–22°C. Extracts were pooled and frozen in aliquots at −20°C until used.

Gel Electrophoresis

SDS-PAGE was performed in 4–15% polyacrylamide gradient gels (Laemmli, 1970) with a 3% 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% 2-mercaptoethanol. The gels were stained by the PAS-silver method (Gradilone et al., 1998).

Purification of L-HGP From EW

L-HGP was isolated from EW with a procedure similar to the described for the isolation of this glycoprotein from oviductal secretion (Arranz et al., 1997). EW (4 mg total ethanol-precipitated protein) was solubilized in 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.6, final concentrations. CsCl was added up to a density of 1.4 g/ml. Isopicnic density gradient centrifugation was performed in a Beckman ultracentrifuge at 150,000*g* for 48 hr at 4°C. After centrifugation, aliquots of 1 ml were removed from the top of the gradient and the density of each fraction was measured. Samples were dialyzed against distilled water for 24 hr at 4°C using a 12,000 cut-off dialysis membrane. Aliquots from the fractions were assayed for proteins and for hexoses. The position of L-HGP in the gradient was determined by SDS-PAGE of aliquots of the fractions.

Preparation of Sperm Suspensions

Sperm suspensions were obtained essentially as described in Martínez and Cabada (1996). Washed spermatozoa were suspended in Ringer solution containing 10 mM Tris-HCl buffer, pH 7.6 (hereafter, Ringer-Tris) to a concentration of 1×10^6 cells/ml. Sperm structure, assessed by immunocytochemical procedure (Martínez and Cabada, 1996), was preserved after 4 hr of incubation in Ringer-Tris.

Sperm Incubation with L-HGP

L-HGP was assayed for its ability to modify the rate of acrosome breakdown induced by incubating the spermatozoa in a hypotonic solution (osmotic shock), a condition that causes sperm to react.

Ten microliters of sperm suspension, obtained as described above, were mixed with 90 µl of L-HGP in distilled water at defined final concentrations, and incubated at 18°C. Control was carried out by incubating 10 µl of sperm suspension in 90 µl of distilled water without L-HGP. Aliquots of 20 µl were taken from each incubation media at 5, 20, 40, and 60 min of incubation and diluted with 500 µl of Ringer-Tris. The resulting suspensions were further centrifuged at 650*g* during 5 min at 4°C. The pellets were resuspended in 20 µl of Ringer-Tris and smeared on a slide in order to evaluate acrosomal status of the spermatozoa by indirect immunofluorescence (see below).

In order to asses specificity of L-HGP, BSA (66 kDa, non glycosylated) or fetuin (66 kDa, 20% glycosylated) were used to incubate spermatozoa instead of L-HGP at the same molar concentrations.

In-Vitro Fertilization

In-vitro fertilization assays were carried out by inseminating a string of about 200 oocytes with a sperm suspension of 10^4 spermatozoa/ml. Previously spermatozoa were incubated (5×10^5 spermatozoa/ml) for different periods.

Indirect Immunofluorescence

Spermatozoa were stained by indirect immunofluorescence with anti-acrosomal matrix antibodies essentially as described (Martínez and Cabada, 1996). Previously, dot blot assays were done to rule out cross-reactivity of the antibodies with L-HGP. Samples were examined with phase-contrast and epifluorescence optics using an Olympus BHS microscope equipped with a mercury fluorescence illuminator.

At least one hundred sperm were counted on each slide, and the percentage of sperm with intact acrosome was estimated.

Binding Assay

Sperm prepared as described above were washed once more in Ringer-Tris without calcium. The binding protocol was essentially the same as described for binding of oviductal proteins to bovine sperm (Lapointe and Sirard, 1996) except that L-HGP was previously

labeled with biotin (see below) instead of $Na^{125}I$. The incubation mixture contained 10 µl of freshly washed sperm resuspended in Ringer-Tris (1.5×10^8 cells/ml), 20 µl of labeled L-HGP (0.020 mg/ml final concentration); 20 µl of unlabeled L-HGP or BSA at a level equal to 0, 20, or 100 times the amount of labeled proteins; and 50 µl of distilled water. The preparation (final volume 100 µl) was then mixed and incubated for 30 min at 18°C. Treatment without calcium was carried out as described (Lapointe and Sirard, 1996). Following incubation, tubes were centrifuged at 4°C during 10 min at 650*g*, the supernatant was removed, and the pellet rinsed three more times with 1 ml of Ringer-Tris. The last pellet was processed as reported (Lapointe and Sirard, 1996) and finally submitted to gel electrophoresis. After running, the proteins were transferred to PVDF membrane and stained with streptavidin/peroxidase (1:1500). The same protocol was also carried out with two other final concentrations of labeled L-HGP (0.002 mg/ml and 0.040 mg/ml).

Biotinylation of L-HGP

L-HGP was biotinylated using D-biotinyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester. Purified L-HGP was incubated for 4 hr at RT in biotin in dimethyl sulfoxide (200 mg/ml) diluted to 0.06 mg/ml in phosphate-buffer saline to labeled the protein. The resulting biotinylated protein was dialyzed for 24 hr at 4°C with 2 or 3 changes of 10 mM Tris-HCl, pH 7.6. Protein concentration of labeled L-HGP was determined once the dialysate was centrifuged. The conjugate was stored at −20°C until used.

Protein and Carbohydrate Determinations

Protein concentrations were estimated by the method of Lowry et al. (1951) using BSA as a standard. Hexose concentration was estimated by the phenol-sulfuric method (Dubois et al., 1956).

Statistics

Statistical evaluation of data consisted in the analysis of variance, considering the experiments as factorial. Differences were considered significant at $P < 0.01$ (Snedecor and Cochran, 1967).

RESULTS

The purification of L-HGP from EW was carried out by submitting 4 mg of total EW proteins to CsCl gradient ultracentrifugation. The procedure rendered a purified glycoprotein focusing at densities between 1.28 and 1.34 g/ml (Fig. 1A).

It was estimated, from recovered proteins after purification, that about 30% of total EW proteins was L-HGP. L-HGP-containing fractions (Fig. 1A, fractions 2–5) showed a diffuse band after SDS-PAGE, with molecular mass ranging between 100 and 135 kDa (Fig. 1B, lanes 2–5). The fractions containing only L-HGP (Fig. 1B, lanes 3–5) were pooled and used in the assays.

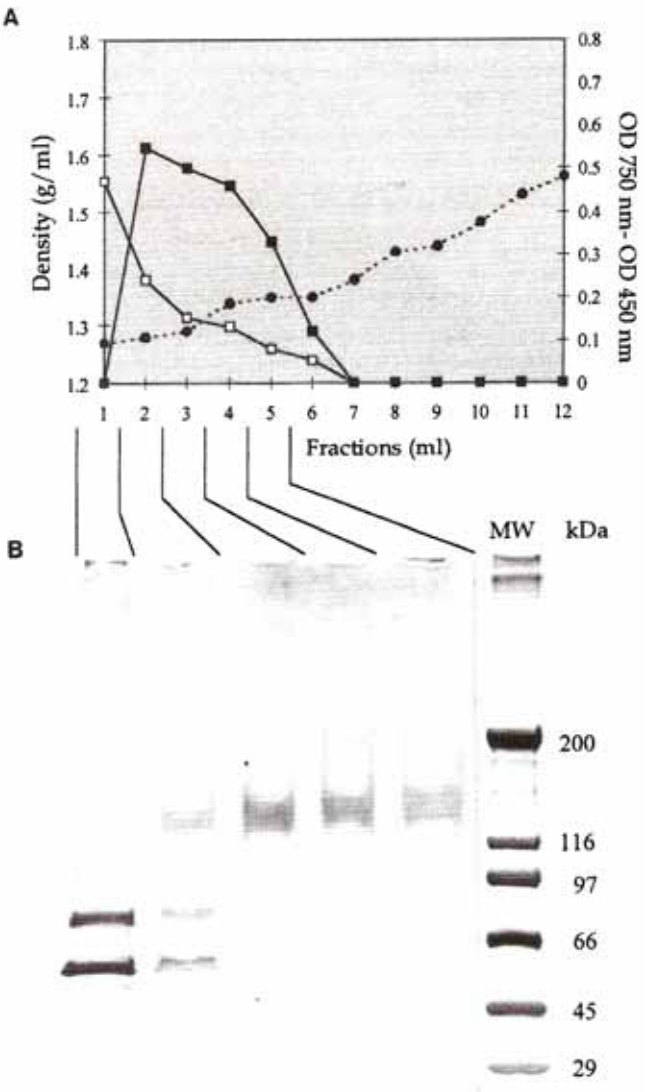

Fig. 1. Purification of L-HGP from EW by centrifugation in CsCl gradient. (**A**) A 4-mg amount of protein precipitated with 5 vol of cold ethanol at −20°C and L-HGP was purified as indicated in Materials and Methods. Twelve fractions (1 ml each) were collected by aspiration from the top of the tube. In each fraction, the density was determined (●). After dialysis, each fraction was analyzed for neutral sugar (■ = OD 490 nm) and protein (□ = OD 750 nm). (**B**) Aliquots (15 µl) of the fractions were submitted to 4–15% gradient SDS-PAGE under reducing conditions with 3% stacking gel. The gel was stained by the PAS-silver method.

Acrosome Status After Incubation With L-HGP

Sperm acrosome status was evaluated by indirect immunofluorescence using antibodies raised against acrosomal matrix content as described (Martínez and Cabada, 1996). This technique shows different staining patterns for acrosome-reacted and intact spermatozoa. Figure 2 shows spermatozoa incubated in 10% Ringer-Tris alone (A and A′) or with L-HGP (B and B′) after 5 min (A and B) and 60 min (A′ and B′) of incubation, respectively. When spermatozoa were treated for 60 min in 10% Ringer-Tris alone, a high percentage of

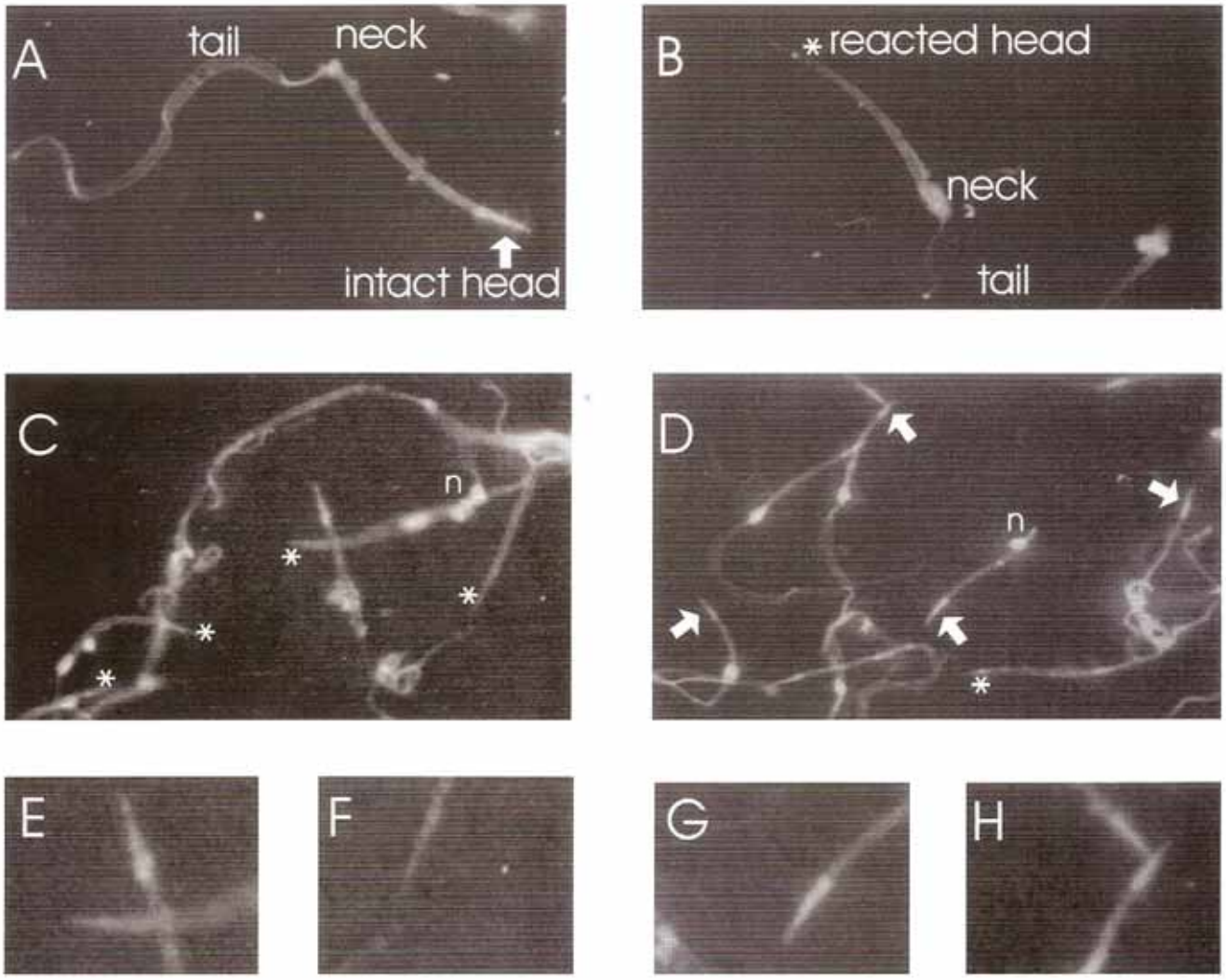

Fig. 2. Acrosomal status of *B. arenarum* sperm evidenced by indirect immunofluorescence after L-HGP incubation. Spermatozoa were stained and observed as indicated in Materials and Methods. (**A**) Intact spermatozoa; (**B**) reacted spermatozoa; (**C**) spermatozoa incubated in 10% Ringer-Tris for 60 min; (**D**) spermatozoa incubated in L-HGP (0.1 mg/ml) in 10% Ringer-Tris for 60 min; (**E, F**) magnifications of sperm heads from C; (**G, H**) magnifications of sperm heads from D. (A, B) 1000×; (C, D) 400×; (E–H) 1500×. The asterisks indicate reacted heads. The arrows indicate intact heads; n, neck.

spermatozoa lost the fluorescence at the anterior tip of the head, indicating the loss of acrosomal matrix. Spermatozoa incubated in the same medium but with the addition of L-HGP (0.1 mg/ml final concentration) showed fluorescence in the acrosomal region after 5 and 60 min of incubation, indicating that most spermatozoa remain unreacted up to this time. Although sometimes a spurious fluorescence in the neck of the spermatozoa is observed, the fluorescence at the anterior tip, associated with changes in the acrosome status, is specific.

The number of acrosome-reacted spermatozoa increased with increasing incubation periods, in a hypotonic solution (Fig. 3). However, when L-HGP was present in the incubation media, about 70% of total spermatozoa had intact acrosome against 23% of total spermatozoa in the media without L-HGP after 60 min of incubation (Fig. 3). In this case, acrosome breakdown was inhibited when spermatozoa were incubated in L-HGP at 0.1 mg/ml, a concentration that is related with the EW protein concentration (0.3 mg/ml) and the estimated percentage of L-HGP/total protein in EW (30% of total protein). Incubation of spermatozoa during 5 min with L-HGP, washing, and incubating 20 min longer showed similar results than incubation of spermatozoa during 30 min with L-HGP (data not shown). In both cases the rate of unreacted spermatozoa was significantly higher than the control reaction without L-HGP. When spermatozoa were incubated with purified L-HGP (0.1 mg/mg final concentration) for 3 hr, the rate of intact spermatozoa decay to 53% compared to 64% observed after 1 hr of incubation, but is still higher than the values found when incubations were carried out in Ringer-Tris 10% (17%).

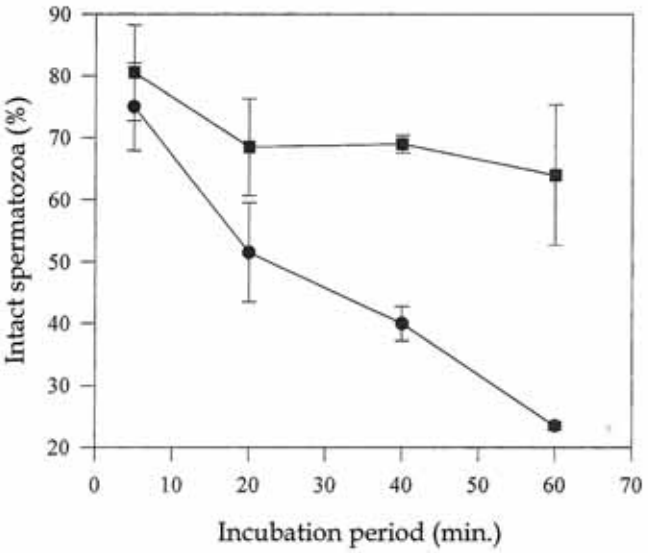

Fig. 3. Effect of L-HGP on the acrosome status of *B. arenarum* spermatozoa. Sperm suspensions (1×10^5 cells/ml) were incubated in either 10% Ringer-Tris alone (●) or with 0.1 mg/ml L-HGP (■) at 18°C. After different periods of incubation aliquots of the suspensions were processed as described in Materials and Methods, and observed under fluorescence microscope. Results are expressed as percentage of intact spermatozoa (mean ± SEM; $N = 3$).

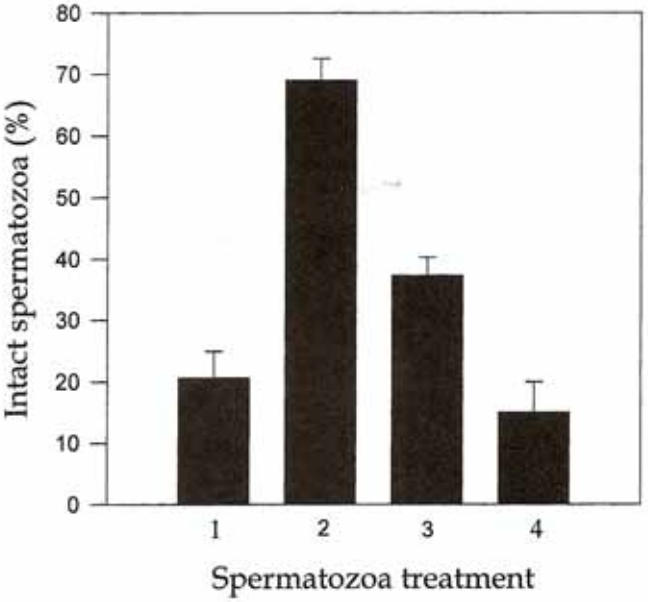

Fig. 4. Effect of different proteins on acrosomal status in *B. arenarum* spermatozoa. Sperm suspensions (1×10^5 cells/ml) were incubated at 18°C for 60 min in 10% Ringer-Tris containing or not different proteins at similar molar concentrations (noted in the figure). After incubations, aliquots of sperm suspensions were processed as described under Materials and Methods and observed under fluorescence microscope. Sperm were incubated with 10% Ringer-Tris alone (1) or containing 100 µg/ml L-HGP (2), 50 µg/ml BSA (3), or 50 µg/ml fetuin (4). Results are expressed as mean ± SEM ($N = 3$).

Specific Acrosome Protection

To verify the specificity of L-HGP protection on acrosome breakdown, spermatozoa were incubated with other glycosylated (fetuin) or nonglycosylated (BSA) proteins.

Spermatozoa incubated in 10% Ringer-Tris with either L-HGP or other proteins (BSA or fetuin) at 0.8 mM final concentration during 60 min were stained by indirect immunofluorescence. The results summarized in Fig. 4 indicate that the percentage of fluorescent acrosomes after 60 min of treatment was significantly lower ($P < 0.001$) in spermatozoa incubated in hypotonic solutions containing equimolar concentrations of BSA (66 kDa, non-glycosylated) or fetuin (66 kDa, 20% glycosylated) than in those treated with L-HGP (100–120 kDa, 50% glycosylated). These results suggest that protection of acrosome in low ionic strength solutions is specific for L-HGP and not a general phenomenon due to the presence of proteins in the incubation media.

Effect of Different Concentrations of EW or L-HGP on Acrosome Breakdown

In order to study if a correlation exists between the rate of acrosome protection with L-HGP concentration in the incubation media, spermatozoa were incubated for 60 min in 0.01, 0.05, or 0.1 mg/ml L-HGP in 10% Ringer-Tris. After incubation, the status of spermatozoa was evaluated. The same procedure was carried out using different concentrations of EW (0.3, 0.15 and 0.03 mg/ml total protein in the medium corresponding to 100%, 50%, and 10% EW, respectively). The concentrations of total proteins in the EW assayed were three times higher than those used when incubating with purified L-HGP in order to obtain similar concentration of L-HGP in both treatments since 30% of EW total protein is L-HGP.

No significant differences were observed in the percentage of intact spermatozoa between purified L-HGP concentrations used (Fig. 5). EW showed similar effect as purified L-HGP although a slight decrease in protection was observed with increasing EW protein concentrations. All the values were significantly higher than the controls without protein ($P < 0.001$), and no significant differences were observed among them. A tendency (although not statistically significant) for the acrosome to rupture during incubation in EW was observed.

Effect of L-HGP on Sperm Fertility

We have also examined the possible influence of L-HGP in sperm fertility. Sperm suspensions were incubated in saline solution containing L-HGP, and, at different periods of incubations, aliquots were used for in-vitro fertilization assays. The results in Fig. 6 indicate that, at least in our working conditions, spermatozoa fertility is not affected by the treatment. Despite a slight decrease in fertilizability that is observed after 40 and 60 min of incubation in 10% Ringer-Tris alone compared with the treatment with L-HGP, this difference is not statistically significant.

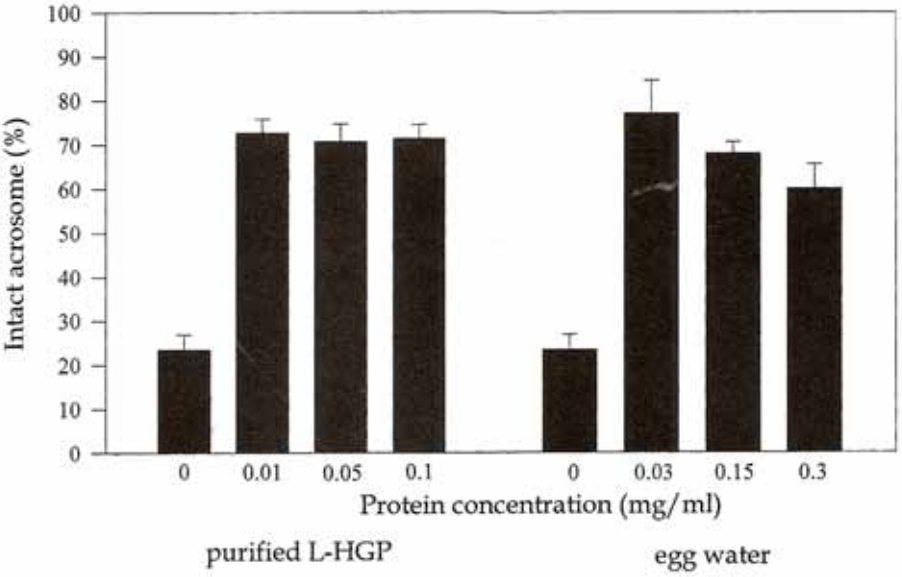

Fig. 5. Effect of different concentrations of L-HGP and EW on the acrosome status in *B. arenarum* spermatozoa. Sperm suspensions (1×10^5 cells/ml) were incubated in 10% Ringer-Tris alone or in different concentrations of L-HGP or EW in 10% Ringer-Tris during 60 min at 18°C. EW-protein concentrations were three times higher than those used with purified L-HGP, to obtain similar L-HGP concentrations in both treatments. The status of the acrosomes was evaluated by indirect immunofluorescence as described (see Materials and Methods) (mean ± SEM; $N = 3$).

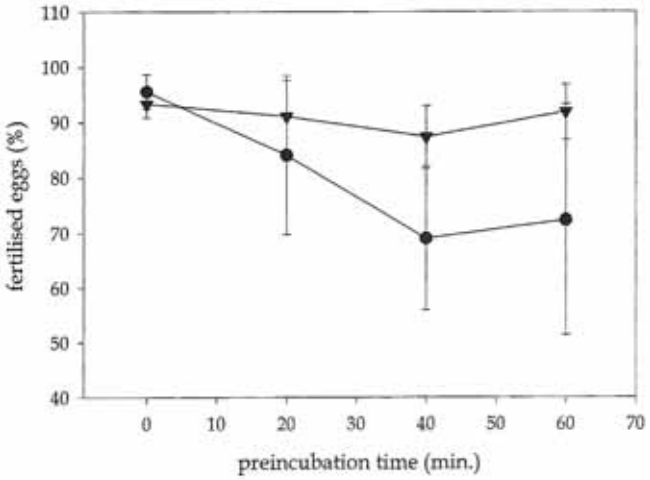

Fig. 6. Effect of L-HGP in sperm fertility. Suspensions of 5×10^5 sperm/ml were incubated in 10% Ringer solution, containing (▼) or not (●) L-HGP (0.1 mg/ml). After incubation, an aliquot of the suspension was used to inseminate strings of about 200 oocytes each. Results are expressed as percentage of oocytes fertilized (mean ± SEM; $N = 6$). The differences between control and treated samples are not statistically significant.

Binding of L-HGP to Spermatozoa

We assayed the binding of L-HGP, previously labeled with biotin (Fig. 7A and B), to spermatozoa. Spermatozoa were incubated with biotinylated L-HGP in 10% Ringer-Tris for 30 min. After exhaustive washings, the resulting pellet of spermatozoa containing associated proteins, was resolved in SDS-PAGE, transferred to PVDF membrane and revealed with streptavidin/peroxidase (Fig. 7C).

No difference in the binding of biotinylated L-HGP was appreciated when competition experiments were carried out with increasing amounts of unlabeled L-HGP in the incubation media to assess specificity. The binding of biotinylated L-HGP to sperm was not affected by the absence of Ca^{2+} in the incubation medium, suggesting that this cation was not an essential requirement for L-HGP binding. As shown in Fig. 8, addition of increasing amounts of labeled L-HGP to the sperm suspension results in an increased binding of the protein to spermatozoa membranes.

The results in Figs. 7C and 8 indicate that L-HGP binds strongly to sperm membranes.

DISCUSSION

In the present study, we report the purification of a major glycoprotein from EW, L-HGP, which constitutes about 30% of EW total protein. This is the only highly glycosylated protein (50% w/w glycosylated) that diffuses from the jelly coat (Arranz et al., 1997).

Studies carried out on the biological activity of the EW have been mainly focused on the fertilizing capacity of spermatozoa. Contradictory results were reported, indicating that factors present in the EW have, in one hand, a beneficial effect on fertilization: when dejellied oocytes are inseminated in 10% Ringer-Tris, no fertilization is observed, while the addition of EW to the fertilization medium results in the fertilization of the oocytes (Barbieri and Raisman, 1969; Katagiri, 1973). On the other hand, when the duration of the incubation of sperm in EW increases, fertility decreases (Elinson, 1971). Díaz Fontdevila et al. (1991) have reported that this effect seems to be related to a component of the EW that can be extracted with agents used to extract lipids. It has also been reported the inhibition of the fertilizing capacity of *B. arenarum* spermatozoa produced by exogenous lipids (Cabada et al., 1984). This phenomenon was explained by the triggering of a premature acrosome breakdown (Díaz Fontdevilla et al., 1988). However, it was difficult to evaluate the acrosome status in a large sperm population by electron microscopy since a reduced number of

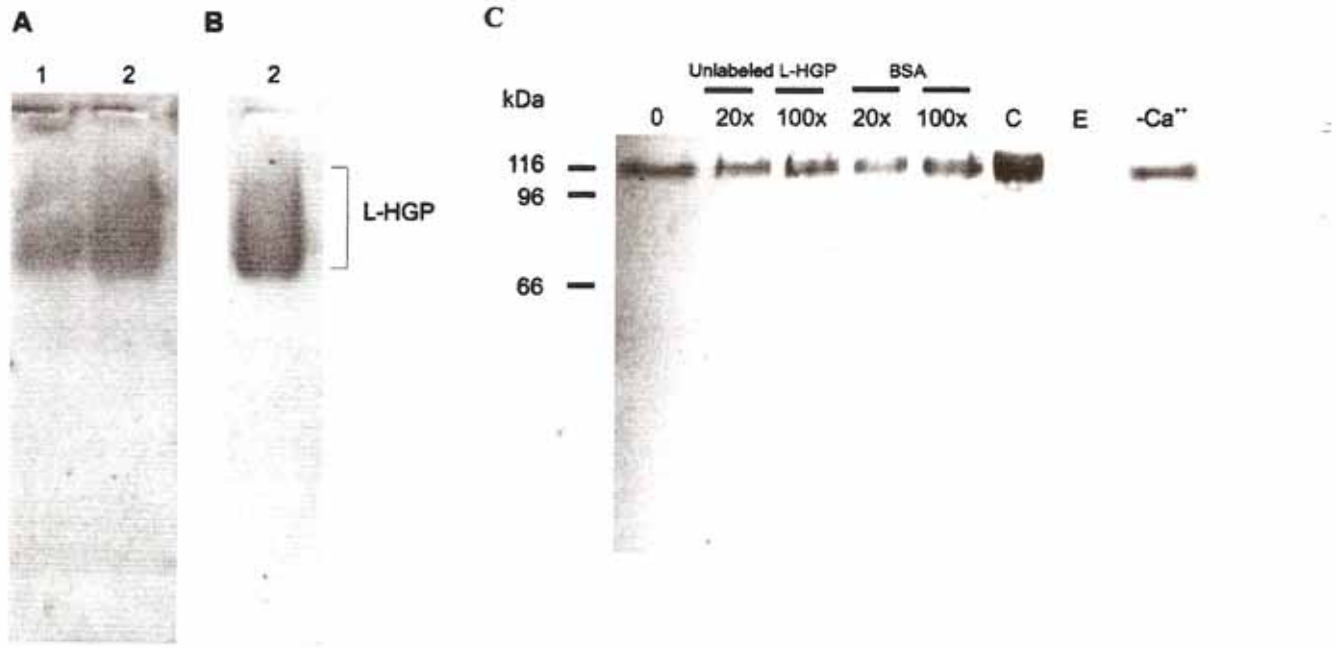

Fig. 7. Labeling of L-HGP with biotin (A, B) and binding of labeled L-HGP to *B. arenarum* spermatozoa (C). Labeling was carried out according to Materials and Methods. One microgram of unlabeled L-HGP (lane 1) or of labeled L-HGP (lane 2) was submitted to SDS-PAGE and transferred to PVDF membrane. (**A**) Immunodetection with antibodies anti-L-HGP (1:1000). (**B**) Revealed with streptavidin/peroxidase (1:1500). (**C**) Spermatozoa were incubated 30 min with biotinylated L-HGP (0.02 mg/ml) alone (0) or with 20× to 100× amounts of unlabeled L-HGP or BSA. After three washes, the final sperm pellet was dissolved and run on a 4–15% gradient SDS-PAGE and revealed by streptavidin/peroxidase. E, spermatozoa incubated without L-HGP in 10% Ringer-Tris; without Ca^{++}, sperm incubated with labeled L-HGP in 10% Ringer-Tris without calcium and with EDTA 1 mM; C, biotinylated L-HGP (1 µg labeled protein) as control.

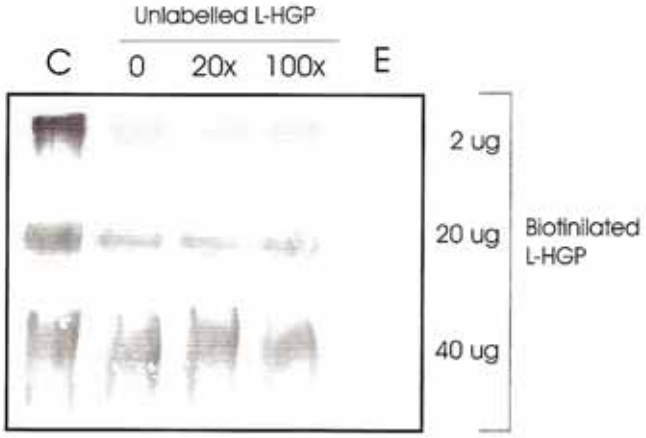

Fig. 8. Effect of different concentrations of L-HGP on the binding of the protein to sperm membranes. Spermatozoa were incubated 30 min with biotinylated L-HGP (0.002, 0.020, or 0.040 mg/ml final concentrations) alone (0) or with 20× to 100× times of unlabeled L-HGP. After three washes by centrifugation, the final pellet was dissolved and run on a 4–15% gradient SDS-PAGE and revealed by streptavidin/peroxidase. C, Biotinylated L-HGP (1 µg labeled protein) as control; E, spermatozoa incubated 30 min without L-HGP in 10% Ringer-Tris.

spermatozoa were observed under TEM in each sample. Recently, a more accessible method for estimating the acrosome status under different experimental conditions in *B. arenarum* sperm populations was reported (Martínez and Cabada, 1996).

We study the effect of L-HGP, a diffusible egg-jelly protein from *B. arenarum*, on acrosome status of homologous spermatozoa.

It was previously demonstrated that a high percentage (75%) of spermatozoa present their acrosome reacted after a short period (20 min) of incubation in hypotonic solution (Martínez and Cabada, 1996). We have found that L-HGP obtained from EW prevents acrosome breakdown when spermatozoa are incubated in low ionic strength solutions, a condition that resembles natural environmental conditions.

Most reports on the site of occurrence of acrosome reaction in Anurans showed that spermatozoa pass through the jelly layer with the acrosome intact and that acrosome reaction takes place near to or at the vitelline envelope (Raisman et al., 1980; Omata, 1993). Premature acrosome breakdown can cause the loss of sperm fertilizing capacity (Raisman et al., 1980), as is the situation in mammal fertilization.

This is the first report in Anurans indicating that a protein purified from EW has the property of inhibiting sperm acrosome breakdown. This activity is especially relevant from the point of view that very few spermatozoa pass through the different jelly coats under natural conditions.

A glycoprotein from seminal plasma of the rabbit was shown to block the induction of the rabbit sperm acrosome reaction in vitro and was named the Acro-

some Stabilising Factor (ASF) (Eng and Oliphant, 1978). It has been suggested that ASF functions as a stabilizer of the sperm membrane during epididymal storage. ASF acts as a decapacitating factor and inhibits fertilization in vivo. Here we report that L-HGP inhibits the acrosome breakdown in *B. arenarum* spermatozoa in hypotonic solutions. However, it seems that the biological activity of L-HGP is different from that of ASF. While L-HGP is present when natural fertilization occurs, the presence of ASF in in-vitro insemination media inhibits acrosome reaction and avoids fertilization.

The effect of protection against acrosome breakdown seems to be specific for L-HGP since neither BSA nor fetuin has a similar activity.

The protective effect was observed when concentrations of L-HGP between 0.1 and 0.01 mg/ml were used. Standard and diluted EW showed similar protection properties. However total EW presented lower protection values than 10% EW. The data correlate well with the loss of fertilizing capacity of *B. arenarum* spermatozoa after 2 hr of incubation in EW previously reported (Díaz Fontdevilla et al., 1991). Protective levels of L-HGP also decline after 3 hr of treatment with purified L-HGP. This suggests the presence of at least two activities of the EW associated to sperm fertility: one of them is the activity of L-HGP in protecting acrosome during a short period of time. The second one is the presence of another factor in the EW that induces membrane modifications in spermatozoa in longer incubation periods. The latter one might be associated to previous findings working with lipid-like substances extracted from the EW (Díaz Fontdevilla et al., 1988).

Sperm incubations with L-HGP did not modify their fertility. This is in coincidence with the results of the protective effect of L-HGP observed when spermatozoa are incubated in this protein. These results also suggest that the protective effect observed on acrosome, is reversible, since the occurrence of the acrosome reaction is a requisite for fertilization. However, a tendency of presenting a fertility-protective effect when spermatozoa are incubated in L-HGP is observed.

Preliminary experiments were done to elucidate the mechanisms of sperm protection. The binding of oviductal proteins to the sperm has been demonstrated in many mammalian species, including human (Lippes and Wagh, 1989), ram (Sutton et al., 1984), stallion (Ellington et al., 1993), and bull (McNutt et al., 1992). In this study, we found that L-HGP, an oviductal protein from *B. arenarum*, binds to homologous spermatozoa. Assays using increasing amounts of unlabeled L-HGP to compete against biotinylated L-HGP for binding showed negative results. In addition, the binding of labeled L-HGP to spermatozoa increases with the concentration of proteins in the incubation medium. The fact that the protection of acrosome breakdown still remains after washing of the sperm is another evidence for the binding of L-HGP. However, these results are preliminary, and more work should be done to determine the localization of L-HGP in sperm plasma membrane. It is known that oviductal mammalian proteins influence sperm survival. However, at present it is not known how oviductal proteins modify sperm metabolism. Some authors speculate that these proteins may stabilize membranes by coating them and that they could protect sperm from reactive oxygen species, as does BSA (Alvarez and Storey, 1993).

In summary, a diffusible protein from *B. arenarum* egg-jelly coat was purified to homogeneity. It has an important activity in maintaining acrosome integrity in hypotonic solutions for at least 1 hr. This transient acrosome protection may allow spermatozoa to pass through the jelly envelopes and reach the vitelline envelope with the acrosome intact. The biochemical characterization of L-HGP may help us to understand the mechanism involved in the inhibition of acrosome breakdown in low ionic strength solutions.

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